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Nota de aplicación

Accurate Collision Cross Sections From Traveling Wave Ion Mobility Measurements in Native Mass Spectrometry – A Structural Investigation of Cas9 Ribonucleoprotein Complexes

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Waters Corporation

Para su uso en investigación únicamente. No se debe utilizar para procedimientos de diagnóstico.

Abstract

Traveling wave ion mobility spectrometry (TWIMS) is a key technology in the native MS toolbox for interrogating biomolecular structure. Central to its role is the ability to determine collision cross sections (CCS), which can be used as a representation of the structure of the biomolecules being analyzed. As TWIMS technology has developed, computational approaches for processing the data must also evolve. Here we describe the increasingly popular IMScal tool, a simple-to-use program for calibrating Waters™ TWIMS instruments to determine CCS values. IMScal was used herein to measure CCS values of Cas9 protein and its ribonucleoprotein (RNP) forms when in complex with an sgRNA, molecules of significant interest in the gene therapy field. A suitability test of the calibration approach gave us high confidence in the CCS values for Cas9 and its ribonucleoprotein complexes. Furthermore, the high-quality data obtained in this application note highlights the

SELECT SERIES™ Cyclic™ IMS mass spectrometer as a powerful instrument for native MS studies.

Benefits

- Determine highly accurate CCS values using the IMScal tool
- Obtain native protein CCS values simply and rapidly
- Perform structural studies on a wide range of analytes with high confidence in results
- Obtain high-quality data using the SELECT SERIES Cyclic IMS mass spectrometer to reduce ambiguities in native MS analyses

Introduction

Since its introduction on the SYNAPT™ HDMS platform in 2006, traveling wave ion mobility spectrometry (TWIMS) has revolutionized mass spectrometry-based analysis of myriad compounds. In particular, the ability to gain structural information on proteins and their complexes was central to the initial success of the technology. The structural information in question is the CCS, a measurable property of an ion-neutral collision complex that directly reports on the shape of the ion in question. This measurement, coupled with *in silico* modelling has helped researchers to access never-before-seen information on biomolecular structure, recently assaying liquid-liquid phase separation and following protein aggregation.[1–2]

In traveling wave ion mobility, CCS values are obtained by calibration of the separation data with standards of known CCS obtained from drift tube measurements. Until recently, the calibration approach for large proteins and their complexes involved tightly bracketing the unknown ion with calibrants.[3] This approach had several pitfalls including the inability to calibrate over a wide range of ions in a single calibration, poor fitting of calibration curves due to insufficient data points, and the inability to extrapolate the calibration curve reliably.

Traveling wave-based ion mobility, in the form of SYNAPT XS and Cyclic IMS instruments, is still highly prominent in the field of MS-based structural biology and native MS and so new calibration tools are needed that can match the advancements of the hardware.

In this application note we describe the IMScal TWIMS calibration software, an increasingly popular tool for the measurement of CCS values. The tool is based on an analytical solution to the physical processes occurring as

ions traverse TWIMS separators and accounts for charge and mass-dependent mobility effects.[4–5] As such the tool can be used to calibrate TWIMS data for a wide range of analyte classes from any Waters ion mobility-enabled instrument. The tool is powered by a graphical user interface (GUI) and is simple to use, particularly in comparison to laborious spreadsheet-based approaches.

Here we demonstrate the use of IMScal for reporting CCS values of native proteins and their complexes. Firstly, we performed a suitability test of the chosen calibrants against proteins of known CCS and obtained excellent agreement between observed and reference values. Secondly, we performed a native MS study of recombinant Cas9 protein from *streptococcus pyrogenes* and its binding to a synthetic guide RNA. Using IMScal we were able to determine CCS values for the protein and the ribonucleoprotein (RNP) complex, gaining structural insights into the protein and its nucleic acid complement. Furthermore, the data demonstrate the capability of the SELECT SERIES Cyclic IMS instrument to study this exciting group of molecules of significant interest in the gene therapy field.

