

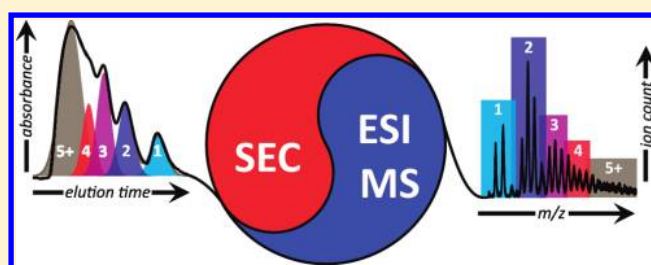
Evaluation of Electrospray Ionization Mass Spectrometry as a Tool for Characterization of Small Soluble Protein Aggregates

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Supporting Information

ABSTRACT: Protein aggregation continues to attract significant interest in many areas of biology and medicine not only due to its pivotal role in the etiology of conformational diseases (such as Parkinson's and Alzheimer's) but also due to its importance in the biopharmaceutical sector, where aggregation of protein therapeutics exerts a deleterious effect on their efficacy and safety. Despite the tremendous success of electrospray ionization mass spectrometry (ESI MS) in a large number of studies of noncovalent protein interactions, application of this technique to study aggregation processes has been very limited so far, and lower resolution techniques, such as size exclusion chromatography (SEC) and analytical ultracentrifugation, remain the default tools in characterizing small soluble protein aggregates. In this work we used heat-stressed human antithrombin III (AT), a 58 kDa glycoprotein, to compare SEC and ESI MS as a means to probe composition of the complex mixture of soluble oligomeric species generated by heat-induced aggregation. SEC allows several oligomeric species to be observed and collected, followed by their identification with ESI MS. The same oligomeric species can be also directly observed in the ESI MS of the unfractionated sample of the heat-stressed AT. The abundance distribution of these small soluble aggregates in ESI MS and SEC cannot be compared directly, since the ESI signal is linked to the molar concentration of the analyte in solution, whereas the UV absorption detection in SEC reports weight concentration. However, once the appropriate corrections are made, the abundance of the small aggregates derived from ESI MS becomes remarkably close to that calculated based on SEC data, suggesting that ESI MS may be directly applied for semiquantitative characterization of soluble protein aggregates.



Formation of protein aggregates is a phenomenon that is frequently encountered in many fields in biomedical research. Aggregation impairs protein activity both directly and indirectly (e.g., by reducing solubility) and is a particularly serious concern in the biopharmaceutical industry, where its occurrence in protein drugs not only affects the economics of the production process¹ but also inevitably raises safety concerns.² Furthermore, over 20 so-called conformational diseases have been identified, where the pathology is linked to either intracellular or extracellular protein aggregation.³ Therefore, understanding the molecular mechanisms of protein aggregation in a variety of settings would greatly catalyze progress in multiple fields in medicine and biotechnology. However, this ambitious goal will remain elusive without the means to detect and monitor the aggregation processes with high resolution. This task remains extremely challenging due to the high degree of complexity and heterogeneity displayed by aggregating proteins, and development of experimental tools and strategies to meet this challenge remains a high priority.

Currently, analytical ultracentrifugation (AUC), light scattering techniques, field flow fractionation (FFF), and size exclusion chromatography (SEC) remain the most commonly used techniques for probing soluble protein aggregates,^{4–6} although their resolution is rarely sufficient to provide adequate

characterization of various species formed during protein aggregation.^{6,7} On the other hand, electrospray ionization mass spectrometry (ESI MS) has been used very successfully in the past decade as a means to characterize noncovalent complexes whose molecular weights extend well beyond 1 MDa with high precision and accuracy.⁸ More recently, the scope of applications of ESI MS in this field has been expanded to include characterization of protein oligomers that are formed during the early stages of aggregation. For example, Kükrer et al. used ESI MS to analyze SEC fractions of acid-stressed monoclonal antibodies and identified a few smaller oligomers based on the molecular weight data provided by ESI MS.⁹ Furthermore, ESI MS has shown a promise of becoming a useful online detection tool for SEC,^{10–14} although it has never been applied in such capacity for characterization of protein aggregation products. An orthogonal approach was recently demonstrated by our laboratory, where ESI MS was applied directly to monitor behavior of proteins subjected to heat stress in solution and ensuing aggregation.¹⁵ While the obvious

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advantage of this approach is its ability to provide a snapshot of the entire population of protein species (based on mass measurements) and their conformational states (based on charge state distributions), there is always a concern that such measurements will discriminate against the higher molecular weight species, thereby introducing a bias against larger oligomeric species.¹²

