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A simple heated-capillary modification improves the analysis of non-covalent complexes by Z-spray electrospray ionization

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Abstract

Rationale—The observation of intact non-covalent complexes by ESI-MS hinges on the ability to minimize in-source activation processes that take place during analyte desolvation. We explored the merits of replacing the sampling cone of a standard Z-spray source with a heated capillary that makes the desolvation process slower and more gradual. We employed well-characterized protein-RNA and DNA-DNA assemblies to compare the alternative configurations.

Methods—Mass analysis evaluated the complexes' integrity, whereas traveling wave ion mobility experiments assessed the stability of biomolecular structure. Analyses were performed back-to-back on the same samples on a Synapt G2 HDMS equipped with either the standard sampling cone or the heated-capillary apparatus. In each configuration, the source/capillary temperature was varied in controlled fashion, while keeping all other desolvation parameters constant to monitor the in-source dissociation of selected DNA duplexes. Ion mobility data were obtained from the same precursor by using the alternative configurations under the same settings.

Results—Monitoring the percentage of associated complex demonstrated that the heated capillary provided softer desolvation that was more conducive to the detection of intact non-covalent interactions. This configuration failed to produce complete dissociation of 14bp and 24bp duplexes, even when the source/capillary temperature was increased well above their solution melting points. Analyzed by IMS-MS, a selected construct displayed just one conformation with the heated capillary, but two with the standard sampling cone.

Conclusions—The heated capillary minimizes in-source activation processes that can lead to unintended dissociation of complexes and perturbation of biomolecular structure, which rely on the integrity of non-covalent interactions. This effect can be attributed to the attenuation of the supersonic expansion typical of the Z-spray geometry and the greater ability to control the energy imparted to the system. This hardware modification will be expected to benefit the analysis of biomolecular structure performed on this particular instrumental platform.

Keywords

electrospray ionization; sampling cone; heated capillary; Z-spray; non-covalent complexes

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Introduction

The detection of intact non-covalent complexes by electrospray ionization (ESI) is made possible by the soft nature of the desolvation process.^{1–3} Solvent evaporation from electrosprayed droplets is promoted by collisions with neutral gas and involves rapid radius contraction and Coulombic fission steps that are essential to the production of solvent-free ions.^{4,5} Depending on hardware design, a combination of factors determines the desired outcome, including the pressure gradient between consecutive regions of the ion source, which are differentially pumped to reach the 10^{-3} – 10^{-10} Torr typical of mass analyzers; the electric field between such regions, which drives charged droplets and nascent ions through orifices, sampling cones, skimmers, or capillary elements; and the temperature of drying (curtain) gas and source surfaces, which help sustain solvent evaporation.³ In addition to achieving desolvation, the process may produce an increase in the ions' internal energy, which may affect their overall stability. The possibility of activating ion fragmentation for structure elucidation was recognized early on in the development of ESI, and was realized by increasing the potential of specific elements involved in desolvation to obtain “in-source” collision-induced dissociation.^{6–9} “Nozzle-skimmer,” “orifice” or “cone” voltage dissociation are typical terms employed to describe this technique in different hardware designs. In the case of non-covalent complexes, increasing the desolvation energy has been employed to achieve their controlled dissociation and assess the strength of the interactions between bound components.^{10–15} In these types of experiments, the stability of the binding interactions in the gas-phase has been expressed in terms of cone voltage or source temperature that induced 50% dissociation of the initial bound complex.^{8,16}

Soft desolvation conditions have enabled the analysis of intact biomolecular assemblies, including protein-protein,^{2,17} protein-nucleic acids,^{18–20} and nucleic acid-nucleic acid complexes.^{21–23} We have regularly benefited from this feature to investigate the interactions of various ligands with selected domains of the packaging signal (Ψ -RNA) of the human immunodeficiency virus type 1 (HIV-1).^{24–26} With proper experimental design, unbiased evaluation of free versus bound species in solution has allowed us to obtain dissociation constants (K_d s) that matched very closely those observed by established solution approaches.^{20,27,28} These types of determinations rely on the ability to obtain faithful snapshots of binding equilibria in solution, which must be acquired in the absence of both in-source dissociation induced by collisional activation,^{10,11,14,15} and non-specific aggregation due to the uncontrolled increases of analyte concentration, which occur upon contraction of the electrosprayed droplets.²⁹ In practical terms, the former can be averted by monitoring suitable non-covalent complexes that can be used to fine-tune the source parameters and achieve the softest possible desolvation conditions. The latter can be avoided by using sufficiently dilute samples to ensure the sequestration of individual free/bound components in separate droplets, which is typically achieved in nanospray mode in the low μ M range.^{30,31}

