2020

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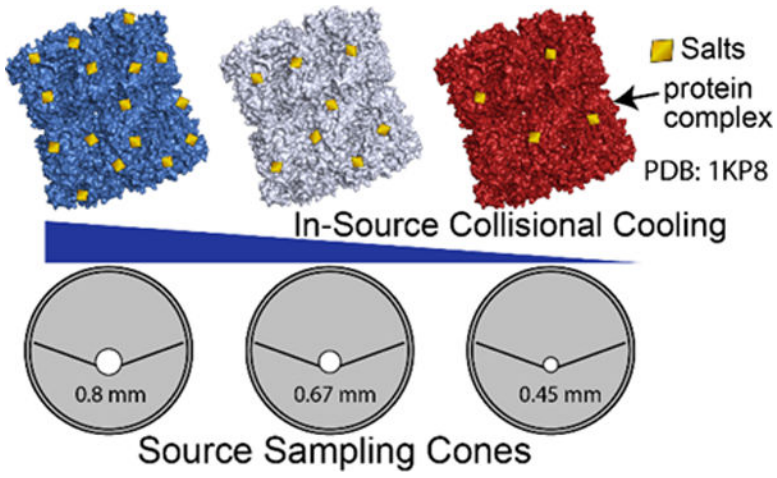
Abstract

Quadrupole-ion mobility-time-of-flight (Q-IM-TOF) mass spectrometers have revolutionized investigation of native biomolecular complexes. High pressures in the sources of these instruments aid transmission of protein complexes through damping of kinetic energy by collisional cooling. Since adducts are removed through collisional heating (declustering), excessive collisional cooling can prevent removal of non-specific adducts from protein ions, leading to inaccurate mass measurements, broad mass spectral peaks, and obfuscation of ligand binding. We show that reducing the source pressure using smaller aperture source sampling cones (SC) in a Waters Synapt G2-S*i* instrument increases protein ion heating by decreasing collisional cooling, providing a simple way to enhance removal of adducted salts from soluble proteins (GroEL 14-mer) and detergents from a transmembrane protein complex (heptameric *Staphylococcus aureus* α-hemolysin, αHL). These experiments are supported by ion heating and cooling simulations which demonstrate reduced collisional cooling at lower source pressures. Using these easily-swapped sample cones of different apertures is a facile approach to reproducibly extend the range of activation in Synapt-type instruments.

Graphical Abstract



Introduction

A significant challenge of native ion mobility-mass spectrometry with nano-electrospray ionization is that cosolutes from solution adduct to protein ions.[1,2] This causes peak broadening that can obscure ligand binding and hinder accurate mass determination.[2] Typically, native-like protein ions are accelerated into neutral buffer gases either in the relatively high-pressure instrument source or in a collision cell within the instrument to aid desalting or to cause unfolding/dissociation. Previous generations of the Waters Q-IM-TOF Synapt instruments used a source “extraction cone” for nozzle-skimmer activation, which can be very effective for desalting and detergent removal from membrane proteins.[3,4] More recent generations of the Synapt instrument replaced the extraction cone with a wide-diameter traveling wave “Stepwave” ion guide between the source region and quadrupole. The Stepwave, which operates in standard configuration at a pressure of ~3.0 mbar for the first segment and ~9.0e-3 mbar at the source turbo pump, is used in combination with a larger sampling cone (SC) than in earlier Synapt series instruments to increase sensitivity at the expense of decreasing the maximum degree of ion activation and desalting for large ions.

It is known that ions generated by ESI can undergo collisional heating and cooling, at times simultaneously, upon transfer into the vacuum of the instrument interior. Here, collisional heating refers to the transfer of energy from neutral buffer gas molecules to internal modes of protein ions, increasing their internal energy and thus effective temperature. Collisional cooling is a related process where internal energy from a protein ion is transferred to a neutral gas molecule, generally after it has already been heated by prior collisions. Heating of protein ions leads to desalting/detergent removal through declustering, where the buildup of internal energy is dissipated by the loss of salts or other molecules. Thus, decreasing the contribution of collisional cooling leads to higher net ion heating that can be utilized to remove salts/detergents. The pressure of the source region is a key determinant of the extent of collisional cooling or heating ions experience.[1,3,6,7] High pressures (low mbar) in the instrument source increase collisional cooling to slow protein ions for efficient transfer.[7,8] However, excessive collisional cooling leads to increased salt adduction.[1,3,8] Thus, a balance

must be struck such that pressure is sufficient to transmit large ions, but not so high as to prevent adduct removal (schematically represented in Figure 1a).