Monitoring protein aggregation directly with ESI MS^{9,15–17} allows the evolution of small soluble oligomers to be observed and characterized in real time; however, the uncertainty regarding the relationship between the fractional concentrations of oligomeric species in solution and the relative abundance of ionic signal in ESI prevents broader utilization of this approach. Dependence of the ionic signal on the analyte concentration in ESI is not straightforward and is known to be attenuated by a variety of factors.¹⁸ Even protein conformation may play a role under certain circumstances,¹⁹ and it is commonly expected that the physical size of the oligomeric species would also exert a strong influence on the signal intensity of the corresponding ionic species in ESI, making MS-based characterization of the aggregation process suspect. Although the mass-dependent attenuation of ESI signal had been examined in the past for relatively monodisperse mixtures of macromolecules (synthetic polymers²⁰), no systematic studies have been carried out with highly polydisperse systems, such as soluble protein aggregates. Another complication arises due to the intrinsic heterogeneity of many proteins (particularly, biopharmaceutical products) due to extensive glycosylation, etc. The presence of multiple oligomers of such proteins in solution usually results in very crowded mass spectra with significant signal overlap, and additional tools are required in these circumstances in order to deduce meaning information from the ESI MS data.²¹

The purpose of this work is to evaluate the suitability of ESI MS for direct profiling of soluble glycoprotein aggregates by comparing the abundance distribution of oligomeric species derived from ESI MS to that obtained with SEC. This evaluation is carried out using antithrombin III (AT), a 58 kDa plasma glycoprotein, which plays an essential role in the blood coagulation cascade.²² Both plasma-derived²³ and recombinant form of human AT²⁴ are currently used to rebalance hemostasis in a number of life-threatening conditions. AT forms aggregates under a variety of stress conditions, such as long-term refrigerated storage, elevated temperature, and elevation of expression level.^{17,25} Analysis of heat-induced AT aggregation carried out in our lab using temperature-controlled ESI MS demonstrated that the protein oligomers undergo progressive growth in terms of both abundance and size as a function of temperature and/or duration of heat exposure,¹⁷ making heat-stressed AT a convenient model system for protein aggregation studies. The primary analysis of AT aggregates is carried out by SEC, where fraction collection followed by ESI MS analysis allows small soluble oligomers to be identified up to the tetramer level. Quantitation of these species based on the UV absorption in SEC produced the abundance distribution that was remarkably similar to that derived from the direct ESI MS measurements, suggesting that the latter is capable of probing the early stages of protein aggregation in a semiquantitative fashion.

MATERIALS AND METHODS

Recombinant human antithrombin III was provided by GTC Biotherapeutics, Inc. (Framingham, MA). All other chemicals

were of analytical grade or higher. Protein solutions for heat-stress treatment were prepared in 150 mM ammonium acetate (pH adjusted to 8.0 with NH₄OH) to a concentration of 30 μ M and were incubated at 70 °C for 1 h. All samples were analyzed at room temperature within 12 h following the completion of heat stress. Samples for ESI MS analysis were diluted to the requisite concentration after incubation.

An Agilent 1100 series liquid chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a TSK Gel 3000 SWXL column (TOSOH Bioscience, King of Prussia, PA) was used for SEC analysis. The separation was performed with 150 mM ammonium acetate (pH 6.8) at a flow rate of 0.5 mL/min and was monitored with 280 nm UV detection. The injected sample volume was 100 μ L for analysis and 250 μ L for fraction collection.

ESI MS experiments were carried out with a QStar-XL hybrid quadrupole/TOF mass spectrometer (ABI/Sciex, Toronto, Canada) retrofitted with a home-built ESI source, where a spray emitter with a smaller diameter (20 μ m) than a normal ESI source was utilized to improve the desolvation of analyte. The flow rate of the sample solution was kept at 0.8 μ L/min during the continuous injection. The position of the ESI emitter and spray voltage were carefully adjusted to optimize the spray efficiency, which directly affected the resolution of the mass spectra. In order to eliminate the bias induced by the instrumental parameters, for semiquantitative analysis the mass spectra were acquired at relatively low, medium, and high declustering potentials, respectively, and the calculated results were averaged. In order to measure masses of proteins represented by highly heterogeneous ion populations in ESI MS, relatively monodisperse ionic populations were mass-selected and subjected to electron capture-induced charge reduction using a 7.0 T SolariX (Bruker Daltonics, Billerica, MA) Fourier transform ion cyclotron resonance (FTICR) mass spectrometer. Because of the high m/z values of AT oligomeric ions, the charge reduction procedure was triggered by electron capture in the ICR cell of the mass spectrometer, rather than electron-transfer reactions in the front-end quadrupole, as described previously.²¹ Postacquisition analysis of both SEC and MS data was carried out using Origin 8.0 (OriginLab Corporation, Northampton, MA) software.

