Video Article

T-wave Ion Mobility-mass Spectrometry: Basic Experimental Procedures for Protein Complex Analysis

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Abstract

Ion mobility (IM) is a method that measures the time taken for an ion to travel through a pressurized cell under the influence of a weak electric field. The speed by which the ions traverse the drift region depends on their size: large ions will experience a greater number of collisions with the background inert gas (usually N_2) and thus travel more slowly through the IM device than those ions that comprise a smaller cross-section. In general, the time it takes for the ions to migrate though the dense gas phase separates them, according to their collision cross-section (Ω).

Recently, IM spectrometry was coupled with mass spectrometry and a traveling-wave (T-wave) Synapt ion mobility mass spectrometer (IM-MS) was released. Integrating mass spectrometry with ion mobility enables an extra dimension of sample separation and definition, yielding a three-dimensional spectrum (mass to charge, intensity, and drift time). This separation technique allows the spectral overlap to decrease, and enables resolution of heterogeneous complexes with very similar mass, or mass-to-charge ratios, but different drift times. Moreover, the drift time measurements provide an important layer of structural information, as Ω is related to the overall shape and topology of the ion. The correlation between the measured drift time values and Ω is calculated using a calibration curve generated from calibrant proteins with defined cross-sections[1].

The power of the IM-MS approach lies in its ability to define the subunit packing and overall shape of protein assemblies at micromolar concentrations, and near-physiological conditions[1]. Several recent IM studies of both individual proteins[2,3] and non-covalent protein complexes[4-9], successfully demonstrated that protein quaternary structure is maintained in the gas phase, and highlighted the potential of this approach in the study of protein assemblies of unknown geometry. Here, we provide a detailed description of IMS-MS analysis of protein complexes using the Synapt (Quadrupole-Ion Mobility-Time-of-Flight) HDMS instrument (Waters Ltd; the only commercial IM-MS instrument currently available)[10]. We describe the basic optimization steps, the calibration of collision cross-sections, and methods for data processing and interpretation. The final step of the protocol discusses methods for calculating theoretical Ω values. Overall, the protocol does not attempt to cover every aspect of IM-MS characterization of protein assemblies; rather, its goal is to introduce the practical aspects of the method to new researchers in the field.

Video Link

The video component of this article can be found at https://www.jove.com/video/1985/

Protocol

The procedure we describe focuses solely on IM-MS analysis of protein complexes. Therefore, we suggest that researchers unacquainted with the field of structural MS refer to the sample preparation steps, instrument calibration and MS and tandem MS optimization procedures described in Kirshenbaum *et al.* 2009 http://www.jove.com/index/details.stp?ID=1954. In general, this protocol involves low micromolar concentrations of complex (1-20 μM) in a volatile buffer such as ammonium acetate (0.005 - 1 M, pH 6-8). Given that 1-2 μl are consumed per nanoflow capillary, we suggest 10-20 μl as a minimum volume, to enable optimization of MS conditions.

Part 1: Acquiring an ion mobility-mass spectrometry spectrum

1. Set the mass spectrometer on the following modes of operation: Mobility-TOF, positive ion acquisition, and V-mode.
2. Turn on all gases (API, Trap and IMS). We use N_2 for IM separation, and Ar for the Trap/Transfer. Recommended initial values are 1.5 ml/min for the Trap region, and a gas flow of 24 ml/min for the IMS device.
3. Set the m/z acquisition range. For an unknown protein complex, we suggest initial use of a wide mass range, which can then be reduced to the desired values. In parallel, adjust the MS profile for maximum transmission efficiency. For large complexes, the acquisition mass range should be set from 1,000 - 32,000 m/z, and the MS profile to Auto. Otherwise, the profile can be set, according to the following chart:

m/z	dwell (%)	ramp (%)

960	10	20
3200	30	40
10667		

4. Check the RF setting and, if necessary, adjust to values appropriate for large protein complexes, thus:

	Source	Trap	IMS	Transfer
RF Offset	450	380	380	380
RF Gain	0	0	0	0
RF Limit	450	380	380	380