Landreh et al. demonstrated on a Synapt G1 HDMS that at high source pressures (up to 7.5 mbar for the backing pressure) membrane protein ions can be poorly resolved due to incomplete detergent removal, while lower source pressures (down to 1.8 mbar) decreased collisional cooling, leading to greater detergent removal.[3] This range of pressures was achieved with an adjustable valve between the source region and its dedicated pumping line, which may on the high end raise pressures throughout the rest of the instrument. The Synapt G2-S*i* does not have a dedicated source pump line. Thus we sought a facile, "hot-swappable" approach to increase ion heating in the source of a Waters Synapt G2-S*i* instrument by reducing the pressure with smaller aperture source SCs, that do not affect pressures in the rest of the instrument (see Table S1 for SC-specific instrument pressures).

Methods:

Source SCs with smaller apertures were obtained from Waters and are commercially available. In addition to the instrument standard (large) 0.8 mm SC, a medium 0.67 mm (Waters SKU: 700009087, Xevo G2-S instrument), and a small 0.45 mm (Waters SKU: 700010289, Xevo TQ-S micro instrument) SC were obtained and tested. Other SC sizes are available from Waters but were not tested here.

GroEL was purchased from Millipore-Sigma and prepared using established protocols.[4] αHL monomers were purchased from Millipore-Sigma and oligomerized in n-tetradecylphosphocholine (FOS-14) detergent solutions as described previously and further described in the Supporting Methods.[9] Waters Synapt G2-S*i* instrumental parameters are listed in the Supporting Methods. GroEL mass spectral peaks were analyzed using Igor Pro (WaveMetrics) to determine excess mass, peak full-width-at-half-maximum (fwhm), and integrated abundances for each charge state. Mass spectra for αHL heptamers were deconvolved using iFAMS and further analyzed with Igor Pro.[10] Ion heating and cooling simulations were performed as previously described,[11] using a Monte-Carlo framework to model the internal energy change of a protein ion due to collisions with neutral gas particles. Simulation details are further described in the Supporting Methods.

Results and Discussion:

Lower pressure in the instrument source can decrease the amount of collisional cooling ions undergo upon transfer from atmospheric pressure to vacuum.[1,3,6,8] Figure 1a shows a theoretical scenario where low and medium pressures (red and green) would completely desalt the protein in the source region while high pressure (blue) would not. Completely desalting a protein in the source region prior to the quadruple may aid experiments performed in the collision or IMS cells, allowing the full range of collision cell voltages to be utilized for unfolding/dissociation if desired or interrogation of bound ligands.

GroEL 14-mer (sequence mass 800,770 Da) is a common protein complex in native MS for assessing instrumental figures of merit for transmission of large ions and various types of ion activation.[1,2,4] We used GroEL to benchmark effects of ion activation in the instrument

source with each SC diameter. The “large” cone refers to the standard 0.8 mm i.d. cone for the Synapt G2-S*i*, while the “medium” and “small” cones refer to 0.67 and 0.45 mm i.d. SCs from Waters Xevo instruments to reduce the backing and source pressures. Instrument pressures beyond the source are unaffected (Table S1). Experimentally determined average excess mass (Figure 1b), abundance weighted drift time (DT) (Figure 1c), peak width (Supporting Figure S1a), and ion abundance (peak area, Supporting Figure S1b) were used to assess GroEL activation with each SC as the cone potential was incrementally raised from 10–180 V. Mass spectra of GroEL for each SC size and replicate are shown in Supporting Figure S2 at a cone potential of 10 V. At low cone potentials, little difference is measured between the cone sizes. Above 60 V, ion activation increases as the cone aperture decreases, with the small cone the most activating and the large cone the least. Selected mass spectra of the GroEL 14-mer for each cone at higher cone potentials (50, 100, 180 V) are shown in Supporting Figure S3. The greatest difference in excess mass between the large and small cone is ~4 kDa at 90 V, whereas the medium cone at 100 V leads to ~2 kDa less excess mass than the large cone. Thus, the medium cone provides the equivalent of an extra ~30 V cone potential beyond the standard configuration (large cone), and the small cone ~50 V. These trends are paralleled in the peak width measures (Supporting Figure S1a), with the smaller cones leading to narrower peaks (more desalted) at lower potentials. The smaller cone reduces the total GroEL ion signal by 20–30% (measured from the combined area of all GroEL charge states) as compared to the standard cone (Supporting Figure S1b) but greatly improves adduct removal capabilities.