In our laboratory, holo-myoglobin with its non-covalently bound heme is utilized as a tuning standard in positive ion mode, whereas the 1:1 complex between HIV-1 nucleocapsid protein (NC) and SL4 RNA (i.e., NC•SL4) is employed for negative ion analysis.^{20,25} In our hands, these systems also serve to monitor the desolvation conditions afforded by the different

platforms available in the laboratory and to ensure that such conditions are as close as possible to enable correlations between related experimental observations. Over time, their regular utilization highlighted intrinsic differences in the ability to observe non-covalent complexes manifested by instruments equipped with either a Z-spray ion source, such as a Synapt G2 HDMS ion mobility spectrometry mass spectrometer (IMS-MS), or a heated-capillary setup, such as a Velos LTQ-Orbitrap and a solariX 12T Fourier transform ion cyclotron resonance (FTICR) mass spectrometer. Stronger interactions can withstand harsher source conditions and may not be able to properly capture the differences between platforms. Indeed, the discrepancies were significant for relatively weak complexes, such as the NC•SL4 assembly, but much more subtle for the stronger heme•myoglobin complex. Representative data obtained for an NC•SL4 sample on either the Synapt G2 HDMS, or the solariX 12T are shown in Figure 1. The spectrum obtained on the former clearly displayed a greater proportion of free unbound SL4 than the latter. This observation was substantiated by determining the partitioning between free and bound species, which indicated that ~9 and ~72% of the total RNA in each spectrum was respectively detected as part of the associated complex (see *Experimental*). Intrinsic differences in vacuum regimes, ion optics, and other key components make any comparison between different platforms perilous at best. However, these results were obtained by using the softest possible tuning allowed by either instrument, thus making it a fair practical comparison. Any attempt to minimize the discrepancies by adjusting all user-accessible parameters, including increasing the pressure of the source region by adjusting the SpeediValve on the backing pump,^{32,33} failed to produce more closely matching results.

The above data were obtained in MS mode, but the Synapt G2 HDMS is more typically used in ion mobility mode to obtain valuable insights into the structure of biomolecular ions.^{34–36} This type of analysis involves determining the time (t_D) taken by ions to travel across a region that contains a low atmosphere of gas, while driven by a moderate electric field.³⁷ This quantity is influenced by the probability of interactions between ions and background gas, which is a function of the size and global topology of the species of interest.^{38,39} Owing to the fact that the stability of biomolecular structure is greatly influenced by their internal energy, the possible effects of any energy imparted to the system by the desolvation process cannot be ignored. For this reason, we examined any possible avenue to minimize the activation effects observed with our standards. Among them, we explored the possibility of modifying the geometry of the original Z-spray design to make it mimic as close as possible a classic heated-capillary setup.^{40–42} This report describes the hardware modification and illustrates its initial characterization by using selected non-covalent complexes.

Experimental

The heated-capillary apparatus shown in Figure 2 was built in house to replace the standard sampling cone present in the source of a Waters (Manchester, U.K.) Synapt G2 HDMS traveling wave ion mobility spectrometer mass spectrometer (IMS-MS). The heart of the device consists of an HPLC-grade stainless steel tube (capillary, 1/16" OD × 0.20 mm ID × 200.0 mm length) mounted on an aluminum flange adapter via an Upchurch Scientific (Lake forest, IL) 1/16" stainless steel nut (Scheme 1). The flange of the adapter includes a groove to accommodate a 13.2 ± 0.01 mm OD Viton o-ring (2.18 ± 0.01 mm thick) that ensures