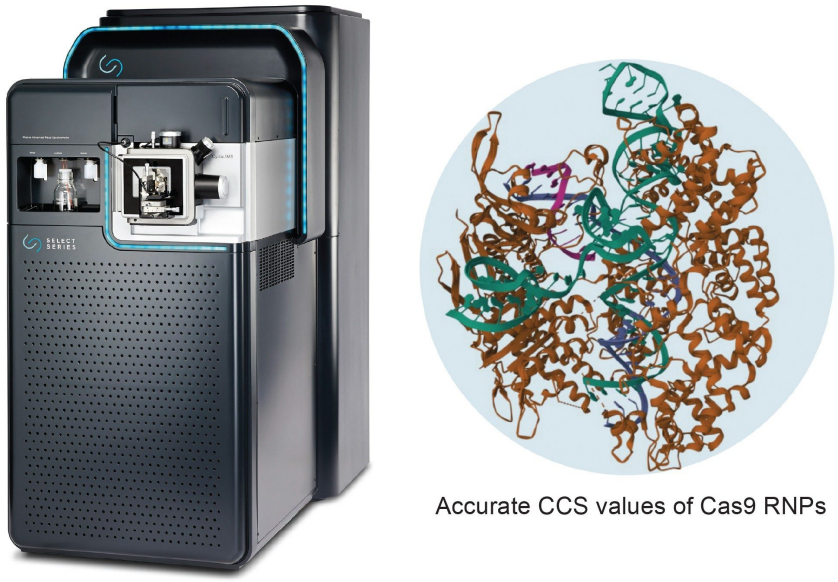

Figure 1. The SELECT SERIES Cyclic IMS mass spectrometer (left) a state-of-the-art solution for native MS. Right – cartoon representation of the crystal structure of Cas9 protein (orange) bound to sgRNA (green) and target DNA (magenta, blue) from PDB entry 4UN3.[6] Structure generated using Mol.[7]*

Experimental

Sample Description

For CCS calibration the polyalanine species were obtained as part of the CCS majormix calibration standard (p/n: 186008113 <https://www.waters.com/nextgen/global/shop/standards--reagents/186008113-major-mix-ims-tof-calibration-kit.html>). Concanavalin A (C2010), bovine serum albumin (A6003), Cas9 protein (CAS9PROT) and a custom sgRNA (HSPD0000004845, targeted to the human CA6 gene) were purchased from Merck-Sigma. Streptavidin was purchased from Pierce (21125). The soluble proteins and the sgRNA were buffer exchanged into 200 mM ammonium acetate using Biorad microbiospin P–6 centrifugal gel filtration cartridges. Final concentrations of the proteins were 1–5 µM. To form the Cas9 RNP, Cas9 protein was incubated with the sgRNA in a 1:2 ratio for 30 minutes at 4 °C before measurement.

Method Conditions

All proteins were measured individually by loading 5 uL of the protein solution into a borosilicate glass nanocapillary (TIP2TW1, World Precision Instruments, FL, USA). Nanocapillaries were mounted onto the source using a static nanospray adaptor kit (p/n: 186006331). Electrospray current was applied to the solution by a piece of platinum wire inserted into the capillary. The cone voltage and collision voltages were minimized to favor measurement of the folded forms of the analytes. A single pass of the cyclic device was performed for the CCS measurements.

MS Conditions

MS system:	SELECT SERIES Cyclic IMS
Ionization source:	Nanolockspray with static needle
Ionization mode:	Positive
Acquisition range:	50–16,000 *m/z*
Capillary voltage:	1 kV

Collision energy (Trap):	6 V
Cone voltage:	40–150 V
Cyclic static wave height:	20 V
Pushes per bin:	3
Array offset (inject step):	55 V
Array wave height (inject):	5 V
Array offset (separate):	55 V
Racetrack bias:	55 V
Array wave height (eject and acquire):	15 V

Data Management

MS software:	Waters Embedded Analyzer Platform for Cyclic IMS v13.2
Informatics:	Masslynx™ v4.2, Driftscope™ v3.0, IMScal tool

Calibration Conditions

For measuring the CCS values of unknown proteins, a set of ion mobility parameters was chosen that allowed optimum transmission and detection of both the calibrants and the unknowns (see MS Conditions section). The IMScal tool, available at imscal.on-demand.waters.com, comes with a list of calibrant reference CCS values to choose from. In this analysis we chose a minimum calibration set made up of polyalanine and bovine serum albumin. The incorporation of both small singly- and doubly-charged ions as well as heavy ions provides the chemical diversity required for a good calibration.