The DT distributions (Figure 1c) for GroEL activation with each SC exhibits the same trend with aperture size. The potential at which unfolding begins with each SC decreases with decreasing cone aperture (large: ~180 V, medium: 150 V, small: 130 V). Independent of aperture size, GroEL unfolding begins with ~400 Da of excess mass remaining (Figure 1b) [1,2,4], suggesting that the smaller SCs do not cause such rapid heating that a different unfolding pathway is followed. Interestingly, the DT distributions for GroEL with each SC indicate aperture-dependent maximal degrees of ion compaction before the unfolding onset voltage (Figure 1c).[12] At high SC potentials (180 V), the medium and small cones can cause dissociation of GroEL without additional activation in the Trap (Supporting Figure S4), whereas the large cone requires further Trap activation to cause dissociation. Together, these GroEL experiments demonstrate that the “hot-swappable,” smaller aperture SCs improve desalting in the source region of the Synapt instrument (i.e., the region before the entrance to the quadrupole).

To demonstrate the advantages of smaller SCs when working with a membrane protein complex that requires significant collisional activation for analysis, we used αHL heptamers formed in FOS-14 detergent.[9] We previously demonstrated that stripping the lipid-like detergent FOS-14 from αHL complexes is difficult, and that dramatic charge reduction occurs when FOS-14 molecules are removed. For each SC, mass spectra without IM were collected at cone potentials of 25 V (Supporting Figure S5a), 100 V (Figure S5b), and 150 V (Figure 2a). Mass spectra of αHL FOS-14 micelle-embedded complexes are highly congested and require deconvolution using Gábor Transform (GT) analysis in iFAMS.[9,10] The average mass, charge, and number of associated FOS-14 molecules are reported in Table S2. At 25 V, the mass spectra for αHL complexes in FOS-14 micelles heavily overlap.

By 100 V, the charge and FOS-14 distributions for αHL complexes with each cone are shifted relative to one another, with the medium and small cone removing more FOS-14 and charge. In triplicate experiments at 150 V, the large cone produces an average charge state of 23.7 ± 0.3 with 116 ± 2 FOS-14 molecules associating with αHL heptamer complexes, while the medium cone results in an average charge of 20.3 ± 0.4 with 111 ± 2 FOS-14 molecules, and the small cone strips the most FOS-14 and charge to 17.0 ± 0.3 and 105 ± 2 FOS-14 molecules (Figure 2b). In terms of signal abundance with these samples, as expected, the higher the degree of activation and charge stripping that occurs, the lower the total signal. (At a cone potential of 25 V, all three cones yielded similar total signal for these complexes in triplicate experiments, whereas, at a cone potential of 150 V, the medium and small cones yielded ~30% and 50% lower total signal, respectively, than did the large cone.) These experiments are consistent with the GroEL data, showing that the medium and small SCs are more activating than the large cone, and that this increase in collisional heating can be useful for removing adducts like detergents and lipids from membrane proteins without altering pressures in other regions of the instrument.

To further explore increased ion activation at lower pressures and identify the main cause of increased activation, protein ion heating and cooling simulations were performed at pressures and potentials that represent the source region with each SC, with smaller cones represented by lower pressures. At low source voltages, all three pressure environments result in similar levels of activation: at high source voltages, a higher fraction of collisions at lower pressures cause heating than at higher pressures. These simulations confirm that variation in ion heating can be explained by changes in the number/frequency of collisions at differing pressures. More details about these simulations are provided in the Supporting Information and Figure S7.

Conclusions:

Here, we demonstrated and quantified the collisional activation effects of using source SCs with smaller apertures on protein ions on a Waters Synapt G2-S*i* instrument. Protein ion activation (heating) follows the trend of increasing activation with decreasing SC aperture size, with only a modest reduction in total ion signal. Through simulations, we confirm the increase in activation is caused by the concomitant decrease in source pressure with the smaller SCs, which reduces collisional cooling of protein ions in the instrument source.[1,3,6–8] Based on excess mass and DT measurements of GroEL, the medium and small cones provide ~30 and ~50 V additional SC potential than the large cone, respectively. This increase in collision energy can be used to strip difficult-to-remove adducts such as detergents and lipids from membrane proteins with the Synapt G2-S*i*'s otherwise very gentle ESI source, as demonstrated here with αHL heptamer complexes in FOS-14 micelles. Distinct additional advantages of these smaller SCs are that they are inexpensive, can be quickly exchanged without venting the instrument, and do not significantly affect pressures beyond the instrument source.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