vacuum-tight installation on the side of the source housing, in place of the standard sampling-cone element. The heating block was obtained by halving an aluminum rod (19.2 ± 0.01 mm OD $\times$ 188.0 ± 0.01 mm length) along the longitudinal dimension. Appropriate grooves were carved onto each half, in such a way as to create a $\sim 1/16''$ -diameter channel through the center of the block once the two halves are reassembled together, which can appropriately accommodate the $1/16''$ OD stainless steel capillary. The same expedient was followed to carve the space necessary to fit the heating block around the flange adapter (Scheme 1a and b). The block also contains appropriate channels for accommodating both an Omega (Stamford, CT) 35 W, 120 V heating element and a short 100 Ω Class A RTD probe thermocouple. The heater's operations are controlled by a Watlow (St. Louis, MO) feedback module capable of regulating temperature in 1 $^{\circ}$ C steps. The entire assembly weighs approximately 148 g, therefore proper mounting can be conveniently accomplished without resorting to additional braces by using the screws employed for the original cone element.

The performance of the heated-capillary modification was evaluated by analyzing a variety of non-covalent complexes. All analyses were performed by direct infusion ESI in nanoflow mode (nanospray).⁴³ Quartz emitters were produced in house by using a Sutter Instruments Co. (Novato, CA) P2000 laser pipette puller. In standard mode operations, each emitter was mounted on the regular xyz-stage provided by the manufacturer. In heated-capillary mode, a micrometric xyz-stage was placed in front of the entrance of the stainless steel capillary, where it was supported by an appropriate brace attached to the body of the instrument (Figure 2). In either mode, a stainless steel wire was inserted in the back-end of the emitter to supply ionizing emitter voltages that ranged between 0.3 and 0.9, or 0.5 and 0.9 kV in standard or heated-capillary mode, respectively. The fact that the device was fastened in direct contact with the source housing ensured that the entire assembly (i.e., capillary and heating block) was held at the source voltage set by the sampling-cone dial of the instrument's software. Therefore, this setting controlled either the sampling cone or the heated capillary in the respective configuration. In either mode of operation, the range of voltages explored in the study spanned between 40 and 85 V, with actual values indicated in the text, as appropriate. For comparison purposes, nanospray analyses were performed also on a Bruker Daltonics (Billerica, MA) solariX Fourier transform ion cyclotron resonance (FTICR) mass spectrometer equipped with a 12T superconducting magnet. In this case, the emitter voltage ranged between 0.80 and 1.1 kV, whereas the capillary exit (the pre-eminent desolvation parameter in this setup) ranged between -110 and -180 V. In all analyses, no nebulizing or drying gas whatsoever was employed on either instrument. All analysis were accomplished in negative ion mode and the reported results were the average of at least triplicate determinations. In either platform, the softest possible tuning conditions employed for comparison purposes were those that maximized the abundance of the complex of interest, while at the same time minimizing the incidence of typical ammonium adducts detected for nucleic acid analytes.^{44,45}

All nucleic acid samples employed in the study were obtained from IDT (Coralville, IO) and used without further purification (Table 1). Duplex constructs were assembled from cognate strands with complementary sequences by following established heat-annealing procedures. Control experiments meant to verify the stability of duplex structures were carried out on a

CARY300 Scan (Varian-Agilent, Palo Alto, CA) UV-Vis spectrophotometer, which allowed the accurate determination of melting points (T_m) by monitoring strand dissociation as a function of temperature (Figure 3). The range of temperatures covered by these determinations spanned from 30 to 80 °C. Each UV experiment was performed in triplicate and the average absorbance at each temperature was represented in Figure 3. As shown in Table 1, the T_m values afforded by these determinations were within ± 2 °C of those predicted by the Unifold server.⁴⁶