5. Apply capillary voltage (1,050-1,400 V) and low nanoflow pressure (0.00-0.03 bar). Once spray is initiated, try to reduce the nanoflow pressure to a minimal value. In addition, adjust the position of the capillary, with respect to the cone.
6. Adjust the MS acquisition parameters, to acquire a well- resolved MS spectrum: the pressure gradient along the instrument, and the sampling cone as well as the extraction cone, bias, Trap and Transfer potential settings, should all be optimized (detailed in the associated JoVE protocol Kirshenbaum *et al.* 2009 http://www.jove.com/index/details.stp?ID=1954). Although these parameters are sample-dependent, the conditions we used for acquiring MS spectra of various ion masses, from peptide to protein complexes, are outlined in Table 1, (see also Fig. 1). To minimize the activation of the complex try to gradually reduce (in steps of ~ 10 V) the sample cone, Trap and bias voltages without changing the position of the peak.
7. Once an optimum mass spectrum is obtained the drift time profile should be adjusted. When analyzing protein assemblies, optimal conditions for both mass and mobility measurements are often incompatible; therefore, it is important to strike the proper balance between the two. Overall, the ion mobility plot should be optimized such that the peaks are distributed over the entire drift time range, and the peak profile is smooth, approaching a Gaussian distribution (Fig. 2A, 2B). Significant peak asymmetry can be related to poor separation of multiple conformations.
8. As a general rule, three parameters, T-wave velocity, T-wave height and IMS gas flow rate can be tuned to optimize the mobility separation. Increasing the T-wave velocity will widen the drift time distribution profile, while increased T-wave height values will narrow it. Similarly, increasing the IMS gas flow will shift the drift time profile toward higher values (the minimal IMS gas flow should be 10 ml/min). We suggest leaving two of the three available variables fixed, and optimizing the third until the IM spectrum is well-resolved (Fig. 2B). To this end, set the T-wave velocity and the gas flow to 250 m/s and 24 ml/min, respectively. Then, as a starting point, set the T-wave height to 3 V and, in a stepwise manner, increase it in 1 V increments. In general, larger ions will require higher wave heights. Usually, there is no need to modify the IMS pressure; however, when high bias voltages are required for tuning, reducing the IMS gas will enable a decrease in the bias voltage value and, consequently, a reduction in protein complex activation. Overall, maximal resolution of 10-12 t/Δt can be reached.
9. When conditions are not optimized (low T-wave height or high T-wave velocity and/or high IM pressure), the ions will not traverse through the IM device effectively, and their journey may take longer than the time required for the next ion packet to be released into the mobility cell. As a result, a new ion packet will be released from the Trap region before the previous packet has been delivered to the pusher region. This will lead to a 'roll-over' effect, in which the peak observed in the first part of the drift time spectrum is identical to that of the ions in the tailing edge (Fig. 2C). This artifact can be eliminated by increasing the T-wave height, and decreasing T-wave velocity and IMS pressure. In addition, the Trap release time can be adjusted. Moreover, it is important to validate that the Transfer T-wave height is set to at least 5 V. To prevent leakage of ions towards the IMS cell, we recommend that the mobility trap height be kept at maximum levels (30 V).
10. Low velocity and high amplitude of Transfer T-waves may lead to the "rippling" of the drift time distribution profile (Fig. 2D). This artifact occurs when the mobility separation of ions (ion arrival/drift time) is not maintained through the Transfer and ToF regions, due to partial synchronization between the pusher frequency and the transfer T-wave velocity. In order to eliminate this effect, either the pusher time or the Transfer T-wave velocity should be adjusted. Since the pusher frequency is related to the mass range, this artifact may reappear when this parameter is changed. T-wave height exerts a minor effect, though its reduction may also help to eliminate ripples.
11. Once the aforementioned parameters are optimized, the IM-MS data can be acquired.

Part 2: Screening experimental conditions to ensure mobility measurements of native structures

To achieve highly resolved MS peaks, protein complexes are often activated within the mass spectrometer, to promote the stripping of residual water and buffer components[11]. However, if the activation energy is increased beyond a threshold value, partial unfolding can be induced forming multiple intermediate states[12], which are unlikely to correspond to the native, solution-state structure (Fig. 3A-C). As a result, the drift time peak may be shifted and broadened, reflecting the heterogeneous population of unfolded structures.
In order to obtain drift time data consistent with solution-phase structures, it is essential to carefully control the voltages used for accelerating ions, prior to IM separation. Moreover, for high MS resolution it is preferable to increase the Transfer rather than the Trap voltage. As the IM device is positioned, first, followed by the Transfer region and the TOF analyzer, hence, the activation follows the IM measurement and the ions remains unaffected, while the MS accuracy can be increased.

