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Running Title: Effects of Electrospray Droplet Size

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Abstract

Spraying solutions of serine under a wide variety of conditions results in unusually abundant gaseous octamer clusters that exhibit significant homochiral specificity, but the extent to which these clusters exist in solution or are formed by clustering during droplet evaporation has been debated. Electrospray ionization emitters with tip sizes between 210 nm and 9.2 μm were used to constrain the number of serine molecules that droplets initially contain. Protonated octamer was observed for all tip sizes with 10 mM serine solution, but the abundance decreases from 10% of the serine population at the largest tip size to ~5.6% for the two smallest tip sizes. At 100 μM, the population abundance of the protonated serine octamer decreases from 1% to 0.6% from the largest to the smallest tip size, respectively. At 100 μM, fewer than 10% of the initial droplets should contain even a single analyte molecule with 210 nm emitter tips. These results indicate that the majority of protonated octamer observed in mass spectra under previous conditions is formed by clustering inside the electrospray droplet, but ≤ 5.6% and ~0.6% of serine exists as an octamer complex in 10 mM and 100 μM solutions, respectively. These results show that aggregation occurs in large droplets, but this aggregation can be eliminated using emitters with sufficiently small tips. Use of these emitters with small tips is advantageous for clearly distinguishing between species that exist in solution and species formed by clustering inside droplets as solvent evaporation occurs.

Introduction

Since the initial report by Cooks and coworkers in 2000[1] that protonated serine octamer is unusually abundant in electrospray ionization (ESI) mass spectra of serine solutions under a wide range of conditions, many groups have investigated both the origin and potential structures of this unusual species.[1-25] Protonated octamer can be even more abundant with sonic spray ionization (SSI), indicating that the formation of this cluster does not depend on direct electrostatic charging of droplets.[2-5,25] Two distinct forms of the octamer have been identified. The most abundant "A" form exhibits extraordinary preference for forming a homochiral structure, and this preference has led to the proposal that this species may have played an important role in the origin of homochirality in living organisms.[3-14] The relative abundances of the two forms of the octamer depend on desolvation conditions,[5,15,16] and the A isomer is exclusively formed with soft source conditions.[5] The structures of various forms of ionic serine octamer have been investigated using a variety of techniques.[1-25] Recently, Scutelnic et al. reported that the A form of the protonated octamer is composed entirely of zwitterionic serine molecules held together by hydrogen-bond interactions in a spherical, asymmetric geometry.[17] The homochirality of the structure was found to be a result of hydrogen-bonding interactions of six serine molecules.[17] Two of the serine molecules have a free hydroxyl group that enables exchange of these residues, consistent with earlier reports that the protonated octamer could exchange up to two of the component serine molecules.[4,5,7,13,14,18-20] A collision cross-section (CCS) value of 191 ± 2 Å^2 for the octamer from ion mobility measurements[17] matches well with the predicted CCS for the proposed structure (189 ± 1 Å^2) and with previously reported CCS values.[7,11,21] Although the gaseous structure of the protonated serine octamer is now well

characterized, there is ambiguity about the extent to which this cluster exists in solution or is formed by aggregation in evaporating droplets.[2-9,14,15,21-24]

Julian and co-workers reported that protonated serine octamer was the predominant species in ESI mass spectra at both high and low ion transfer capillary temperatures, but abundant high mass clusters, corresponding to sixty serine molecules or more, were also observed under the former condition.[15] This led to the conclusion that the protonated octamer was formed by ion evaporation and that it either exists in solution or is formed at the surface in the charged droplets.[15] Cooks and co-workers reported evidence for a neutral form of the serine octamer in experiments where droplets were nebulized, heated, and ions deflected before remaining neutral molecules/clusters were subsequently ionized by solvent droplets formed by electrospray ionization or by atmospheric pressure chemical ionization where analyte enrichment in droplets should not occur.[9] Protonated serine octamer was the predominant ion in the resulting mass spectra and tandem MS data were consistent with these ions having the same structure as those ions formed directly by ESI. Based on these results, the authors concluded that the neutral octamer is formed during solution nebulization and that the octamer is the only neutral cluster species in the droplet.[9] Remarkably, neutral serine octamer can also be formed by sublimation, indicating that octamers are preferred structures even without solution droplets.[3,8]