Recombinant nucleocapsid protein (NC) from HIV-1 was produced in house by over-expression in *E. coli* BL21,²⁰ and then desalted by ultrafiltration against 150 mM ammonium acetate (pH adjusted to 7.0) in Microcon devices from Millipore (Billerica, MA). The stemloop 4 (SL4) domain of the HIV-1 packaging signal was also purchased from IDT (Coralville, IO) and employed after ultrafiltration desalting. Aliquots of SL4 and NC solutions were mixed in a 1:1 molar ratio in 150 mM ammonium acetate and incubated at room temperature for at least 15 min. Immediately prior to analysis, each sample was diluted to a final concentration of 1 μ M and then added with a 10% volume of 2-propanol to facilitate stable spray. A typical ~ 5 μ L aliquot was loaded onto each quartz emitter to complete the analysis as described above. The extended duplex (ED_{a,b}) sample was prepared in similar fashion and analyzed at a 5 μ M concentration. For IMS-MS determinations, the signal of most abundant charge state was isolated in the mass-selective quadrupole and then injected in the traveling wave region of the instrument to determine the respective arrival time (t_D).^{35,36} For each experiment the wave height and velocity were maintained at 650 V and 40 m/s, respectively. Sampling-cone and heated-capillary voltages employed in specific experiments are indicated in the text, as appropriate. Data interpretation was carried out by using MassLynx (v4.1, SCN781). In order to accurately identify the apex of each time-domain signal, the observed t_D profile was submitted to Gaussian fitting in PeakFit (v4.2 SeaSolve Software Inc., Framingham, MA) by using the Savistky-Golay smoothing algorithm. The deconvolution parameters were kept constant for all samples to avoid bias.

The percentage of complex association (%A), which refers either to the proportion of bound RNA over the total free and bound detected in NC •SL4 analyses, or to the proportion of duplex DNA over the total free and annealed strand in duplex samples, was monitored as a function of source temperature to evaluate the effect of the desolvation process. This quantity was calculated by using the intensity of each signal detected for all free and bound species, which was divided by the respective charge state.^{20,27} The baseline for these determinations was established by initially equilibrating the source at 30°C (the minimum temperature at which the instrument can exert any control), and then tuning all user-accessible parameters in such a way as to maximize the signal of intact duplex. Actual settings used to obtain the data shown here are reported in the text, as appropriate. The temperature was typically increased in predetermined increments to cover a range spanning the melting temperature observed by the UV melting experiments and corroborated by Unifold predictions. The mass spectra were acquired after 3 min temperature equilibration at each interval. Reported temperatures are uncalibrated readouts provided by the instrument's software and Watlow controller, as appropriate. All the other settings were kept constant across the entire range of temperatures explored in the experiments, with the upper limits representing the settings that induced either full duplex dissociation, or complete signal

deterioration, whichever was reached first. The experiments did not use any nebulizing or curtain gas that may impart additional energy to the system. At the same time, no attempt was made to enact collisional cooling by increasing the pressure of the source or other regions of the instrument. For these reasons, only the applied voltages and temperature settings defined the experimental conditions explored in the study.

Results and Discussion

Apparatus description

A rather simple device was developed with the goal of replacing the standard sampling cone of the Z-spray source with a heated capillary (Figure 2).^{40–42} A flange adapter was designed to fit exactly on the original port utilized by the cone (Scheme 1), in such way as to enable the seamless transmission of any electrosprayed droplets and nascent ions to the first vacuum region of the source. For this reason, the device is mounted directly onto the side of the source housing, where two socket screws are sufficient to sustain its limited weight (Figure 2). The heated capillary consists of a stainless steel HPLC tube (capillary) with a length of 200.0 mm, which matches that of the glass transfer line present in the source of our solariX 12T. The internal diameter of 0.20 mm was selected in empirical fashion by striving to match the normal pressure readings obtained with the standard sampling cone in place (i.e., ~2 torr uncalibrated reading). The heater block comprises two identical halves that can be fitted snugly around the adapter and capillary tube for best possible heat-exchange. The placement of the mount and the length of the apparatus situate the entrance of the capillary off on the side of the instrument, away from the standard emitter stage. For this reason, a small brace was built to enable the proper location of a xyz-stage micromanipulator right in front of the capillary entrance (Figure 2). The fact that the flange adapter of the apparatus is in direct contact with the housing makes it possible to control the capillary voltage by adjusting the software dial that is normally responsible for the sampling cone. In contrast, either a thermocouple-feedback module or the standard instrument setup controls the temperature of the heated capillary or source housing, respectively. In all heated-capillary experiments, the housing temperature was kept constant at the lowest possible setting allowed by the instrument's software, while only the heated-capillary temperature was varied as discussed below. Overall, the described features make the apparatus easy to install and to control.