To validate that data acquisition is performed under conditions that maintain the native structure of the complex, it is recommended that data be recorded over a range of experimental and solution conditions, rather than according to a single, optimized set of parameters:

1. Increase capillary and cone voltages in a stepwise manner, while monitoring the effect on the drift time spectrum.
2. As in Step 1, increase the Trap collision voltage in a stepwise manner, and acquire data at 10 V intervals.
3. To identify the unfolded conformations and assess the acquired data, manually induce dissociation of the protein complex by titrating the sample with acetic acid over a pH range of 2-7, and record the data (Fig. 3B).

Part 3: Correlating between drift time values and cross-sectional areas

Unlike conventional IM measurements, in which the measured drift time values are linearly related to Ω, in the T-wave IMS system, the cross sectional area is defined by a calibration approach. Thus, rather than an absolute measurement, a relative exponential correlation is generated between the measured drift times and Ω[1,13]: $\Omega \sim t_D^X$

where t_D is the measured drift time, and *X* is the proportion constant that can be extracted from a calibration curve. The calibration is performed by measuring the drift times of ions with known Ω (measured from conventional IM experiments).

1. Drift time measurements are calibrated using denatured proteins equine cytochrome C, horse heart myoglobin and bovine ubiquitin with known collision cross-sections. To this end, solutions of 10 µM in 49/49/2, volume ratio, water/methanol/acetic acid should be prepared (reagents used are outlined in Table 3).
2. Acquire IM-MS data for the calibrant proteins under exactly the same instrument conditions used for the target protein or protein complex: $t_D' = t_D - \frac{c\sqrt{\frac{m}{z}}}{1000}$ (Part 1). All the voltages and pressure values should be identical, to preserve the IM separation settings.
3. For each charge state of the calibrant proteins extract the experimental drift time value (t_D) (described in Part 4).
4. Correct each of the calibrant drift times (t_D) (Table 2) using the following equation: $t_D' = t_D - \frac{c\sqrt{\frac{m}{z}}}{1000}$, where m/z is the mass-to-charge ratio of the observed ion, and c is the Enhanced Duty Cycle (EDC) delay coefficient[1]. Its value, typically between 1.4 and 1.6, is instrument-dependent. The EDC value is indicated within the System | Acquisition Settings | Acquisition Setup tab.
5. Using Prof. David Clemmer's Cross-Section Database: http://www.indiana.edu/~clemmer/Research/cross%20section%20database/Proteins/protein_cs.htm[14] correct each of the calibrant cross-section for both ion charge state and reduced mass. $\Omega_C = \frac{\Omega}{z\sqrt{\frac{1}{m}+\frac{1}{M_G}}}$, where Ω_C is the corrected cross-section, Ω is the literature cross-section, z is the ion charge state, m is the molecular weight of the calibrant ion, and M_G is the molecular weight of the IM background gas (typically N_2).
6. Plot ln(t_D) against ln(Ω_C) (Fig. 4A).
7. The resulting curve corresponds to the following equation: $ln(\Omega_C) = Xln(t_D') + A$ The parameters *X* and *A* can be extracted by fitting the plot to a linear relationship. The slope *X* corresponds to the exponential proportion factor and A represents the fit-determined constant.
8. Calculate the fit correlation coefficient r^2, using the Pearson's equation: $r = \frac{\sum(x-\bar{x})(y-\bar{y})}{\sqrt{\sum(x-\bar{x})^2(y-\bar{y})^2}}$. Acceptable values for r^2 are greater than 0.95 (Fig. 4B). A lower correlation coefficient value may be due to:
   a. Incomplete unfolding of the protein calibrants. This will lead to peak widening due to the heterogeneous assembly of intermediate states.
   b. In our experience, an aged sample can deteriorate the IM spectra.
   c. Dissimilar experimental conditions used for the different calibrant proteins. In this case, plotting the data for each protein separately should generate diverse correlation coefficients, though each of them should be higher than 0.95.
   d. Noisy data and incorrect smoothing and centering of the drift time distribution.
   e. Calculation error.
9. Recorrect the calibrant drift time using the determined exponential factor, *X*, derived in step 7: $t_D'' = zt_D'^X\sqrt{\frac{1}{m}+\frac{1}{M_G}}$
10. As a validation step, replot Ω_C vs. t_D'' and define the correlation coefficient. Higher values than 0.95 are to be expected.
11. According to the procedure described in Step 4, correct the measured drift time of the target protein or protein complex: $t_D' = t_D - \frac{c\sqrt{\frac{m}{z}}}{1000}$
12. Calibrate the drift time of the target protein/protein complex using the exponential factor, *X*, defined in Step 7: $t_D'' = zt_D'^X\sqrt{\frac{1}{m}+\frac{1}{M_G}}$
13. Calculate Ω of the target protein/protein complex using the fit-determined constant, *A*, defined in Step 7: $\Omega = At_D''$.
14. For each experimental condition, Steps 2 to 13 should be repeated. When defining the cross sectional area of the unknown protein or protein complex, we recommend that each experiment be repeated at least three times, and the standard deviation of these triplicate measurements determined.