Results from NMR and IR spectroscopy of serine solutions by Vandenbussche et al. do not show evidence for serine clusters in solution at the concentrations used in most MS studies.[22] The diffusion coefficients of serine in solution were measured using diffusion ordered spectroscopy NMR at 25 °C and a diffusion coefficient corresponding to that of the serine monomer was reported. Based on these results, the authors reported an upper limit to the existence of clusters in solution of less than 4%. Results from IR spectroscopy of L-serine in

D_2O revealed symmetrical C=O stretching, a bond that is expected to be sensitive to clustering, suggesting the presence of only the serine monomer in solution. This conclusion is consistent with earlier work characterizing serine in D_2O in which all IR bands could be assigned solely to the serine monomer.[26] Additional mass spectrometry studies performed by Vandenbussche et al. on serine solutions in solvent mixtures of different polarities displayed the same clustering pattern, indicating that cluster formation does not depend on solvent conditions.[22] The authors concluded from these combined experiments that serine octamers do not exist in solution and that they are likely formed during the droplet evaporation process.

The number of analyte molecules in an initial droplet depends on the droplet size and the solution concentration. The size of electrospray droplets depends on a number of factors, but droplet size can be controlled by varying the emitter tip diameter or by changing the backing pressure on a nanospray emitter of a given size to control solution flow rates.[27-34] Droplets formed either at small tip sizes or with low or no backing pressure have lifetimes as short as 1 μs.[29] ESI emitters with small tip diameters have been used to reduce adduction of non-volatile salts to biomolecules, such as sodium or potassium chloride, even when the concentration of these salts is ≥ 150 mM.[35-37] This occurs under conditions where there is on average fewer than one analyte molecule per initial electrospray droplet. The desalting effect improves with decreasing tip size so that most droplets contain salt but no analyte molecule. Thus, the analyte molecule is separated from the majority of the salt ions during droplet formation. Under these conditions, non-specific aggregation of analyte molecules that can occur inside of electrospray droplets is also reduced or prevented. For example, both dimers and monomers of β-lactoglobulin are produced by ESI from a 10 μM solution in aqueous 100 mM ammonium acetate.[34] The dimer signal is 35% of the overall protein signal with 4.4 μm emitters, but it is less than 9% with 317

nm tips.[34] These results indicate that the dimers produced with the larger tips are predominantly formed by non-specific protein aggregation during solvent evaporation inside the droplet and that they do not exist in significant abundance in solution at this concentration. This result is consistent with the known dimerization characteristics of this protein wherein the dimer is the predominant species at concentrations >50 μM in physiological conditions.[38] Droplets formed with the smallest nanoelectrospray emitters are sufficiently short lived that they do not enter the mass spectrometer.[34] Under these conditions, the droplet temperature is unaffected by heated interface capillaries that can increase the temperature of larger droplets, so that solution-phase equilibria of non-covalent complexes should not be adversely affected with the smallest tips.[34,39,40]

Here, the average number of serine molecules inside of the initial electrospray droplet is varied by changing the diameter of nanoscale ESI emitters and by changing the concentration of serine in solution. By constraining the number of serine molecules inside of the initial droplets to on average, fewer than one, information about the extent to which the serine octamer exists in solution is deduced.

Methods

Borosilicate capillaries (1.0 mm outer diameter, 0.78 mm inner diameter, Sutter Instruments, Novato, CA) were pulled using a Flaming/Brown P-87 micropipette puller (Sutter Instruments) to produce nanoelectrospray emitters with five different tip sizes with inner diameters between 210 nm and 9.2 μm. Tip diameters were measured using a Hitachi TM-1000 scanning electron microscope (Schaumburg, IL) in the Electron Microscope Laboratory at the University of California at Berkeley for each set of puller parameters. Four tips were pulled for

each set of pulling parameters to measure the standard deviation of the resulting diameters. The five tip sizes used in this experiment had inner diameters of 210 ± 11 nm, 377 ± 17 nm, 1.2 μm ± 0.1 μm, 3.6 μm ± 0.1 μm, and 9.2 μm ± 0.3 μm.

