# Do Ionic Charges in ESI MS Provide Useful Information on Macromolecular Structure?

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Multiple charging is an intrinsic feature of electrospray ionization (ESI) of macromolecules. While multiple factors influence the appearance of protein ion charge state distributions in ESI mass spectra, physical dimensions of protein molecules in solution are the major determinants of the extent of multiple charging. This article reviews the information that can be obtained by analyzing ionic charge state distributions in ESI MS, as well as potential pitfalls and limitations of this powerful technique. We also discuss future areas of growth with particular emphasis on applications in structural biology, biotechnology (protein-polymer conjugates), and nanomedicine. (J Am Soc Mass Spectrom 2008, 19, 1239–1246) © 2008 American Society for Mass Spectrometry

The idea to use electrospray ionization (ESI) as a means to generate ions of polar and thermally labile (bio)polymers in the form suitable for MS analysis [1] and even its experimental implementation [2–4] preceded the triumphant entry of this ionization technique into the mainstream of biological mass spectrometry by some time. One particular feature of ESI that seemed to be a major impediment for its application to macromolecules was multiple charging, a phenomenon that seemingly complicated the appearance of mass spectra by crowding them with multiple ion peaks corresponding to the same analyte. Although ionic species carrying more than one elementary charge are not uncommon in mass spectra of macromolecular ions generated by means of fast atom bombardment (FAB) or matrix-assisted laser desorption/ionization (MALDI), they typically account for only a small fraction of the overall signal. Furthermore, typical ionic charge density (defined here as the average number of charges per unit mass) is much lower for the macromolecular ions produced by FAB and MALDI compared with ions generated by ESI. Charge reduction strategies via ion-molecular reactions in the gas phase provided an opportunity to simplify ESI mass spectra of polypeptides by eliminating peaks of multiply charged ions. However, it was the realization that multiple charging is an intrinsic property of ESI process and must be dealt with using mathematical tools, that made it a practical tool for the analysis of macromolecules [5].

In retrospect, it seems almost ironic that the multiple charging of macromolecules in ESI MS is now viewed not as a liability, but an extremely valuable asset, which provides a new important dimension to the analysis of biopolymers. Indeed, it was not long after the acceptance of ESI MS as a tool for measuring masses of macromolecular ions that observations were made linking the dramatic changes of ionic charge state distributions to protein denaturation in solution [6, 7]. These observations brought about the realization of the potential of ESI MS as a means of probing protein higher order structure and detecting large-scale conformational transitions in solution.

Natively folded proteins by definition have stable, compact cores sequestered from the solvent and undergo ESI to produce ions carrying a relatively small number of charges. This is because the compact shape of a tightly folded polypeptide chain in solution does not allow the accommodation of a significant number of protons on their surface upon transition from solution to the gas-phase. For this reason, ion peaks in ESI mass spectra of proteins in aqueous solutions at neutral pH typically dominate the high $m/z$ regions of the mass spectra and are almost always characterized by having narrow distribution of charge states. Unlike folded proteins, conformers lacking native structure (i.e., those that are either partially or fully unfolded in solution as a result of denaturation), as well as intrinsically disordered proteins, give rise to ions carrying a significantly larger number of charges and their charge-state distributions are significantly broader. This is because once a protein loses its compactness upon denaturation (or unfolding), a significantly larger number of charges can be accommodated on its surface. Native and non-native protein states often coexist at equilibrium under mildly denaturing conditions. In such situations, protein ion charge state distributions become bimodal, reflecting the presence of both native and denatured states. Dramatic changes of protein charge-state distributions therefore often serve as gauges of large-scale conformational changes [8].

In the nearly two decades that passed since the publication of the initial reports linking protein confor-

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mation in solution and the extent of multiple charging in ESI MS, analysis of ionic charge state distributions became a potent tool in biophysics. However, certain aspects of this methodology remain controversial, and the consensus is still lacking as far as the exact meaning of the protein ion charge in ESI MS. The purpose of this article is to discuss some of the controversial issues, highlight current capabilities of ESI MS experimental strategies relying on ionic charge state distribution analysis, and to identify the areas of growth where new and exciting developments are likely to occur in the very near future.