Part 4: Defining drift time values

Required software: MassLynx and Driftscope (Waters).

1. Open the IM-MS spectrum using the Driftscope software.
2. From the main menu, select View and uncheck Chromatogram, Drift Time and Spectrum (optional), leaving active only the 2D map displaying drift time vs. m/z.
3. From the menu bar, choose Display |Options |Display Editor Panel and adjust Intensity Threshold values to minimize background noise (in most cases, this setting can be defined as Min=30-40% and Max=100% counts).
4. Choose Display |2D Map Intensity Scale, and three options will appear: Linear Scale, Log Scale and Square Root Scale. A choice of Log Scale (logarithmic data transform) will compress the intensity color code, and enable the simultaneous appearance of a broad range of intensities (as opposed to the linear and square root options, with which only the most intense peaks will become visible).
5. From the toolbar panel, Use Selection Tool button. This option will activate the different selection options, and enable selection of the relevant region within the spectrum. The most precise tool is Enable Region of Interest Selections, by means of which a border may be drawn around the region of interest, thereby excluding all unnecessary data and noise peaks. Likewise, the Orthogonal and Band Selection options are useful, when the region of interest is not surrounded by redundant peaks.
6. Once the region of interest is selected, use the Accept Current Selection command to remove unnecessary information.
7. Export the data to MassLynx, while retaining the drift time information.
8. Within MassLynx, open the chromatogram of the saved drift time spectrum, and combine the time bins. The corresponding mass spectrum will open automatically.
9. Apply the smoothening function by defining the window size and number of smooth parameters (should be tuned specifically for each spectrum, using minimal values).
10. Apply baseline subtraction, if necessary.
11. Center the spectrum and measure the mass, to validate protein identity and mass accuracy.
12. For each charge state, combine the m/z range. The corresponding drift time spectrum will automatically appear. Smooth and center the drift time profile, and define the drift time value by indicating the centroid of each peak.