Evaluation of apparatus performance with non-covalent complexes

Initial experiments aimed at testing the performance of the new apparatus were accomplished on the familiar NC •SL4 standard. The spectrum shown in Figure 4 was obtained by optimizing all user-accessible settings to reach the highest possible percentage of associated complex (i.e., highest %A, see *Experimental*), while minimizing the incidence of typical ammonium adducts. The desired goal was achieved by employing an emitter voltage of 0.7 kV, a heated-capillary voltage of 52 V, and source/capillary temperature of 30°C. Typical indicators of spectral quality, such as signal intensity, signal-to-noise ratio, absence of solvent or ammonium adducts, etc., were comparable to those observed for the same sample with the standard sampling cone. However, this spectrum displayed a %A of ~73%, which was not only much closer to the value obtained for the same sample by the

heated-capillary source of the solarix 12T (i.e., ~72%), but represented also a 64% improvement over that provided by the standard sampling-cone configuration under best possible tuning conditions (i.e., ~9%, Figure 1). This outcome is significant in light of the fact that, except for source geometry and associated settings, the respective data were obtained on the same instrument under identical experimental conditions.

The encouraging outcome observed for the NC •SL4 standard prompted us to perform additional studies to evaluate the performance of the heated-capillary apparatus. Based on their predictable dissociation behavior, DNA duplexes represent ideal non-covalent complexes that can be used to investigate the effects of gas-phase activation.^{47–50} In fact, the stability of duplex structures depends largely on the number of base pairs and percentage of GC versus AT base pairs. For this reason, their association energy and melting temperature (T_m) can be accurately predicted in solutions of any concentration and ionic strength.⁵¹ The irreversible nature of gas-phase dissociation versus the reversible one of solution melting prohibits the direct utilization of T_m to describe their gas-phase energetics. Additional precluding factors are the absence of stabilization by solvent and counter-ions and the fact that dissociation is influenced not only by the thermodynamics of the system, but also by the kinetics of the activation step.⁴⁹ Nevertheless, T_m information can be still effectively used to rank the stabilities of suitable duplexes and guide their selection to properly cover the range of activation conditions accessed by the new apparatus. In our case, the choice fell upon the 14_{a:b} and 24_{a:b} duplexes that shared very similar GC content, but displayed T_m values of 42.6 and 58.0°C, respectively (Table 1). The constructs were employed to perform in-source dissociation experiments in which the source temperature was systematically increased from 30 to 90°C in 5°C increments to induce gas-phase denaturation of duplex structures into individual single-stranded components (see *Experimental*). In each dissociation experiment, all tuning parameters were optimized for maximum yield of intact complex at 30°C, and then kept constant across the entire range of temperatures considered. This allowed us to generate self-consistent dissociation curves by monitoring the proportion of intact duplex (%A) left after each increment, and by plotting the corresponding percentage of dissociation as a function of temperature. These experiments were not performed to obtain a gas-phase T_m , but rather to identify the range of source conditions that preserved the integrity of duplex assembly. In order to enable direct comparisons between the various configurations, these experiments were accomplished by using both the original sampling cone and the new heated capillary on the Synapt G2, as well as the regular heated-capillary source on the solarix. The determinations were performed back-to-back on the same samples, as described earlier for NC •SL4 analysis.