RESULTS AND DISCUSSION

Profiling Small Soluble Aggregates of Heat-Stressed AT with SEC. Unstressed AT exhibits no pronounced tendency to oligomerize under near-physiological conditions, as suggested by both MS and SEC measurements. The SEC profile of AT is consistent with the protein existing mostly in the monomeric form (peak at 17.5 min in Figure 1a), with higher molecular weight species contributing less than 2% of the total protein signal (eluting between 11 and 16 min in the chromatogram shown in Figure 1a). Likewise, the ESI mass spectrum of intact AT consists of a narrow distribution of ions with mass 57.3 ± 0.1 Da (data not shown). Incubation of AT at 70 °C for 1 h results in a dramatic change in the appearance of the SEC chromatogram (Figure 1b), with the monomeric species (represented by a distinct peak at 17.5 min) becoming a minor fraction of the entire protein population. The rest of the species elute within a 11.5–16.5 min time window, and even though the presence of several peaks is evident based on the convoluted shape of the chromatogram (black trace in Figure 1b), their contributions are not completely resolved. This partially resolved chromatogram was processed under the

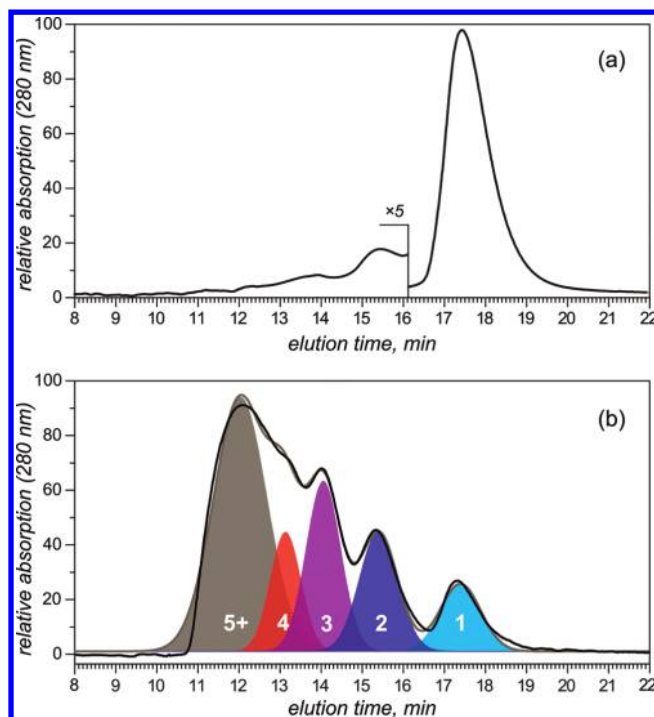


Figure 1. Size exclusion chromatogram of intact (a) and heat-stressed AT (b) in 150 mM ammonium acetate, pH 8.0. The results of deconvolution of the SEC profile of the stressed protein are illustrated with the five colored Gaussian curves representing distinct contributions of various putative protein species formed during protein aggregation; the sum of these individual contributions is shown with a gray line.

assumption that an individual contribution of each species can be represented with a Gaussian curve. The number of such curves that would be needed to fit the experimental SEC profile was determined to be five, based on the number of extrema and inflection points on this curve.

The results of such curve-fitting are shown in Figure 1b with colored and numbered bell curves. While the first four curves are numbered numerically, the one corresponding to the earliest elution time is labeled as “5+” to reflect the fact that it is too close to the void volume of the column and is, therefore, likely to contain contribution from several unretained species. Identification of the protein species corresponding to the individual components of the total chromatogram was carried out with ESI MS using an approach similar to that employed recently by Kükrer et al. in the studies of acid-induced aggregation of monoclonal antibodies.⁹ Specifically, fractions were collected at times corresponding to the relative retention time of each individual contributor (ranging from 0 min for species “5+” to 6.5 min for species “1”) in the SEC run shown in Figure 1b, but the sample injection volume was increased from 100 to 250 μ L, and a guard column was employed to prevent introduction of insoluble protein aggregates to the analytical column.