Part 5: Representative Results

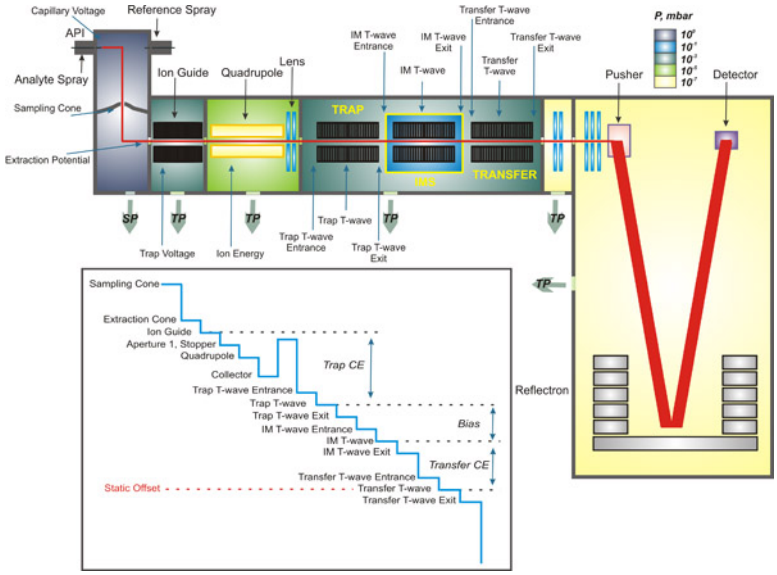

Figure 1. Schematic representation of the Synapt HDMS instrument indicating the major tunable parameters of IMS-MS acquisition. Experimental parameters used for IM-MS measurements are labeled according to their position within the instrument. The ion beam is colored in red, and the pressure in each region is designated using a color code. The panel on the bottom illustrates the potential gradient along the instrument and the potential differences defining the Trap and Transfer collision energies as well as the Bias potential. All potentials read-backs are referenced to the Static Offset voltage which is usually set to 120V.

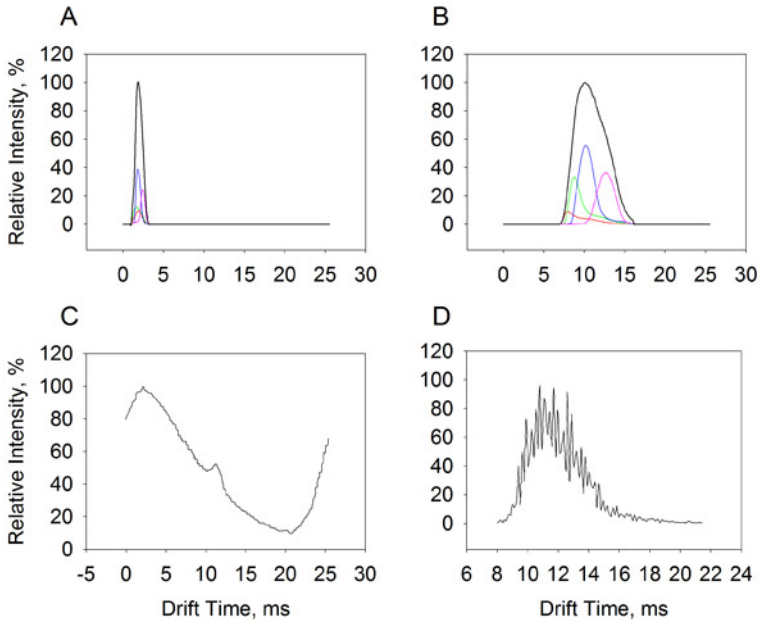

Figure 2. Ion mobility arrival time distributions for the Gβυ protein.
A. A high T-wave velocity leads to a narrow distribution of the drift time profile. The plot illustrates the arrival time distribution of the 16+ (red), 15+ (green), 14+ (blue), and 13+ (magenta) charge states, as well as the total drift time profile (in black) of the $G_{\beta\upsilon}$ protein.
B. An optimized drift time spectrum with a smooth Gaussian peak shape. Similar color labels as in **A**.
C. A 'roll-over' effect, which occurs when the time taken for ions to traverse the mobility cell is slower than the interval between the injections of new ion packets into the device. As a result, the extended drift time peak appears at the beginning of the spectrum. This effect can be eliminated by increasing the T-wave height, and decreasing T-wave velocity and IMS pressure.
D. Artificial 'ripples' are caused when the Transfer T-wave velocity and pusher frequency are partially synchronized. This effect can be overcome by adjusting either the pusher frequency or Transfer T-wave velocity.