Solutions of 10 mM or 100 μM L-serine (Sigma-Aldrich, St. Louis, MI) in 49.95:49.95:0.1 methanol:water:acetic acid (Sigma-Aldrich, St. Louis, MI) were electrosprayed and signal was monitored between *m*/*z* 50 – 1500 using a Finnigan LTQ mass spectrometer (Thermo Fisher Scientific, San Jose, CA). Data was acquired in the linear ion trap. Instrument parameters were optimized for the protonated octamer signal using the automated tuning feature of the control software while spraying a 10 mM solution from a 1.2 μm diameter emitter tip. The same instrument parameters were then used for all subsequent experiments. The transfer capillary voltage, tube lens voltage, and transfer capillary temperature were set to 23 V, 135 V, and 100 °C respectively. Automatic gain control was turned off on the instrument and the injection time was maintained at a constant value of 2.5 ms that was optimized to prevent space-charge distortion of the mass spectra.

Nanoelectrospray was initiated by applying a voltage of approximately 0.4 – 1.4 kV to a 0.127 mm diameter platinum wire that is inserted inside of the capillary and that is in contact with the solution. The electrospray voltage was increased until the spray was stable and then allowed to equilibrate for one minute before data acquisition. Stable sprays are obtained at lower voltage for emitters with smaller tips. Five replicate measurements using different emitters for each tip diameter were acquired by averaging for one minute at a scan rate of 8.33 Hz.

The abundance of each ion that is >0.1% of the protonated monomer is reported relative to the abundance of the protonated monomer, which is the most abundant ion in each spectrum. Variance from the mean is reported as the standard error of five replicates for each tip size at

each concentration. Overlapping clusters with different charge states were identified using high-resolution data obtained with an FT-ICR mass spectrometer (Thermo Fisher Scientific, San Jose, CA) for each tip size. Automatic gain control was used to obtain sufficient signal to isotopically resolve any overlapping contributions at each cluster *m*/*z*. Some replicate data were acquired on a Velos Pro mass spectrometer (Thermo Fisher Scientific, San Jose, CA) and a Q-TOF Premier quadrupole time-of-flight mass spectrometer (Waters Corporation, Milford, MA) using conditions reported in Supporting Information.

Results and Discussion

Effects of Emitter Tip Size at 10 mM. Mass spectra were obtained by electrospray ionization of a 10 mM solution of L-serine using emitters with tips sizes of 9.2 μm, 3.6 μm, 1.2 μm, 0.38 μm and 0.21 μm. A 10 mM concentration was chosen because it has been commonly used in prior studies.[1-3,5-10,15,17,21-25] The relative abundances of the most abundant cluster ions formed by ESI as a function of tip size is shown in Figure 1a. The identity of the peak at *m*/*z* = 841 was confirmed by high-resolution measurements with an FT-ICR instrument to be predominantly protonated serine octamer (>95%) at all tip sizes. The abundances of doubly protonated 16-mer and triply protonated 24-mer metaclusters were an average of 2.5 ± 0.4% and 0.6 ± 0.1%, respectively. The abundance of protonated octamer obtained from the lower resolution data was corrected for the presence of these metaclusters.

The protonated octamer is the most abundant cluster at all tip sizes, consistent with results from previous ESI studies of serine solutions at this concentration. At larger tip sizes, the relative abundances of the protonated octamer and other clusters are significantly higher than at

smaller tip sizes. The relative abundance of the protonated octamer decreases from an average of 23.6 ± 1.7% for the two largest emitter tips to 9.1 ± 1.6% for the two smallest tip sizes (Figure 2, top). These data indicate that the majority (≥ 61%) of the protonated octamer that is observed at the larger tip sizes is formed by aggregation of serine molecules inside of the electrospray droplet as a result of solvent evaporation. To the extent that no aggregation inside of the droplet occurs in the two smallest tip sizes, these results suggest that ≤ 5.6% of the serine population exists in the octameric form in 10 mM solution.