ESI MS analysis of these five fractions (Figure 2) yields mass spectra with resolution ranging from good (species 1 and 2) to adequate (species 3), to poor (species 4), to completely unresolved (species 5+). Although the mass spectra obtained for fractions 1 and 2 have adequately resolved peaks corresponding to different charge states of the respective species, charge state assignment and mass calculation based on these data alone are not straightforward. In fact, the application

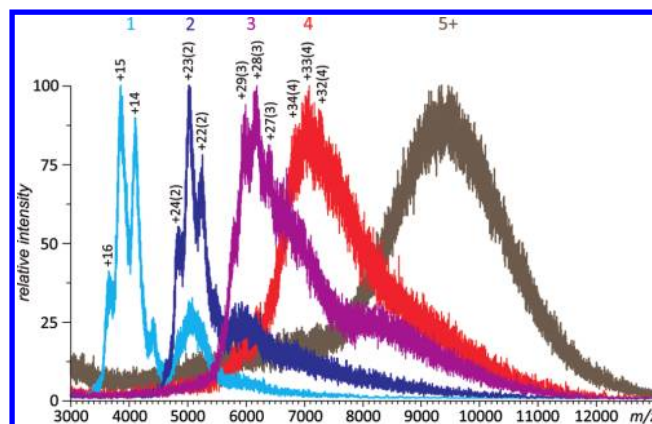


Figure 2. ESI mass spectra of SEC fractions representing the putative individual species of heat-stressed AT indicated in Figure 1b with Gaussian curves. Charge state assignments and oligomer size (in parentheses) are shown for each fraction with the exception of 5+. See the text for more detailed information on charge state assignment.

of the statistics-based charge-assigning approach pioneered by Tito et al.²⁶ to deconvolute the ESI mass spectrum of fraction 1 (whose SEC retention time corresponds to AT monomer) yields 65.6 kDa as the “most probable” mass (see the gray curve in Figure 3d). Recently we have shown that the misassignment of charges using this approach is common in heterogeneous systems,²¹ such as extensively glycosylated proteins, due to the dependence of the ionic charge on the physical size of biopolymers.^{27,28} Glycoforms with higher carbohydrate content have larger surface area and, therefore, will accumulate more charges upon transition from solution to the gas phase during the ESI process. As a result, they will be overrepresented at the lower m/z end of the charge state distribution envelope, while the glycoforms with lower carbohydrate content will be overrepresented at the opposite end of the envelope.

To circumvent this problem, we have recently introduced a technique which reduces the heterogeneity of the protein sample by mass-selecting a relatively narrow slice of the entire ionic population in MS, followed by its partial reduction using electron-transfer reactions in the gas phase. The resulting ladder of the charge states can be used for confident charge assignment and protein mass calculation.²¹ A similar approach was employed in this work to obtain charge state assignment for protein ions in ESI mass spectra of fractions 1–3. Since the m/z values of ionic species fall outside of the region where mass selection can be achieved using a front-end quadrupole filter in FTICR MS, isolation of relatively homogeneous ionic populations was carried out in the ICR cell by selecting an appropriate waveform, and the partial charge reduction was triggered by electron capture, rather than electron transfer (the latter can be done only outside of the ICR cell). The resulting mass spectrum for SEC fraction 1 shows a ladder of well-resolved peaks representing the mass-selected “parent” population (at m/z 3820) and the products of partial charge reduction at m/z 4100, 4410, and 4780. Processing this ladder using the statistics-based charge-assigning routine²⁸ yields 57.3 kDa as the protein species mass, a value which is within 0.1 kDa of the mass of AT monomer. The same approach was used to determine the masses of protein species 2 (114.4 kDa) and 3 (172.0 kDa), which were assigned as AT dimers and trimers, respectively (Figure 3).