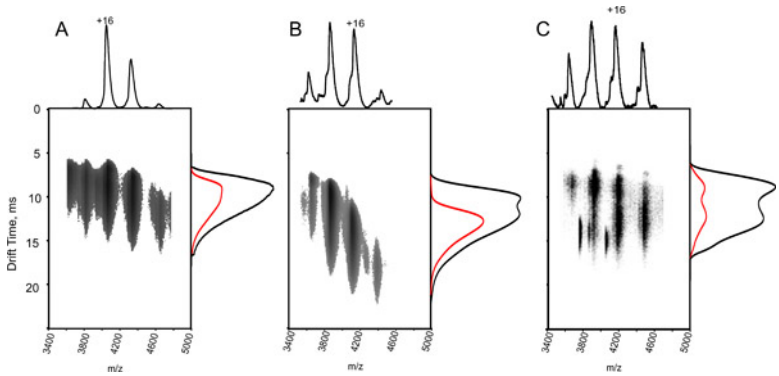

Figure 3. The effect of ion activation and partial denaturing conditions on IM-MS spectra of hemoglobin. Plot of drift time versus m/z for the tetrameric hemoglobin complex, using an aqueous solution of 10 mM ammonium acetate (pH=7.6) (A, C) and the addition of 0.1 % acetic acid (B). Data acquired using Trap collision energy voltage of 13 V (A, B) and 35 V (C) Although in all three panels the mass spectrum (projected on the top) looks similar, with a tetrameric charge series centered at 4,000 m/z, the drift time profile (projected on the sides) is different (total drift time distribution is in black, and the 16+ profile is in red). The longer drift time of the partially denatured sample, obtained in B, and the gas-phase activated ions, obtained in C, is indicative of some degree of unfolding. This observation illustrates that even though the measured mass corresponds to an intact complex, its solution structure is disrupted. As a consequence, careful control of experimental conditions is required.

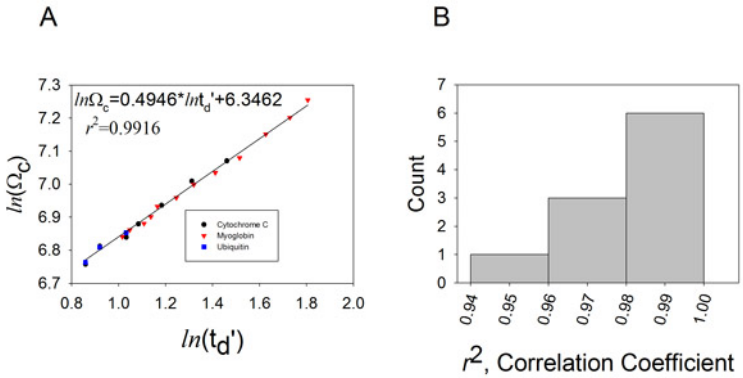

Figure 4. By generating a calibration curve, drift time measurements and collision cross-sections can be correlated.

A. Measured drift time values of the multiple charge states of equine cytochrome *C* (circles), horse heart myoglobin (triangles) and bovine ubiquitin (squares) were plotted against literature Ω values corrected for both ion charge state and reduced mass. The fit yields a linear function corresponding to: $\ln(\Omega_C)=X\ln(t_{D'})+A$. The determined exponential factor (*X*), fit-determined constant (*A*), and correlation coefficient are displayed on the plot for data acquired at a T-wave velocity of 350 m/s, and a static wave height of 11 V. **B**. A histogram of the correlation coefficient distributions obtained from 10 consecutive calibration experiments.

Protein sample / Technical parameters	**GluFibrino-peptide monomer 1.6 kDa**	**Myoglobin monomer 17 kDa**	**Hemoglobin tetramer 67 kDa**	**Transferrin monomer 80 kDa**	**GroEL 14-mer 801 kDa**
Backing pressure, mBar	4.4	5.0	5.1	5.1	6.5
Trap pressure, mBar	$1.6x10^{-2}$	$2.4x10^{-2}$	$2.4x10^{-2}$	$2.6x10^{-2}$	$2.8x10^{-2}$
IMS Pressure, mBar	$4.4x10^{-1}$	$4.4x10^{-1}$	$4.4x10^{-1}$	$4.4x10^{-1}$	$4.2x10^{-1}$
Sampling cone voltage, V	46	80	80	80	118
Extraction cone voltage, V	1.7	1	1	1	3
Bias voltage, V	20	20	25	25	50
Trap collision energy, V	20	15	15	15	80
Transfer collision energy, V	5	12	12	12	15

Table 1. Experimental conditions used for analyzing macromolecules.