The research reported in this publication was supported by the National Institute of Allergy and Infectious Diseases (award number R21AI125804-02) and the National Institute of General Medical Sciences (training grant award number 2T32GM007759-39 to J.W.W.) of the National Institutes of Health. This content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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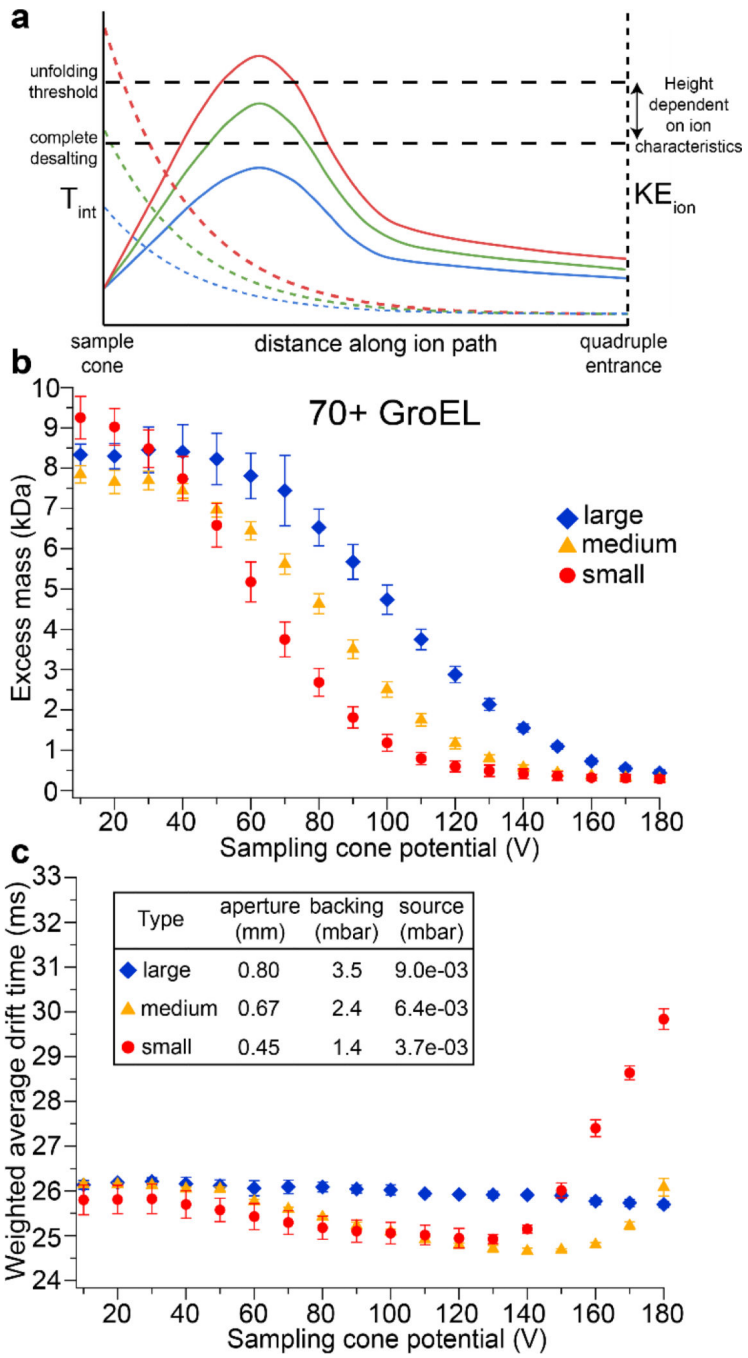

Figure 1:
(a) Simple model of protein ion heating and cooling (effective ion vibrational temperature, T_{int} (solid lines) after ions are accelerated in the instrument source (with initial laboratory-frame kinetic energy KE_{ion} (dashed lines)) under varying source pressures to demonstrate the balance between ion heating, desalting/declustering, and unfolding/dissociation at low (red), medium (green), and high (blue) pressures (note: not to scale). (b) Excess mass and (c) weighted average DT for GroEL^{70+} as the SC potential is raised with each SC size. Data were collected in triplicate on separate days with error bars representing one standard

deviation in the triplicate measurements. Inset table for (c) provides aperture size for each cone and corresponding instrument pressure readbacks.