Calibrant	*n*	*M / Da*	*z*	*m/z*	Reference $^{TW}CCS_{N2}$ / Å^2	Drift time / ms*
Polyalanine	7	515.27	1	516.28	211	14.6
	8	586.30	1	587.31	228	17.4
	9	657.34	1	658.35	243	19.9
	14	1012.53	2	507.27	333	9.3
	15	1084.20	2	542.79	344	10.3
	16	1155.28	2	578.31	357	11.1
BSA	1	67000	14	4780	4490	49.1
	1	67000	15	4450	4490	43.0
	1	67000	16	4170	4470	38.1

Table 1. Calibrants used in this work. A minimal calibration set was chosen to include small singly- and doubly-charged ions (polyalanine) and a native protein (BSA).

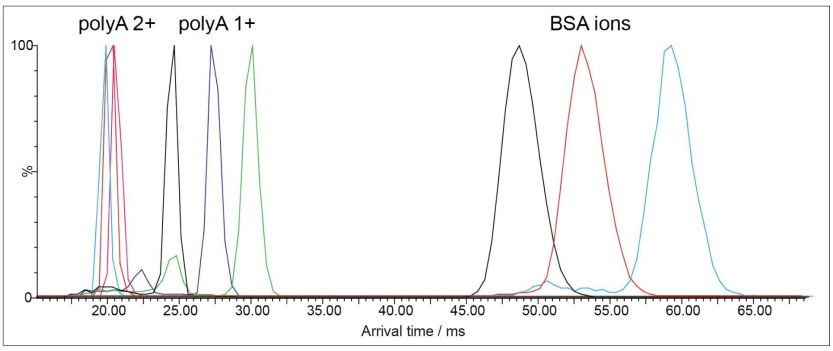

Figure 2. Observed arrival time distributions for the calibrant ions.

As well as reference CCS values and measured drift times of calibrants and unknowns, the IMScal tool requires a 'default_settings.dat' file to describe properties of the instrument and the analysis parameters (Table 2). Parameters such as the length of the travelling wave separator (0.254 m for SYNAPT XS, 0.980 m for a single pass of the cyclic device), the wave velocity, wave height, wavelength and gas pressure must be noted and used in the default settings file. Note that for the SELECT SERIES Cyclic IMS mass spectrometer, the voltage (wave height) parameter is scaled by a factor 0.75 to account for the radial geometry of the mobility device.

Parameter	Description	Value
length	Length of the IMS device / m	0.980
velocity	Traveling wave velocity / ms^{-1}	375
voltage	Traveling wave height / V	15**
pressure	IMS cell pressure readback / mbar	1.77
lambda	Traveling wave length / m	0.012
accuracy	Reference CCS accuracy / %	2.0

Table 2. A description of default settings for use with IMScal.

Once the default settings were defined, at least two input files are created, one to describe the calibrants and another to describe the unknowns. Once defined these are browsed into the GUI (Figure 3) and the calibration can be executed. The resulting output file lists the CCS values of the unknowns along with their associated uncertainties.