Representative data obtained from a 4 μ M solution of the 14_{a:b} sample in 150 mM ammonium acetate displayed signals corresponding to the multiply charged duplex and its single-stranded components (Figure 5, see *Experimental*). An emitter voltage of 0.5 kV and cone voltage of 40 V were employed for the sampling-cone experiments, whereas 0.59 kV and 60 V were employed respectively for the new heated-capillary ones. A rapid glance immediately revealed that the signals corresponding to the intact duplex were clearly more intense in the spectra afforded by the new apparatus than in those afforded by the standard cone design. At the highest temperature explored (i.e., 90°C), duplex signal could be still

detected in the new apparatus spectrum (Figure 5d), but was conspicuously absent in its sampling-cone equivalent (Figure 5b). The $24_{a:b}$ sample was analyzed in analogous fashion by using emitter and cone voltages of 0.5 kV and 50 V on the standard configuration, emitter and capillary voltages of 0.8 kV and 60 V on the new apparatus, and 0.9 kV and -170 V on the solariX's heated-capillary source. The results showed a combination of free and bound species analogous to those afforded by $14_{a:b}$, but with different partitioning (not shown). Consistent with its larger size, the $24_{a:b}$ duplex provided greater charging than the $14_{a:b}$ counterpart (i.e., 5- thru 9- versus 4- thru 5-, respectively). However, the determination of %A from free/bound species factored in all the respective charge states to enable proper comparisons (see *Experimental*). In analogy with the NC •SL4 experiments, the new apparatus provided results that mimicked very closely those afforded by the heated-capillary source of the solariX (not shown).

The gas-phase dissociation curves in Figure 6 summarize in comprehensive fashion the data obtained from the three different sources. For both $14_{a:b}$ and $24_{a:b}$, the sampling-cone configuration provided full-fledged curves in which the initial duplexes were completely dissociated at the higher end of the temperature range. With this configuration, the source temperatures at which 50% of the initial duplex dissociated were found to be 42 and 55°C for $14_{a:b}$ and $24_{a:b}$, respectively. As expected, these values did not match the corresponding solution T_m , but satisfied the ranking order determined by the respective duplex stability in solution (i.e., $24_{a:b} > 14_{a:b}$). In contrast, the heated-capillary configuration did not produce full-fledged dissociation curves. The more labile $14_{a:b}$ construct provided only a maximum 38% dissociation at the higher end of the temperature range. The more stable $24_{a:b}$ displayed virtually no dissociation across the entire range, in spite of a T_m of 56.5°C observed in solution (Figure 3). The fact that spectral quality did not vary significantly across the range suggests that efficient desolvation was maintained during the entire experiment. Therefore, the absence of dissociation was not likely due to poor energy transfer from the heated capillary, but rather to the considerably gradual nature of the process, which may hamper effective strand dissociation. Surprisingly, the solariX's heated-capillary produced very little variations of dissociation levels for either sample. When comparing data from different platforms, one must consider that the overall results depend not only on initial source effects, but also on additional factors contributed by the remaining elements of the instrument, the discussion of which is beyond the scope of this work. In this context, the fact that the performances of the sampling-cone and heated-capillary configurations were evaluated on the exact same instrument ensured the validity of the comparison. The outcome of this exercise clearly confirmed that the new apparatus is significantly less energetic than the standard cone configuration.

Implications for ion mobility determinations

The MS detection of meaningful non-covalent complexes requires that any component that is bound in solution must remain bound during the course of the analysis, whereas anything that is unbound must remain unbound. When this requirement is met, the results provide an accurate representation of the binding equilibrium in solution. This tenet applies also to intramolecular interactions that define the global topology of biopolymers, which can be studied by IMS-MS analysis.^{23,52–54} Perturbation of such interactions can induce the

transition of compact conformations associated with native structures into extended unfolded counterparts, which can be revealed by significant increases of the observed t_D values.^{55,56} In the case of nucleic acids, structure topology is largely defined by base pairing and stacking interactions, which stabilize not only discrete elements of secondary structure, but also long-range tertiary contacts and quaternary assemblies.^{57–59} For this reason, unintended base-pair dissociation induced by in-source activation could lead to substantial perturbation of the structure under investigation. We explored this possibility by analyzing a duplex construct ($ED_{a:b}$) obtained by annealing mutant replicas of the dimerization initiation site of HIV-1, which is the structural domain responsible for the dimerization and packaging of the viral genome.^{60,61} The complementary strands were mutated from their wildtype form to preclude intramolecular folding into stable hairpin domains that could establish loop-loop intermolecular binding.^{25,62} A standard heat-refolding procedure was employed to assemble the desired duplex construct (see *Experimental*).