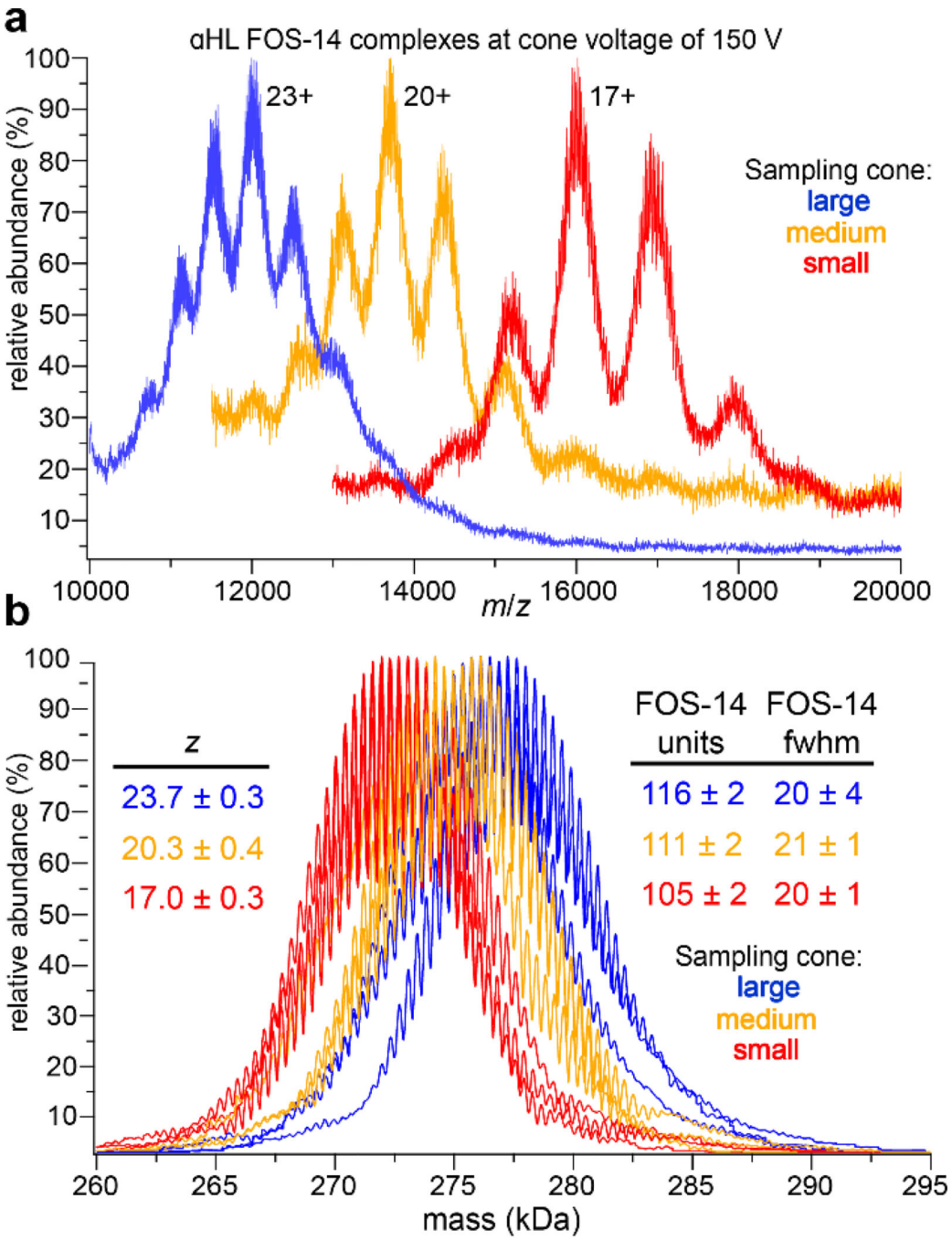

Figure 2:
(a) Overlaid mass spectra of αHL complexes in FOS-14 micelles with each SC at a cone potential of 150 V. Lower *m*/*z* portions of the mass spectra are truncated for clarity due to increasing signal from empty FOS-14 micelles (full mass spectra in Figure S6). The highest-abundance charge state for each spectrum is indicated. Lower charge states reflect greater removal of FOS-14. (b) Overlaid triplicate data for GT-deconvolved mass distributions of heptameric αHL in FOS-14 micelles with each SC at a cone potential of 150 V. The abundance-weighted average charge (*z*), average number of FOS-14 detergent molecules,

and fwhm in the number of detergent molecules is given for each cone. The ± value represents the standard deviation of the triplicate measure. Note: hexameric complexes are detected from the mass spectra but are not included in this analysis for simplicity.[9]