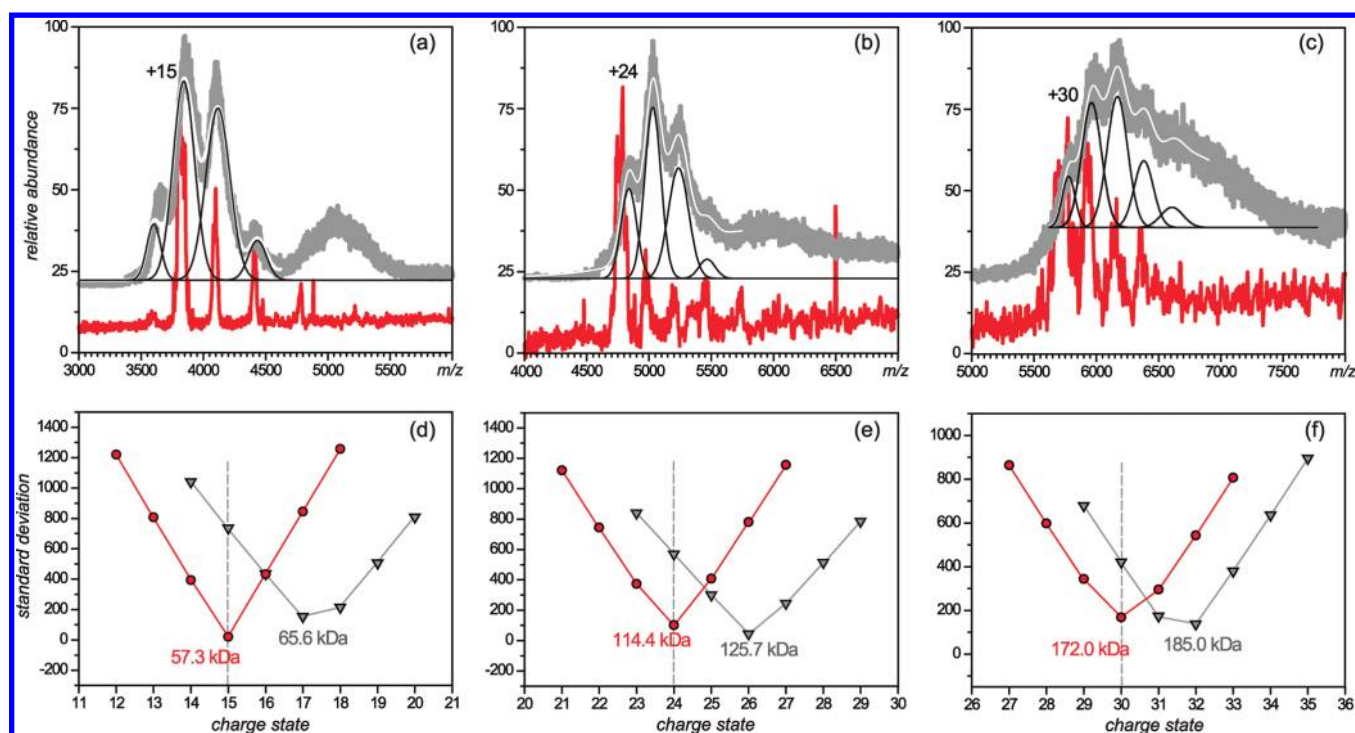


Figure 3. Charge state assignment for protein ions in ESI mass spectra of SEC fractions 1–3. Panels a–c show unprocessed mass spectra (gray traces) and the results of partial reduction of ionic charges triggered by electron capture following mass selection of relatively homogeneous populations of protein ions in the ICR cell of the FTICR MS. Panels d–f show the result of statistics-based charge assignment for raw ESI MS data (gray triangles) and charge state ladders produced by partial charge reduction (red circles). The latter was used to calculate the masses of AT species in each of these three fractions and fit the raw ESI MS data with expected contributions from several charge states: individual contributions are represented with black Gaussian curves in panels a–c, and their sums are represented with white curves.

Unfortunately, the signal-to-noise ratio in the mass spectrum of the products of partial reduction of protein ions from fraction 4 was too low to obtain confident charge state assignment. In this case the charge state assignment was carried out by calculating the theoretical m/z values for ions of various oligomeric states of AT falling within the 6000–8000 m/z region. These m/z values were used to generate a series of drop-lines that were overlaid with the unprocessed ESI mass spectrum of species 4 and searched for the best match between a series of drop-lines for a specific AT oligomer and the profile of the ionic signal (see the Supporting Information for more detail). A tetrameric form of AT was determined to provide the best match with the mass spectral features of SEC fraction 4, suggesting that AT tetramer is likely to be the major component of this fraction. The ESI mass spectrum of fraction 5+ (gray trace in Figure 2) is completely unresolved, barring any possibility to determine its composition mass spectrometrically. However, since this fraction was collected very close to the void volume of the column, and all other (later eluting) fractions were assigned as tetramer through monomer, fraction 5+ has been assigned as unresolved higher oligomers (pentamers and higher).

ESI MS-assisted identification of AT oligomers contributing to the SEC profile of the heat-stressed protein allows the relative abundance of each small soluble aggregate to be determined based on the area of the corresponding “bell curve” in Figure 1b. Whereas the higher oligomers (fraction 5+) represent 44% of the total protein population in solution, the fractional concentrations of smaller AT species are 12% (tetramers), 20% (trimers), 15% (dimers), and 9% (monomers). This nonmonotonic tendency implies that AT trimer is

the most abundant species in this oligomerization system, while the apparent abundance of peak 5+ in the SEC profile is a result of contributions from a large number of unresolved soluble protein aggregates.