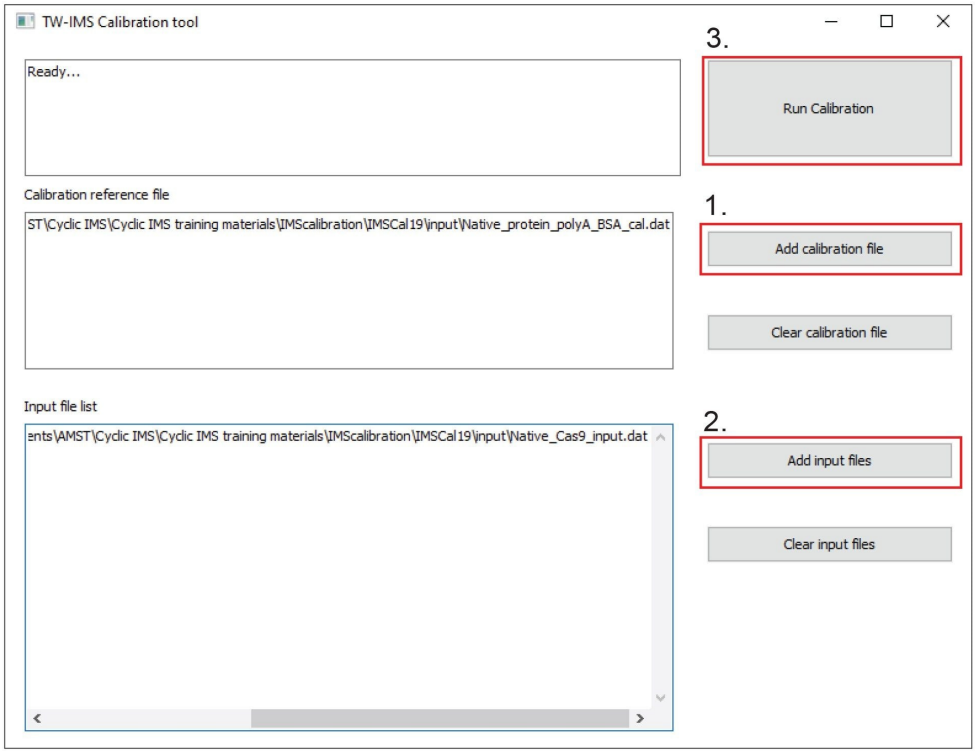

Figure 3. The graphical user interface of the IMScal tool. This easy-to-use program requires two input files – the calibration file and the unknown file - in addition to the defaults settings file. Results are written to an output file.

Results and Discussion

The first step of the workflow was to perform a quality check of the chosen calibration. To do so we analyzed the readily available protein complexes streptavidin and concanavalin A under the same conditions as the calibrants. Streptavidin has been measured previously on drift tube systems and concanavalin A is often used as a calibrant itself, meaning reference CCS_{N2} values are available for both proteins. Our measured CCS values were in excellent agreement with the reference values, all within 0.3 % or less (Table 3). This gave us high confidence in the calibration when applying it to unknowns.

Calibrant	*MW / Da*	z	Observed $^{TW}CCS_{N2}$ / Å²	Reference CCS_{N2} / Å²	ΔCCS_{N2} / %
Streptavidin	53216	14	3882	–	–
		15	3881	3884†	-0.08
		16	3962	3949†	0.3
Concanavalin A	102700	19	6076	6060‡	0.3
		20	6090	6080‡	0.2
		21	6111	6090‡	0.3
		22	6159	–	–

Table 3. Measured $^{TW}CCS_{N2}$ values of streptavidin and concanavalin A compared to drift tube reference values.

To demonstrate the measurement of CCS_{N2} values for an unknown protein target we chose the commercially available Cas9 endonuclease protein. This protein is of significant interest currently due to its involvement in CRISPR-based gene-editing therapies, one of which has been recently approved in the form of CASGEVY™, currently marketed for treatment of beta-thalassaemia. Methods of characterization of the constituents or precursors of CRISPR therapies are highly sought after and here we provide a means to obtain structural information on the protein and its binding to its sgRNA complement.

Firstly, we acquired native ion mobility mass spectra of the Cas9 protein (Figure 4 Top, Red spectrum). A charge state distribution centered around 6,000 *m/z* was observed, with the major species being the 27+. The measured mass for this group of signals was 162 kDa, in agreement with the expected mass. The mobility data (Figure 4 bottom), showed each charge state to have a single conformational family, indicating the protein to be in a folded state. Secondly, we acquired a native ion mobility mass spectrum of the sgRNA (Figure 4 Top, blue), observing a charge state distribution centered on the 8+ with each charge state appearing as a single conformational family by virtue of its arrival time distribution. Thirdly, we acquired native IM-MS data of the Cas9 protein after incubation with the sgRNA (Figure 4 Top, black). Strikingly, we observed a complete shift of the observed signals to higher *m/z*, centered over 7,000 *m/z*. The predominant mass in this spectrum was measured at 194.5 kDa corresponding to the Cas9 protein in complex with a single sgRNA. Upon close inspection of the higher *m/z* region of the spectrum we also observe a second charge state distribution corresponding to a higher mass. The ions responsible for these signals are also separated in mobility (Figure 4 bottom, indicated by white markers). The measured mass of these ions is 227 kDa, corresponding to a second sgRNA binding to the Cas9 protein. This observation is interesting as Cas9 should only bind a single sgRNA. But, given that the protein has