A similar decrease in abundance with decreasing emitter tip size occurs for the larger clusters. For example, the abundance of the 26^{3+} cluster decreases from an average of 3.4 ± 0.1% at the largest two tip sizes to 1.6 ± 0.2% at the smallest two tip sizes (Figure 1b). The two-fold lower abundance at small tip size indicates that a substantial population of the larger clusters are also formed by aggregation within the bigger droplets formed by emitters with larger tips.

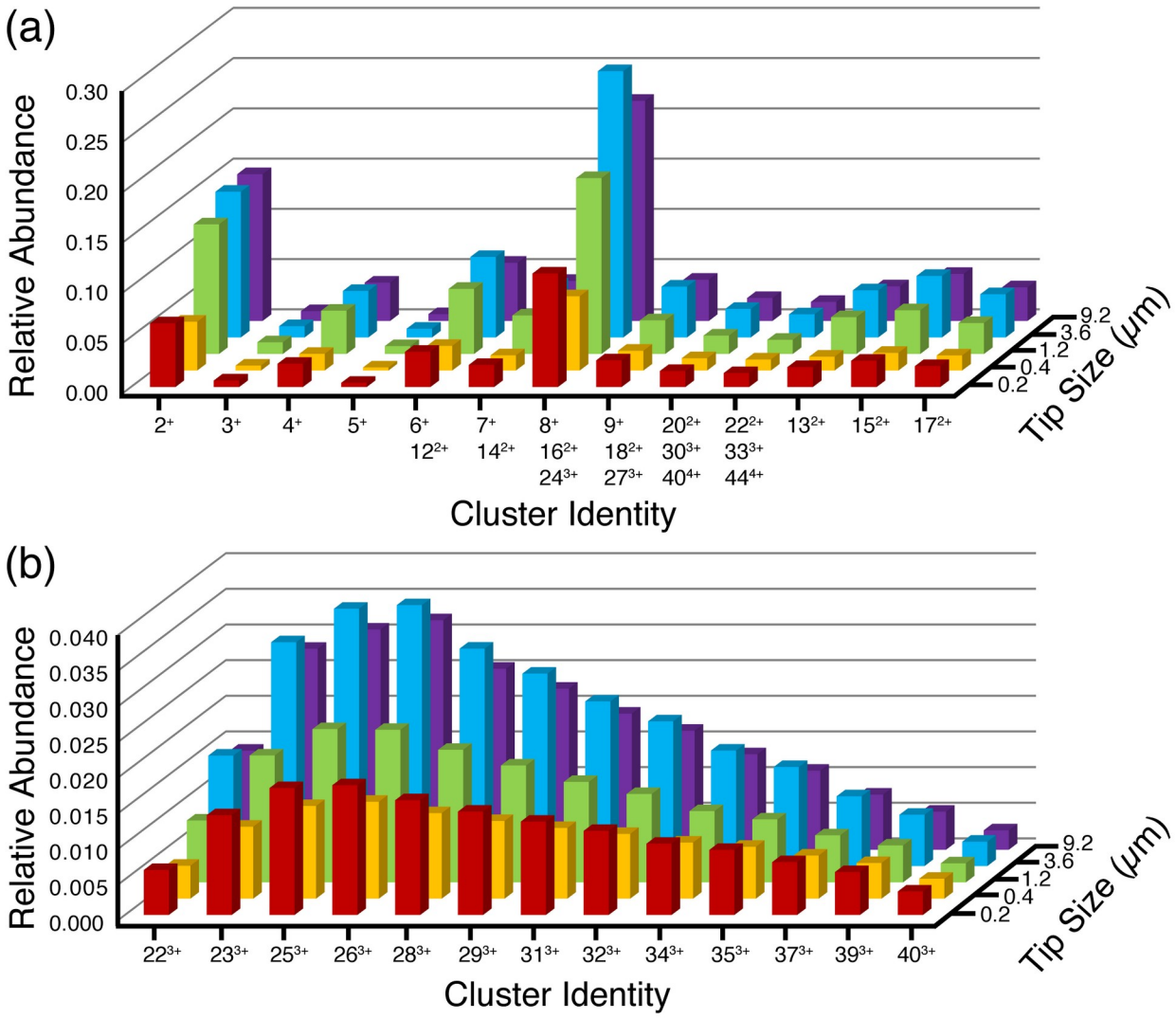

Figure 1. Relative abundances of protonated serine clusters formed by electrospray ionization of a 10 mM L-serine solution as a function of electrospray emitter tip diameter. Clusters shown are (a) the most abundant singly and doubly charged clusters or (b) the most abundant unambiguously identifiable triply charged clusters. A comprehensive list of the cluster assignments is given in Table S1.

Droplet Size and Initial Number of Serine Molecules. The initial size of droplets produced from nano-ESI emitters depends on the diameter of the emitter tip.[27-34] The number of

analyte molecules that are contained in each of the initially formed droplets also depends on droplet size and concentration. Davidson et al. reported that the initial droplets produced with 1 – 3 μm emitter tips were, on average, 60 nm in diameter or ~1/17th the emitter tip diameter.[27] They also showed that the percentage of droplets that contain a single analyte molecule increases from 24% to 98% when the solution concentration is reduced from 40 μM to 0.4 μM at this same tip size.[27] A similar estimate of initial droplet diameters produced by micron size and smaller emitters was inferred from the effectiveness of desalting protein ions to be between 1/14th and 1/20th the size of the emitter tip diameter.[35]

A rough estimate of the number of analyte molecules that are contained in the initial droplets produced by electrospray ionization at each tip size was obtained using an average droplet size of 1/17th the tip diameter.[27,35] This estimated average number of analyte molecules per droplet is determined using equation 1:

$$\#\ of\ molecules = \left(\frac{D_E \times \left(\frac{1}{17} \right)}{2} \right)^3 \times \frac{4}{3}\pi \times 10^{-24} \times M \times N_A \qquad (1)$$

where D_E is the emitter tip diameter in nm, 10^{-24} is a unit conversion from nm^3 to L, M is the concentration of the solution in M, and N_A is Avogadro's number. The average number of molecules in droplets produced from various tip sizes at a solution concentration of 10 mM and at 100 μM are given in Table 1. At 10 mM, even an emitter tip size of 210 nm will produce droplets that contain an average of approximately six analyte molecules. Because of the variability of droplet sizes that are formed, it is not possible to rule out aggregation inside these droplets as a potential source of protonated octamer in ESI mass spectra. For this reason, these experiments were repeated with a 100-fold less concentrated serine solution. At this same 210

nm tip size, only one in about 17 droplets should contain an analyte species from solution. Even with a wide distribution of droplet sizes, these conditions should significantly reduce any potential for aggregation inside an electrospray droplet. Any clusters observed under these conditions would provide strong evidence that the cluster exists in solution prior to droplet formation.

Table 1. Approximate Average Number of Molecules in Each Initial Droplet Produced by NanoESI.

Tip Size	10 mM	100 µM
210 nm	6	0.06
377 nm	34	0.34
1.2 µm	1,109	11
3.6 µm	29,943	299
9.2 µm	499,754	4,998

Effects of Emitter Tip Size at 100 µM. In order to ensure conditions where there are substantially fewer than one analyte molecule per initial droplet at the smallest tip size, electrospray mass spectra were acquired from 100 µM solutions at each emitter tip size. Under these conditions, clusters up to a triply protonated 26-mer were observed at >0.1% relative abundance with the largest tip size (Figure 3). Protonated dimer is the most abundant cluster and the abundance decreased from 6.1 ± 0.9% to 2.7 ± 0.6% at the largest and smallest tip sizes, respectively.