Direct Profiling Small Soluble Aggregates of Heat-Stressed AT with ESI MS. The ESI mass spectrum of heat-stressed AT obtained under the ESI source conditions that minimize ion solvation without inducing dissociation contains a series of well-resolved ion peaks in the m/z region below 7000; more distinct peaks can be seen in the higher m/z region, although several of them are not baseline-resolved (Figure 4). Compared to the spectrum of intact AT (inset in Figure 4), additional clusters of peaks populate the mass spectrum of the heat-stressed protein. Assignment of these peaks to specific AT oligomers was carried out using an approach similar to that described in the preceding paragraph for SEC fraction 4 (see the Supporting Information for more detail) using the masses derived from ESI MS analyses of SEC fractions of heat-stressed AT. No assignment was attempted beyond the pentamers due to the insufficient resolution of ionic signal in the high m/z range (above 8000 in Figure 4). In a few cases, the same peak was assigned to more than one oligomeric species due to the close match of two calculated m/z values; all such possible double assignments are clearly labeled in Figure 4. It is noteworthy that these assignments might not cover some “shoulder contributions”, i.e., ionic species which contribute signal to those peaks where apexes are close enough to the m/z values of these species so that significant peak broadening occurs (see the Supporting Information for more detail). The actual contributions of these species can be taken into account upon completion of the deconvolution procedure.

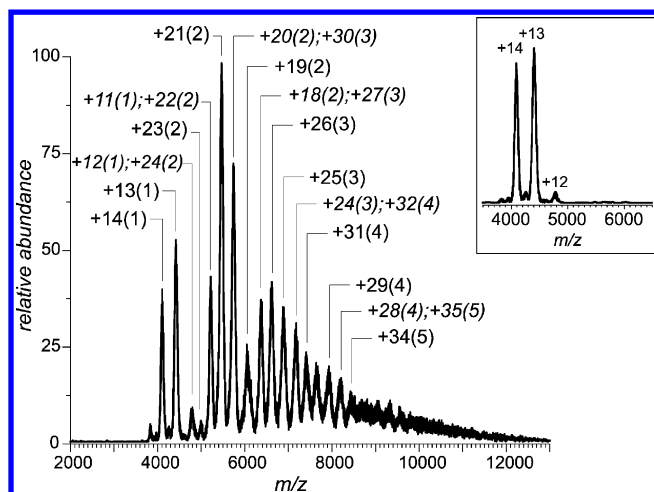


Figure 4. ESI mass spectrum of heat-stressed AT obtained without chromatographic separation. Charge state assignment is shown for most peaks (numbers in parentheses indicate the size of AT oligomers). The inset shows a reference mass spectrum of the intact protein (3 μ M in 150 mM ammonium acetate, pH 8.0).

Although a quick examination of the mass spectrum of heat-stressed AT may suggest that the protein dimer is the most abundant species (followed the monomer and then the trimer), which is in apparent disagreement with the results of SEC analysis (trimer > dimer > monomer, see Figure 1b), one needs

to realize that even in the absence of any instrumental biases the signal intensities in ESI MS and SEC correlate with different types of protein concentration in solution. Indeed, the UV signal in the SEC experiment is proportional to the total number of aromatic residues within the eluting protein species and, therefore, reports mass concentration of the analyte. On the contrary, the MS signal is proportional to the number of ions reaching the detector; therefore, in the absence of any discrimination during ionization, transmission, and detection, the ionic signal reports molar concentration of the analyte in solution. In other words, under the ideal circumstances (no bias), an equimolar mixture of monomers, dimers, and trimers would produce an MS readout showing equal relative abundance for these species, whereas the SEC profile will show a 1:2:3 intensity ratio for peaks representing these same oligomers. Therefore, in order to have a meaningful comparison of the size distribution of AT oligomers produced by SEC and ESI MS, the way of expressing the analyte concentration in solution should be identical. In most aggregation studies weight concentration is the preferred method of expressing the size distribution of small soluble aggregates, and in order to adopt this approach, MS data must be converted to reflect weight concentration, rather than mass concentration.