potential to bind also to target DNA, in this *in vitro* setting we may be forcing a binding of the second sgRNA given there is no DNA to compete for the binding site.

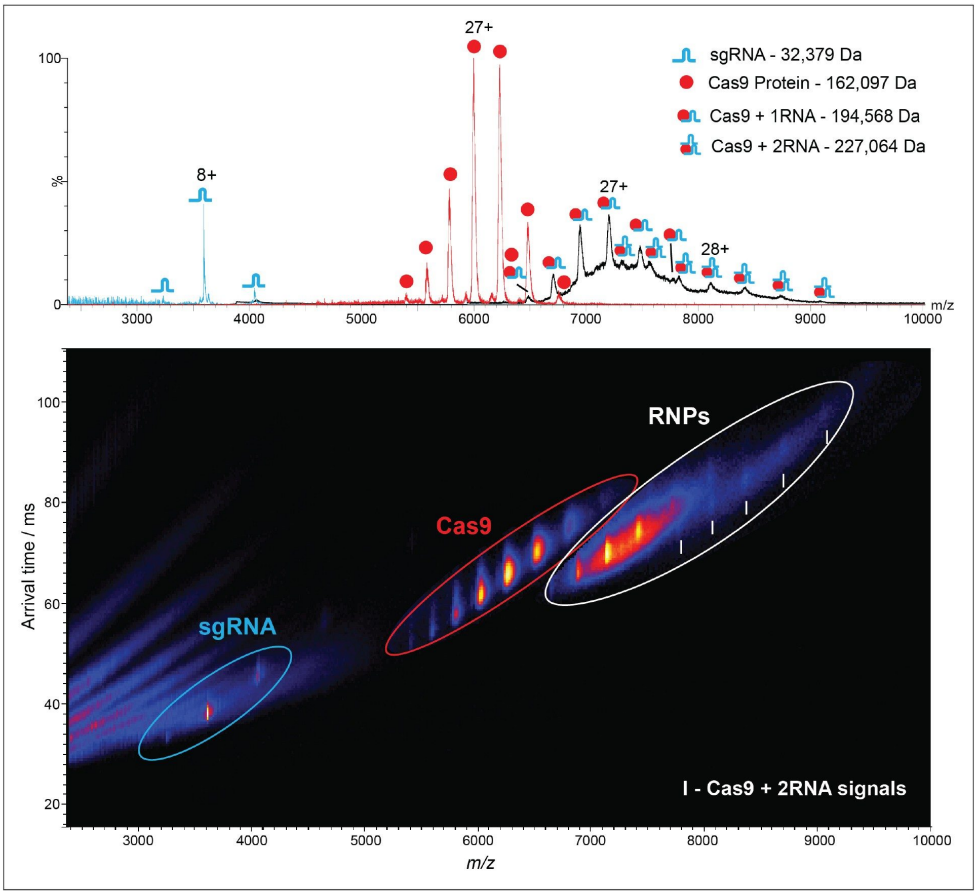

Figure 4. Native ion mobility-mass spectra of CRISPR-related species. Top – overlaid spectra of the sgRNA (blue), Cas9 protein (red) and Cas9 incubated with sgRNA (black). The observed mass for the sgRNA was 32,379 Da, in excellent agreement with the sequence mass. The Cas9 spectrum (red) exhibited a single major species consistent with the expected mass (162,097 Da). The Cas9+RNA spectrum exhibited a multitude of signals corresponding to Cas9 ribonucleoprotein complexes (RNPs) with 1 and 2 bound copies of sgRNA. Bottom – mobilogram of the three overlaid spectra from the top panel. Each species appears as a single arrival time distribution indicating a single conformational family. The arrival times of each species were used to determine their CCS values.