Standard Protein	**Molecular Mass (m)**	**Charges (z)**	**m/z**	**Collision Cross-Section (in 2)**
Cytochrome C	12213	10	1222.3	2226
		11	1111.3	2303
		12	1018.8	2335
		13	940.5	2391
		14	873.4	2473
		15	815.2	2579
		16	764.3	2679
		17	719.4	2723
		18	679.5	2766
Myoglobin	16952	11	1542.1	2942
		12	1413.7	3044
		13	1305.0	3136

		14	1211.9	3143
		15	1131.1	3230
		16	1060.5	3313
		17	998.2	3384
		18	942.8	3489
		19	893.2	3570
		20	848.6	3682
		21	808.2	3792
		22	771.6	3815
Ubiquitin	8565	8	1071.6	1442
		8	1071.6	1622
		9	952.7	1649
		10	857.5	1732
		11	779.6	1802

Table 2. Calibrant proteins and their collision cross sections values determined by conventional IMS measurments[14].

Devices	**Company**	**Catalogue number**
Synapt HDMS-32K RF generator	Waters Ltd.	
P-97 Flaming- Brown micropipette puller	Sutter Instruments	P- 97
Sputter coater	Electron Microscopy Sciences	EMS550
Binocular microscope	Nikon	
Reagents	**Company**	**Catalogue Number**
Ammonium Acetate	Sigma- Aldrich	Sigma, A2706
CsI 99.999%	Sigma- Aldrich	Aldrich, 203033
Methanol	Sigma- Aldrich	Fluka, 34966
Acetic Acid	Fisher Scientific	AC12404
Equine myoglobin (from horse heart)	Sigma- Aldrich	M1882
Equine cytochrome c (from horse heart)	Sigma- Aldrich	C-2506
Bovine ubiquitin (from red blood cells)	Sigma- Aldrich	U6253
Hemoglobin	Sigma- Aldrich	H2625
Gas	**Comments**	
Nitrogen, 99.999% pure	8 cubic meter cylinder	
Argon, 99.999% pure	8.8 cubic meterscylinder	

Table 3. Reagents and equipment.

Discussion

The protocol described here enables to define the collision cross section of proteins or protein complexes with an unknown three dimensional structure, with the aim of providing information on their overall shape, subunit packing and topology. To this end once collision cross section values are depicted it is necessary to convert these values to structural details. This process requires additional experimental efforts as well as computational analysis, which are briefly discussed below.

To begin with, it is recommended to analyze proteins or protein complexes with known structures. These measurements can provide a useful quality control of the methodology and will enable accuracy assessment of the acquisition parameters by comparing theoretical and measured Ω values. The theoretical cross sectional areas can be calculated from the crystal structure coordinates using the MOBCAL[15,16] software, which is an open source FORTRAN based software allowing code editing according to the operator needs. For running such calculations it is required to modify the program such that the number of iterative calculations performed per input structure is increased and that coordinate files containing large number of atoms are accepted[1].

An IM-MS strategy for defining topological arrangements of subunits within multicomponent assemblies has been recently proposed[4,6]. The method involves the monitoring of dissociation pathways of protein assemblies to smaller components. This dissociation is achieved through

controlled adjustment of solution phase conditions, which gives rise to a distribution of subcomplexes reflective of the "building blocks" of the assemblies. The simultaneous measurement of Ω values of both the intact complex and disassembly products generates structural restraints which are then used for calculating topological models of the protein complexes. The basic assumption underlying this methodology is that the generated subcomplexes retain their native-like confirmations, and indeed recent studies have demonstrated that the solution structure of the disassembly products is maintained and no major rearrangement in either solution or gas phases have occurred[4,6].

The last step in the assignment of quaternary structure to gas-phase protein complex ions is fitting the collision cross section values to computer generated models. Modeling approaches are employed in order to explore the different possible topological arrangements of subunits and their *in silico* Ω values are calculated and compared to the experimental ones. Currently only a few computational approaches are used, like the spheretype coarse-grained method that approximates the diameter of subunits[1,8]. On the whole, this field is still in its early years and further development is required to make this approach generic, and applicable to a wide range of complexes.

Disclosures

No conflicts of interest declared.

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