Should the ionic contributions of various oligomers to the mass spectrum of heat-stressed AT be completely resolved, MS data conversion would be very straightforward: one would only need to multiply the total ionic signal of each oligomer by its

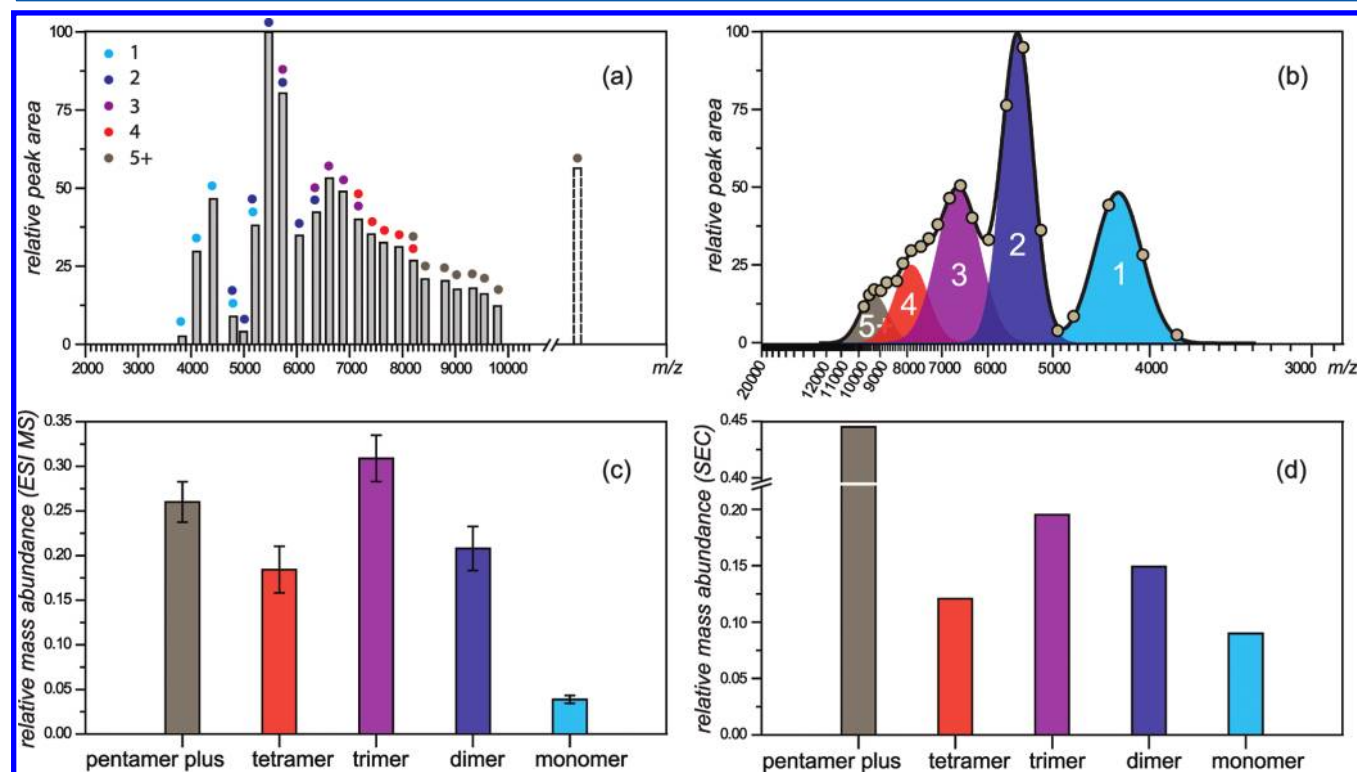


Figure 5. Deconvolution of the ESI mass spectrum of heat-stressed AT and conversion of the raw data to reflect mass concentration of small soluble AT oligomers in solution. Panel a shows peak areas of various ionic species in the raw ESI mass spectrum in the bar diagram format. Contributions of individual AT species are indicated with colored dots above each bar. Panel b shows a plot of the data shown in panel a on reversed m/z scale and deconvolution of this data set by finding the best fit with five Gaussian curves. Panel c shows in the bar diagram format sums of bar heights of ionic species from panel b multiplied by the size of the corresponding oligomer (i.e., number of incorporated AT monomers). Panel d shows the areas of Gaussian curves used to fit the SEC profile of the heat-stressed AT (from Figure 1b) representing relative mass concentrations of the monomer and the small soluble aggregates.

size (number of incorporated monomer units). Unfortunately, a few peaks in the mass spectrum of the heat-stressed AT can be assigned to more than one oligomeric species (*vide supra*), making this easy approach unfeasible. In order to circumvent this problem, the following strategy was used to tease out individual contributions of AT oligomers from the mass spectrum shown in Figure 4. First, a raw mass spectrum was converted to a bar diagram where bar heights represent peak areas in the raw mass spectrum (Figure 5a). Each bar in this diagram was assigned as representing a specific oligomer, unless the corresponding peak in the raw mass spectrum could contain contributions from two different species (in which case it was assigned as representing two different species, as indicated by colored dots above each bar in Figure 5a). AT pentamer peaks, as well as unresolved peaks in the high m/z region of the mass spectrum ($>10\,000$), were counted cumulatively as representing a "pentamer plus" species (pentamers and higher oligomers); the total contribution of the unresolved ions is shown in Figure 5a as an unshaded column at the end of the m/z scale.