Analyzed in MS mode by utilizing either the sampling-cone or heated-capillary configuration, a 5 μ M solution of $ED_{a:b}$ sample in 150 mM ammonium acetate provided nearly identical spectra that displayed signals corresponding exclusively to the intact duplex (not shown). A source/capillary temperature of 35°C was employed in both modes of operation. The emitter/cone voltages used for the sampling-cone experiments were 0.72 kV and 80 V, respectively, whereas emitter/capillary voltages of 0.73 kV and 82 V were used for the new apparatus. In each case, the intense 9- precursor ion was isolated in the mass-selective quadrupole of the Synapt G2, and then injected into the traveling wave to obtain the t_D profiles displayed in Figure 7. The results showed that sampling-cone analysis provided two distinct populations, P1 and P2, with t_D values of 6.17 and 7.39 ms, respectively, which were stable within the experimental timeframe. In contrast, the heated-capillary apparatus provided a unique, well-defined population with a value of t_D matching that of P1. It must be emphasized that the analyses were completed back-to-back on the same sample and on the same platform. Additionally, any effort to optimize the full range of accessible tuning parameters failed to eliminate, or at least minimize, the observed discrepancies.

When the putative conformational landscape of the $ED_{a:b}$ construct was examined by using structure prediction algorithms, such as mFold⁴⁶ and sFold,⁶³ no stable secondary structures were observed outside the expected double-stranded duplex. In addition, earlier IMS-MS studies have shown that duplexes of this size (i.e., 28 bp) adopt a distinctive B-type helix in the gas-phase, with no ambiguities with other possible topologies.⁶⁴ The fact that the t_D exhibited by P2 was greater than that of P1 indicates that the former possesses a more extended conformation, consistent with possible rearrangement or partial unfolding of the latter. The elucidation of the structures of these populations is currently being pursued by using a combination of structure-prediction algorithms and molecular dynamics (MD) simulations. While the results of this study will be discussed in a dedicated report, what is important here is that the detection of the P2 population is unambiguously linked to the utilization of the standard cone configuration. Therefore, in light of the results of duplex dissociation experiments, it is not difficult to attribute its origin to the unintended destabilization of the P1 structure by in-source activation.

Conclusions

The analysis of well-characterized protein-RNA and DNA-DNA complexes has clearly shown that the sampling-cone configuration of the standard Z-spray source can produce unintended in-source activation even when the softest possible tuning parameters are employed. In contrast, the heated-capillary configuration limits in-source activation and minimizes the destabilization of complex association and biomolecular structure.

Replacing an 8.34 mm cone with a 200.0 mm capillary can affect the desolvation process in many different ways. The longer channel afforded by the capillary minimizes the effects of the supersonic jet expansion typical of the Z-spray setup by reducing the pressure gradient between the atmospheric emitter region and the source's first vacuum compartment. The lower gradient decreases the evaporation rate of electrosprayed droplets and decelerates the motion of atmospheric gases, droplets, solvent molecules, and nascent ions toward the lower pressure region, thus decreasing the energy of possible collisions between any of these components. The longer channel afforded by the capillary increases the surface area available for exchanging heat with droplets and nascent ions. In this way, a comparatively lower temperature can be employed to achieve complete solvent evaporation and ammonium-adduct reduction. In addition, the longer channel increases the reaction time of the desolvation processes by extending the residence of droplets and nascent ions in a relatively high-pressure region of the instrument. Therefore, the overall outcome is determined by the combination of lower energy imparted to the system and longer time available for desolvation to take place.

Biomolecular complexes are typically stabilized by numerous weak interactions between components, which are statistically unlikely to dissociate all at once. In fact, it has been demonstrated that duplex dissociation upon collisional activation involves the multistep unzipping of contiguous base pairs.⁴⁹ The process is entropy driven and favored by fast activation regimes. Depending on the number of base pairs, strand dissociation may compete with concomitant covalent fragmentation (i.e., base losses and backbone cleavages), which is enthalpy driven and favored by slow activation regimes.⁴⁹ As a first approximation, the former could be associated with the fast jet expansion of standard cone operations, whereas the latter could be more favored by the longer residence time afforded by the heated-capillary apparatus. However, the absence of any covalent fragmentation in either mode indicates that the stability of the selected duplexes was sufficiently low that their dissociation threshold was consistently lower than the activation barrier of covalent fragmentation. Therefore, the greater power to preserve the integrity of duplex association displayed by the heated capillary is likely a direct consequence of the greater ability to control the energy imparted to the system during the desolvation process.