Finally, using the IMScal tool we were able to obtain CCS values for each of the observed species (Figure 5). The

$^{TW}CCS_{N2}$ values of the lowest observed charge states of the sgRNA, Cas9, Cas9+1sgRNA and Cas9+2sgRNA were 2280 ± 13, 7893 ± 119, 8573 ± 170, and 9891 ± 202 Å^2, respectively. The modest increase in the CCS of Cas9 versus Cas9 + 1RNA (+680 Å^2) agrees with the reported binding mode where the majority of the sgRNA appears threaded into the cavity of Cas9. The second RNA binding produces an increase of 1318 Å^2 versus the singly-bound Cas9, suggesting the binding is more external in nature. In summary, these accurate and reliable CCS values give valuable insights into the structure of the Cas9 protein and the nature of its binding to sgRNA, demonstrating the power of the high-quality data from the SELECT SERIES Cyclic IMS mass spectrometer in combination with state-of-the-art CCS calibration from the IMScal tool.

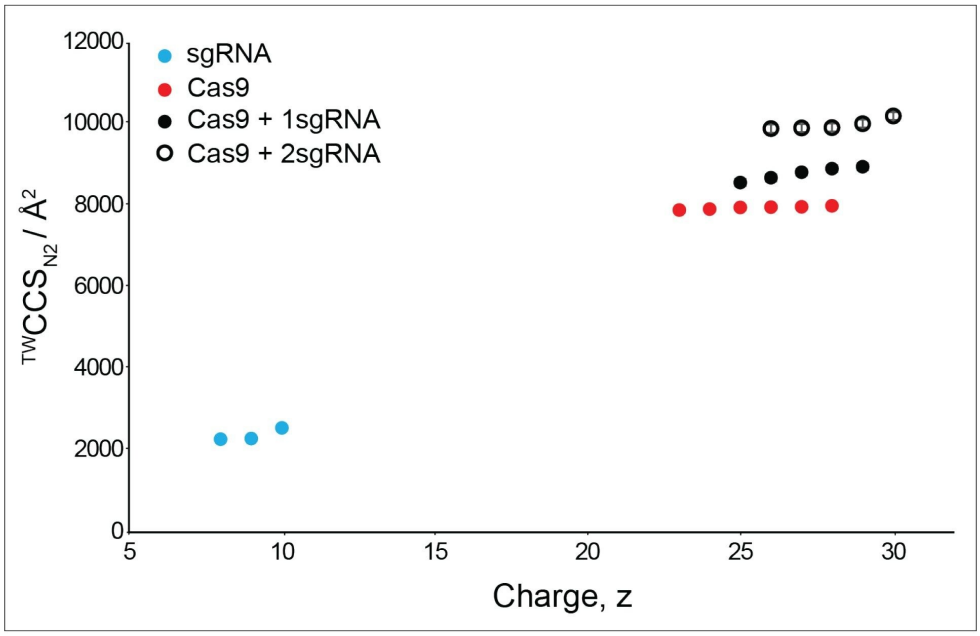

Figure 5. CCS values obtained from the spectra in Figure 4. Errors bars are shown but are smaller than the point markers.

Conclusion

The ability to obtain accurate collision cross section values from ion mobility mass spectrometry is a cornerstone of MS-based structural biology. Here we describe the implementation of the IMScal tool for obtaining CCS values

of native proteins from data acquired on a SELECT SERIES Cyclic IMS mass spectrometer. We applied the IMScal tool to a structural study of the recombinant Cas9 protein from ***streptococcus pyrogenes*** and its binding to sgRNA. The accurate CCS values offer the potential to gain high confidence structural insights into native proteins and their assemblies. The work here also highlights the potential of the SELECT SERIES Cyclic IMS instrument to characterize these important molecules that are part of the growing market of gene-based therapeutics.

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720008461, August 2024