In a second step, this diagram was replotted using an inverse m/z scale (Figure 5b) so that the spacing between the adjacent peaks within each cluster representing the same oligomeric species would be uniform. This is similar to intensity-as-a-function-of-charge plots used previously in our laboratory to extract information on protein conformers from ESI MS data,^{29–32} although a single graph now represents several species and, therefore, allows the overlapping contributions of different oligomeric species to the total ionic signal to be accounted for. This combined data set was processed to find an optimal fit with five Gaussian curves in the same way as was done previously for the SEC profile of the heat-stressed protein (Figure 1b), the only difference being that the MS data set is represented by discrete data points, rather than a continuous curve. The results of this deconvolution procedure are shown in Figure 5b with five colored bell curves, which clearly correspond to AT monomer (labeled "1" in Figure 5b), dimer (2), trimer (3), tetramer (4), and larger oligomers (5+). These curves were used to deduce the individual contribution of each oligomer to peaks representing more than one species by dividing the height of the bar in proportion to the ordinate values of the two curves at the appropriate location on the inverse m/z axis. The sum of bar heights adjusted for contributions from the interfering species reflects its fractional molar concentration in solution (unadjusted for any bias that may be introduced by the mass spectrometer). These values can now be easily converted to fractional mass concentrations by simple multiplication as discussed in the preceding paragraph using multiplication factors ranging from 1 to 4 for monomers, dimers, trimers, and tetramers, respectively. The multiplication factor for the higher oligomers (5+ in Figure 5b) was set as 5, since AT pentamer was expected to be the most abundant species in this group in terms of molar mass (based on the observed monotonic decrease of abundance of the smaller oligomers from dimers to tetramers). We note, however, that this is expected to result in the fractional mass concentration of the larger oligomers being somewhat underestimated.

The result of the conversion of ESI MS data to the distribution of AT oligomers based on their mass concentrations is shown in Figure 5c, and the same distribution derived from the SEC data is shown in Figure 5d (see the Supporting Information for numeric values). Comparison of the two distributions leads to a striking conclusion that the

order of abundance of lower molecular weight AT species in solution provided by the two techniques is the same (trimer > dimer > tetramer > monomer). The only disagreement between the two distributions is the relative abundance of the higher molecular weight species (pentamers and higher), which appears to be the most abundant species in solution (accounting for nearly 45% of the total mass) according to the SEC data but only the second most abundant species (accounting for less than 30% of the total mass) according to the ESI MS data. As has been discussed earlier, underestimation of the fraction of the high molecular weight species was not unexpected, since a conversion of molar concentration to mass concentration for this entire group of large AT oligomers was carried out using a conversion factor for pentamers, the smallest species in this group.

CONCLUSIONS

Despite the spectacular success of ESI MS in numerous studies of noncovalent associations of proteins and biopolymers, application of this technique for quantitative or even semiquantitative profiling of various oligomeric species present in solution is still frequently met with skepticism due to a commonly held belief that instrumentation bias is so significant that any conclusions derived from such measurements would be unreliable. One particular area where such limitations were expected to be particularly unforgiving is protein aggregation, where the enormous heterogeneity of the protein species manifests itself in a wide distribution of soluble oligomers. As a result, most applications of ESI MS in this field were confined to qualitative analysis of soluble protein aggregates, whereas quantitation of such species is done by lower resolution techniques, such as SEC and AUC. The results of the present study are rather surprising in this regard, as the remarkable agreement between ESI MS and SEC in ranking lower molecular weight oligomers of heat-stressed AT with respect to their fractional concentrations in solution suggests that ESI MS can be used at least for semiquantitative characterization of such heterogeneous systems. This may open up new exciting opportunities in many areas of life sciences where protein aggregation is very prominent (although not particularly desirable), since the ability to characterize the aggregates both qualitatively (in terms of size and conformation) and quantitatively (concentration) is critical for understanding the molecular mechanism of this complex biophysical phenomenon.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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