## Can Charge State Distributions be Used to Provide Meaningful Information on Large-Scale Protein Dynamics Beyond Detection of the Onset of Unfolding? Pitfalls and Limitations

In the seminal work published a decade ago, Konermann and Douglas provided convincing evidence that appearance of high charge density protein ions in the ESI mass spectra coincides with either decrease or complete loss or tertiary structure in solution [8]. Therefore, bimodal appearance of protein ion charge state distributions always indicates that at least two forms of protein molecules coexist in solution, compact (highly structured and usually natively folded) and less structured (denatured). A shift from a narrow distribution of low charge-density ions to a bimodal one signals the onset of protein unfolding.

While the natively folded protein conformations are usually unique, very few proteins possess only one unstructured state. It is, therefore, not surprising, that the ionic signal representing non-native protein species is very heterogeneous (compared with the low charge-density ions representing folded proteins) and evolves as the solution conditions change. Indeed, individual domains of larger proteins are often relatively autonomous, and their unfolding is not necessarily triggered simultaneously. As a result, intermediate charge-density ions can be observed in ESI mass spectra, which correspond to protein molecules in which some domain(s) is (are) unfolded, while other may retain their native structure. An example is shown in Figure 1, where denaturation of a 58 kDa glycoprotein anti-thrombin III reveals the existence of at least three non-native states. These states are represented in ESI MS by ionic distributions centered around charge states +20, +32, and +38, which become variably populated as the solvent conditions change from mildly to strongly denaturing.

In the course of unfolding, even most single-domain proteins usually populate multiple conformations retaining varying levels of native structure. In most cases these non-native conformations do not give rise to distinct ion signals in ESI MS because of the insufficient differences in the solvent-accessible surface area (SASA) among individual conformers. This results in either

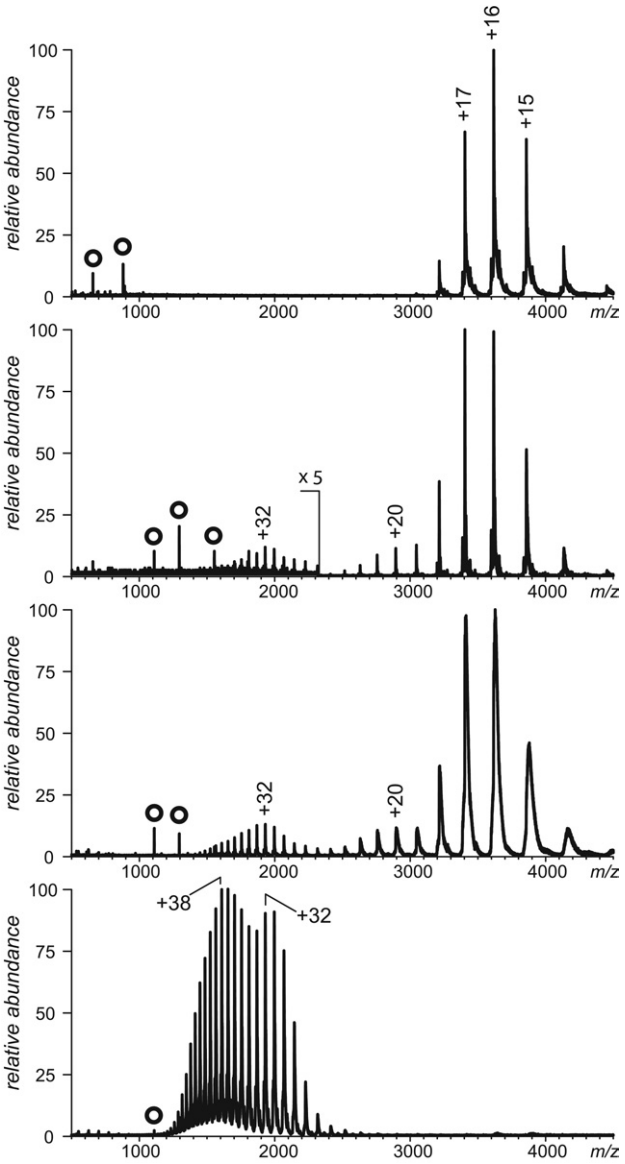


**Figure 1.** ESI mass spectra of anti-thrombin III acquired under the following conditions (from top to bottom): 20 mM ammonium acetate; 20 mM ammonium acetate/methanol (50:50); 20 mM ammonium acetate/methanol/acetic acid (49:50:1, vol:vol:vol); 20 mM ammonium acetate/methanol/formic acid (45:50:5, vol:vol:vol). Ion peaks labeled with circles correspond to low molecular weight impurities.

unresolved or poorly resolved charge-state distributions, where two or more different protein conformers may produce ions carrying equal numbers of charges in the gas phase. In many cases, however, the information on individual protein states can be extracted from such unresolved charge state distributions using chemometric tools [9, 10].