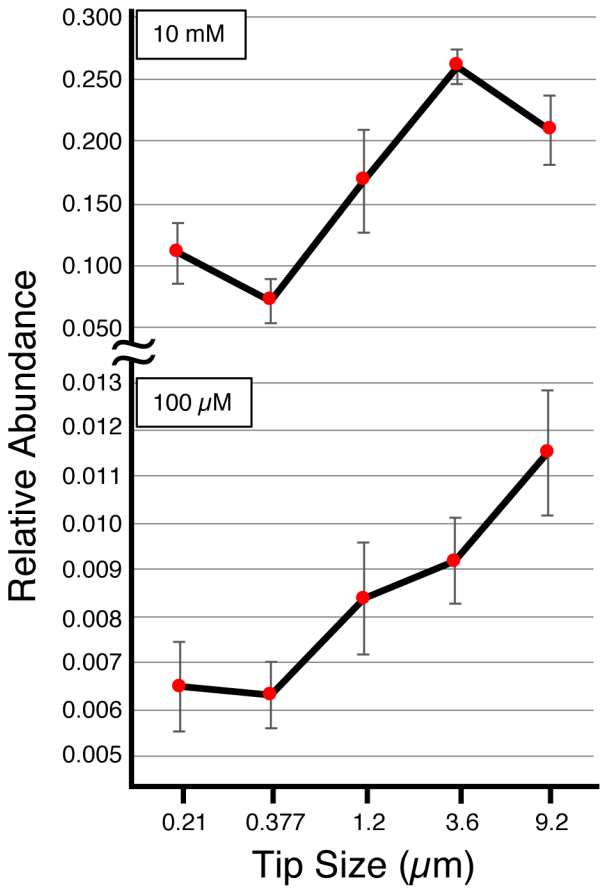

Figure 2. Relative abundance of protonated octamer formed by electrospray ionization of a L-serine solution at 10 mM (top) and at 100 μM (bottom) concentration as a function of electrospray emitter tip diameter.

The relative abundance of the protonated octamer decreases with decreasing tip size from 1.2 ± 0.1% to 0.7 ± 0.1% (Figure 2, bottom). As was the case at 10 mM, the peak at m/z = 841 consists of >95% protonated octamer with 16-mer and 24-mer at an average of 4.4 ± 1.4 % and 0.5 ± 0.1%, respectively. There is no significant difference in protonated octamer abundance at the smallest two tip sizes for which each droplet should contain either one or zero analyte molecules on average. These results indicate that the protonated octamer is only 0.6 ± 0.1% of

the total serine population in 100 μM solution. This value is much lower than the upper limit of 5.6 ± 1% determined for a 10 mM solution. At 10 mM, there are on average six serine molecules per droplet at the smallest tip size, so aggregation inside the evaporating droplets may contribute to the protonated octamer signal. Thus, the 5.6% value derived from the 10 mM data is an upper limit to the true solution abundance of the octamer. The significantly lower value of solution-phase octamer obtained for the 100 μM experiments, however, can also be explained by a shift in equilibrium towards the monomer at the significantly lower concentration.