These observations can have important consequences on the IMS-MS investigation of biomolecular structure on this instrumental platform. Different solutions have been investigated to minimize structural perturbation before ESI-MS analysis, for example, by lowering the surface temperature of the source or other elements,⁶⁵ or by increasing the pressure in such elements to achieve collisional cooling.^{32,33,66} In addition, it has been shown that ions traversing the traveling wave can undergo significant increases of their

effective temperature.^{67,68} The possible implications of this intrinsic phenomenon in the elucidation of biomolecular structure are the objects of intense investigation.^{23,69,70} This report addresses the possible destabilization introduced at the very front-end of the experiment, during the desolvation process. The data shown here suggests extreme caution in interpreting experimental results and emphasize the importance of appropriate controls to evaluate the significance of the contributions of unintended in-source activation.

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Figure 1. ESI-MS data obtained from the NC•SL4 complex by utilizing either a Z-spray **a)**, or a heated-capillary **b)** source. See text for details.

Figure 2.

Pictures of the heated-capillary apparatus. **Panel a)** shows the positioning of the assembly on the side of the standard Z-spray source. **Panel b)** shows the entrance of the capillary in relation to the emitter holder controlled by a micrometric xyz stage.

Figure 3.
UV-melting from the 14_{a:b} **a)** and 24_{a:b} **b)** duplex. Estimated T_m s are indicated.

Figure 4. ESI-MS data obtained from the NC •SL4 complex by utilizing the heated- capillary apparatus built in house.

Figure 5. ESI-MS analysis of 14_{a,b} duplex performed at **a)** 30°C and **b)** 90°C with the original cone configuration and **c)** 30°C and **d)** 90°C with the new heated-capillary apparatus.

Figure 6.

MS dissociation curves obtained for the 14_{a:b} **a)** and 24_{a:b} **b)** duplex by using the three different source configurations employed in the study. The ■ symbol represent the standard cone configuration; ◆ the new apparatus; and ● the heated-capillary source of the solariX (see *Experimental*).

Figure 7.

Traveling wave ion mobility arrival time (t_D) distributions obtained from the 9- charge state of the $ED_{a,b}$ duplex by using the standard cone **a)** or the heated-capillary **b)** configuration. Insets show corresponding Gaussian-fitted traces (see *Experimental*).

Scheme 1.

Details of the heated-capillary modification for the Z-spray source. Perspective views from the **a)** exit and **b)** entrance of the capillary assembly. **c)** CAD drawings with dimensions. The flange adapter is secured with screws onto the source housing in place of the standard sampling cone. Viton o-rings and a standard HPLC-type stainless steel nut ensure vacuum-tight sealing. The nanospray emitter is placed in front of the entrance of the heated capillary by a xyz-stage micromanipulator (not shown).

Nucleic acid constructs employed in the study. Name, sequence, mass, %GC content, and solution T_m obtained by both UV melting determinations and thermodynamic predictions are provided.

Table 1

Strand	Sequence (5' → 3')	Mass (Da)	Duplex %GC	UV-exp. T_m (°C)	Calc. T_m (°C)
14 _a	TAATACGACTTAAC	4230.8	28.6	44.5	42.6
14 _b	GTAAAGTCGTATTA	4292.9			
24 _a	TCACTATATAATACGACTCACTAT	7254.8	29.2	56.5	58.0
24 _b	ATAGTGAGTCGTATTATATAGTGA	7445.9			
ED _a	CUCGGCUUGCUGAAGCAGGCAGUCGUUCAGGGCUC	11501.1	62.9	N/A	64.0
ED _b	GAGCGGAACGGCAAGCCUGCACGGCAAGAGGGCGAG	11187.7			