The ability of ESI MS to "visualize" individual conformers is unique among biophysical techniques that are commonly employed to study protein structure. Naturally, it poses a very intriguing question: can the fractional concentrations of various protein states be estimated based on the relative abundance of ions

representing these states in ESI MS? Many early studies implicitly assumed that the answer to this question is "yes," although some reservations were also expressed [9]. A recent elegant study by Konermann and coworkers explored the relationship between the fractional concentrations of different conformers and their respective ionic signals [11]. Convincing evidence was presented that non-native polypeptide chains generate higher signal response compared to the natively folded species. In some unfavorable cases, the ionic intensity ratios may deviate from the actual concentration ratios by as much as two orders of magnitude. The higher ionization efficiency of unfolded proteins is attributed to their increased hydrophobicity, which increases their surface activity. As a consequence, these species are much more likely to be located at the solvent/air interface in the charged droplets produced by electrospray and will have a much higher probability to be transferred to the ion-producing progeny droplets during Coulomb explosion events. Fortunately, the study also found that suppression of the ionic signal corresponding to the natively folded protein species can be minimized if the experiments are carried out in a charge surplus regime [11].

Artificial enhancement of the ionic signal of (partially) unfolded protein species in ESI MS or indeed occurrence of false-positive signals of protein unfolding may also be observed in a situation where significant quantities of protein complexes or aggregates exist in solution [12]. Dissociation of such complexes in the gas phase usually proceeds via the so-called asymmetric charge partitioning, where a single polypeptide chain is ejected from a metastable complex and carries a disproportionately high number of charges [13]. An example of such a process is presented in Figure 2, where mild collisional activation of large protein complexes (14-meric molecular chaperon GroEL) in the ESI interface results in ejection of a highly charged monomeric ion from the complex. The remaining part of the complex (13-mer) retains a disproportionately low fraction of the initial number of charges (average charge +43), giving rise to ionic signal at the high end of the *m/z* scale. The monomeric products of dissociation populate the low end of the *m/z* scale (average charge +29), and their presence in the mass spectrum alongside the low charge density monomeric ions (average charge +16) falsely suggests the existence of unstructured monomers in solution at equilibrium with the natively folded species.

Protein ion charge state distributions can be affected by a variety of other processes occurring in the ESI interface region. One particularly common situation is the apparent reduction of the number of protons carried by protein ions in the gas phase, which is frequently encountered when acid unfolding of proteins in solu-