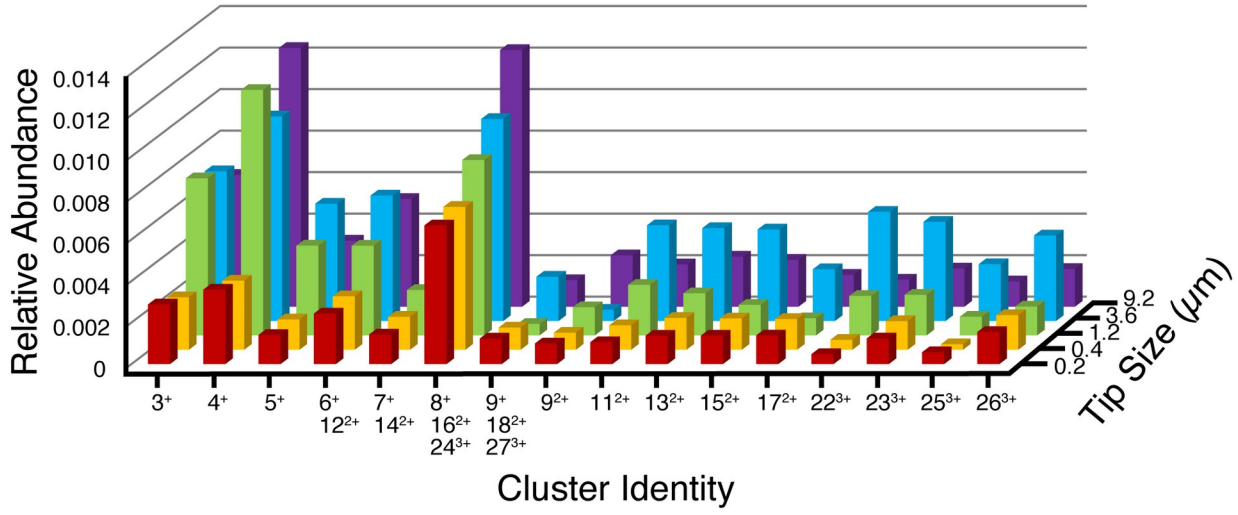

Figure 3. Relative abundances of protonated serine clusters larger than the dimer formed by electrospray ionization of a 100 μM L-serine solution as a function of electrospray emitter tip diameter.

The abundances of clusters larger than the octamer are all less than half that of the octamer. The abundance of these clusters is lowest for the smallest two tip sizes (377 nm and 210 nm) which produce clusters with similar abundances. These tip sizes correspond to conditions

where each droplet is expected to contain one or zero analyte molecules on average. The similar abundance of clusters at these two tip sizes indicates that these molecular assemblies entered the droplet from solution, indicating that a number of these oligomers exist in solution at low abundance. Assuming that the abundances of the clusters obtained from the 210 nm emitter tips reflect their corresponding abundances in solution, then clusters larger than the dimer, excluding the octamer, each compose <0.35% of the total serine population and, combined, represent only 2.2% of the population.

To determine the extent to which these results are instrument dependent, additional data were acquired using a Thermo Fisher Velos Pro mass spectrometer and a Waters Q-TOF Premier quadrupole time-of-flight mass spectrometer. The population abundances of the protonated octamer determined with the Q-TOF instrument at the smallest tip size are 5.8 ± 0.2% and 2.8 ± 0.3% at 10 mM and 100 μM, respectively. In these experiments, clusters as large as 57^{4+} were observed. Values of 5.6 ± 0.1% and 0.8 ± 0.2% were obtained using a Velos Pro mass spectrometer. The similar results with three different instruments indicate that, although there is some instrument dependent variability, serine octamer exists in solution at low abundance.

Protonated octamer can also be formed by gas-phase dissociation of larger clusters[15] and interface conditions can affect the distributions of a variety of different clusters.[41-43] In order to determine the extent to which source conditions affect the population abundance of the protonated octamer in these experiments, the transfer capillary temperature, transfer capillary voltage and the tube lens voltage were varied and ions were formed from a 100 μM serine solution with a 210 nm emitter. The transfer capillary temperature was varied between 60 °C and 220 °C. There were no significant trends in the abundances of the protonated octamer or higher order clusters over this temperature range (Figure S1, supporting information). The

transfer capillary temperature used in these experiments (100 °C) is at the lower end of this temperature range indicating that gas-phase dissociation of higher order clusters that may be induced by higher transfer capillary temperatures does not contribute substantially to the protonated octamer abundance under these conditions.

The extent to which the potential applied to the transfer capillary induces dissociation was investigated over a range between 5 V and 120 V. The population abundance of the protonated octamer at 5 V and 23 V (the potential used in these experiments) is similar, but decreases substantially at the higher voltages, consistent with collisional dissociation of this and other higher order clusters (Figure S2a, supporting information). The population abundance of the protonated octamer varies with tube lens voltage with a minimum value of 0.4% around 90 V (Figure S2b, supporting information). The origin of the higher abundance at higher and lower tube lens voltages is unclear, but may be related to relative ion transmission efficiencies of clusters at different *m/z,* which would affect the calculated ion population abundances. In sum, these data indicate that the protonated octamer observed with the 210 nm tip size under the operating conditions of these experiments, is not a result of in-source activation and dissociation of larger clusters, consistent with these ions originating from solution.