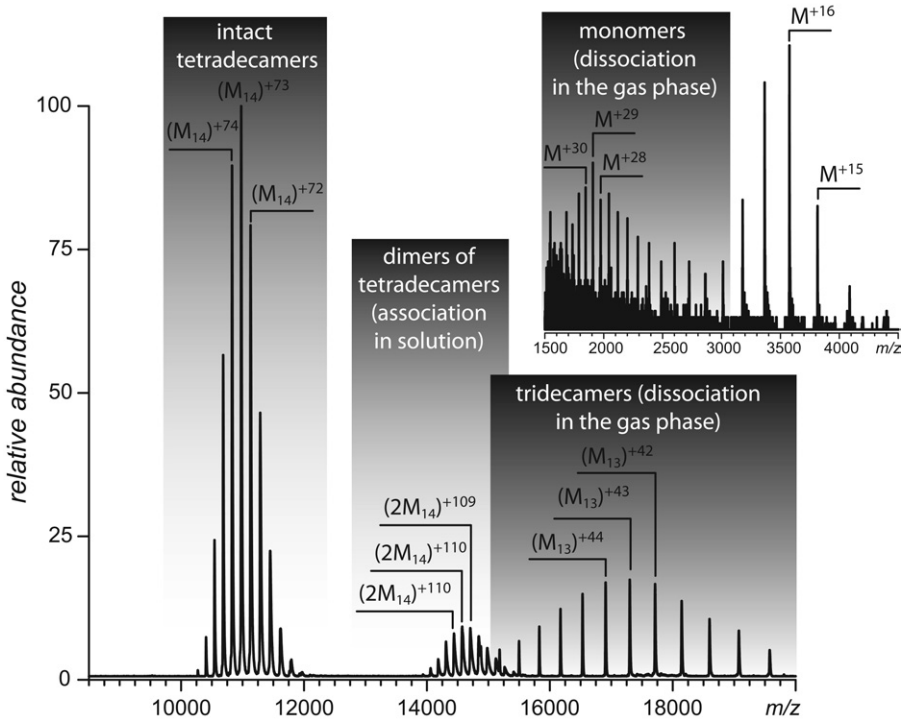


**Figure 2.** ESI mass spectrum of GroEL acquired under near-native conditions in solution (100 mM ammonium acetate) and mild collisional activation in the ESI interface. The low *m/z* region of the spectrum is shown in the inset. Highly charged monomers and low charge density tridecamers are products of dissociation of tetradecameric structures in the gas phase. Oligomerization of GroEL tetradecamers (formation of $2M_{14}$ species) is likely caused by increased protein concentration in ESI droplets as a result of solvent evaporation.

tion is induced by adjusting the pH of weak buffer solutions (such as ammonium acetate, $CH_3CO_2NH_4$) with acids (such as acetic acid, $CH_3CO_2H$). Evolution of charge state distributions following mild acidification (down to pH = $pK_a$ of acetic acid) does not reveal any noticeable contributions of gas-phase ion chemistry [14]. However, continuous acidification of protein solution (below the $pK_a$) often results in a detectable decrease of the average charges of ionic species representing native protein conformations [14]. This is due to protein-anion adduct formation in solution (e.g., $MH_n^{n+}\cdots CH_3CO_2^-$). At pH equal to that of the $pK_a$ of acetic acid the $CH_3CO_2^-/CH_3CO_2H$ pair acts as a strong buffer and large quantities of $CH_3CO_2H$ are required to bring about even a relatively small pH decrease. This results in a dramatic increase of absolute $CH_3CO_2^-$ concentration, leading to more efficient $MH_n^{n+}\cdots CH_3CO_2^-$ complex formation. This complex dissociates in the gas-phase to produce $MH_{(n-1)}^{(n-1)+}$ and neutral $CH_3CO_2H$, since charge separation in the gas-phase (to produce $MH_n^{n+}$ and $CH_3CO_2^-$ ions) would carry a very significant enthalpic penalty. The result of these processes is a reduction of the protein ion charge density, which can be incorrectly interpreted (if the gas-phase processes are ignored) as a result of tightening of the protein structure in solution [14].

Finally, it must also be noted that even selection of data acquisition parameters in ESI MS may (and almost always does!) introduce bias in the ionic charge state distribution by altering the transmission/detection efficiency as a function of *m/z*. Obviously, analysis of charge state distributions of protein ions in ESI MS provides reliable information on protein conformational dynamics only if the observed changes in the extent of multiple charging are due to the changes of protein compactness in solution. Ignoring the gas-phase processes that may alter the ionic charge distributions can introduce a significant systematic error in the evaluation of protein conformational heterogeneity and solution dynamics.

## Can Charge-State Distributions Provide Information on Protein Geometry in Solution?

The importance of tertiary structure in determining the extent of multiple charging of proteins in ESI MS raises another intriguing question: is it possible to use ionic charge state distributions to assess the geometry of proteins in solution? To answer this question definitively, one needs to have very good understanding of mechanistic aspects of protein ion production in ESI MS. All suggested models predict strong dependence of ionic charge on physical dimensions of protein molecules in solution, e.g., average molecular cross section in solution in the ion evaporation model [15] or surface of protein molecules encapsulated in the progeny droplets in the charged residue mechanism [16, 17]. Although there is still a disagreement in the literature as to what particular parameter is reflected by the protein ion charge in ESI MS (reviewed in [18]), recent systematic studies provide strong evidence that it is the solvent-exposed surface area (SASA) of a natively folded protein in solution that dictates the extent of multiple charging of corresponding ions in the gas-phase [16]. In fact, it is possible to use the surface-charge correlation to obtain estimates of SASA of natively folded proteins and their complexes and to evaluate solvent-shielded surface at protein–protein interfaces within macromolecular assemblies in solution [16]. Although evaluation of SASA based on the measurements of average charges of protein ions in ESI MS cannot presently rival the established techniques as far as measurement precision, it may be extremely useful for characterizing protein assemblies in solution that are not amenable to structural analysis using traditional biophysical tools due to their transient nature or heterogeneous character.