Serine Octamer in Solution. The observation of protonated octamer at the 100 μM concentration with the smallest tip size, where only one in roughly 17 droplets initially formed by electrospray ionization contain a single analyte molecule, provides compelling evidence for the existence of the octamer in solution at ~0.6% of the serine population. Even at this low concentration, the octamer is a magic number cluster, which is indicative of its special stability

in solution. It is interesting to speculate about differences in results and conclusions from prior experiments. There was no signal detected for any clusters in the NMR study by Vandenbussche et al., but the authors report a detection limit of 4% relative abundance if the octamer is the only cluster present in solution.[22] The value of ≤ 5.6 ± 1% serine octamer abundance in a 10 mM solution is consistent with the NMR results. We cannot rule out that some aggregation occurs inside the droplets at this concentration even with the smallest tip size. There are also effects of ion transmission and detection efficiency that are not taken into account in our study.

Data from many previous mass spectrometry studies show the protonated octamer is the most abundant form of serine in the spectra, yet our results show an upper limit of ~5.6% of serine octamer in solution at similar concentrations. Previous studies have typically been done using much larger emitters compared to those used here. These include the commercial Thermo Finnigan/Thermo Fisher electrospray source (76 or 102 μm in diameter depending on needle gauge)[6,7,15,44], homebuilt SSI sources (100 μm in diameter)[2-4,14,25] and homebuilt nebulization sources (200 μm in diameter)[9]. A notable exception to extensive formation of protonated octamer are results from Myung et al., which showed that clusters containing over 600 serine molecules can be generated using sonic spray with large diameter capillaries, high flow rates, and soft source conditions.[25] Under these conditions, protonated octamer comprises only ~0.3% of the total ion distribution. Based on our results where aggregation is observed to form the octamer with our largest tip, which is less than 10 μm, it is expected that the even larger emitters used in many prior studies will produce much larger droplets with a corresponding significantly larger number of serine molecules in each droplet. Formation of even larger clusters and subsequent dissociation of these larger clusters may also contribute to the abundant protonated octamer signal observed in prior studies.[15]

Conclusions

Under readily achievable conditions, the number of analyte molecules that are inside of an initially produced electrospray droplet can be reduced below an average of a single molecule per droplet. This can be achieved by lowering analyte concentration or by decreasing the size of the electrospray emitter tip diameter. With a 210 nm emitter tip diameter and 100 μM serine solution, on average, only one droplet in 17 contains an analyte molecule from solution. Under these conditions, protonated octamer is a magic number cluster and is about 0.6% of the serine population. Although the abundance is low, these results provide compelling evidence that the serine octamer is a special cluster that exists in solution even at this low concentration. The upper limit of 5.6% protonated serine octamer population obtained from a 10 mM solution is consistent with results from a prior NMR solution study that reported a population that must be below the detection limit of ~4%. These results indicate that aggregation of analyte molecules inside of evaporating electrospray droplets can lead to a significant abundance of clusters in electrospray mass spectra that are not indicative of the concentrations of these species in the original solution. By reducing the emitter tip size, aggregation can be significantly reduced and even eliminated, making it possible to establish the concentration of noncovalent complexes that exist in solution and that are not formed as an artifact of the electrospray ionization process. These results should be equally applicable to studies of protein-substrate and higher order macromolecular interactions where careful control studies of different analytes are typically required to provide evidence for the extent of specificity that may exist between binding partners in solution.

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Supporting Information

Cluster assignments for each peak in the mass spectra. Derivation of equation one. LTQ transfer capillary temperature results. In-source activation of larger clusters at various electrospray source conditions. Waters Q-TOF Premier Mass Spectrometer Instrument Parameters. Thermo Fisher Velos Pro Mass Spectrometer Instrument Parameters.

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