While the ability of ESI MS to provide quantitative information on dimensions of natively folded proteins and their assemblies in solution is very useful, a prospect of extending the scope of this technique to include non-native protein states seems even more exciting. Indeed, currently there are no reliable biophysical methods to characterize structure of distinct partially unfolded states due to the impossibility of separating them physically from each other and/or from the native state. ESI MS may be the only technique capable of closing this gap due to its unique ability to make a distinction among various protein states based on the analysis of ionic charge state distributions. However, even though it is possible to detect the non-native protein states using ESI MS and even obtain estimates of their relative abundance in solution (vide supra), it is still too early to say with certainty that their physical dimensions (e.g., SASA) may be reliably estimated based on the number of charges accommodated by protein ions in ESI MS. For example, it is not uncommon to hear an argument that the extent of multiple charging of unfolded polypeptides in ESI MS must not be determined solely by their physical dimensions, but is also likely to be affected by the number of available ionizable sites.

Multiple charging of proteins in ESI MS most commonly occurs in the positive-ion mode in the form of protonation (the attachment of multiple protons, $H^+$), although other types of polycation formation can also occur. Formation of polyanionic species in the negative ion ESI MS usually proceeds via deprotonation of polypeptides. It is the intimate involvement of protons in generating multiply charged ions of biopolymers that led some to believe that the charge state distributions observed in ESI MS must reflect the cumulative charge on the acidic and basic residues in solution [19, 20]. In this view, the large-scale conformational dynamics of the polypeptide chains in solution influenced the charge state distributions indirectly, by modulating the $pK_a$ values of individual amino acid residues.

Although the acid-base paradigm is a convenient way of thinking of the protein ions in solution, it is a very poor predictor of the extent of multiple charging of proteins in ESI MS. Fenselau and coworkers demonstrated that both polycationic and polyanionic species could be generated from a protein solution at any given pH, proving that evolution of the charge distribution reflects conformational transitions, rather than titration of basic or acidic side chains [21]. Although the notion of the solution phase acid-base chemistry as a determinant of the extent of multiple charging in ESI MS was abandoned long time ago, acid-base chemistry is still frequently invoked as a factor influencing protein ion charge state distributions. For example, it is often argued that the extent of multiple protonation of an unstructured polypeptide chain should be limited by the number of basic residues in its sequence [22]. Specifically, proton affinity of the solvent molecules is suggested to provide a "cut-off" level for amino acid residues that can be protonated in the gas phase [23]. Since most proteins possess a high number of basic sites, the requisite number of positive charges can almost always be distributed among the available basic sites in the unstructured polypeptide chain. However, there are several examples of highly acidic proteins, which contain very few basic sites.

One particularly interesting example of such a system is pepsin, a 33 kDa protein containing 41 acidic and only four basic residues (see the inset in Figure 3). It is tempting to argue that there are simply not enough basic sites in this protein to afford adequate protonation

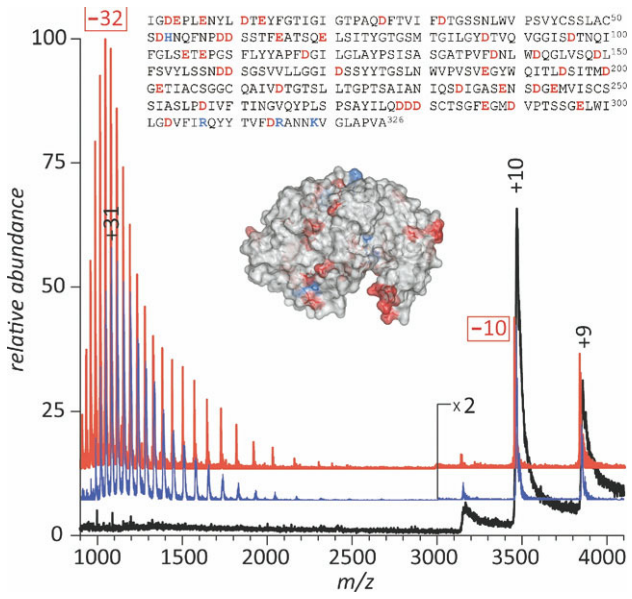


**Figure 3.** ESI mass spectra of porcine pepsin acquired under near-native (10 mM ammonium trifluoroacetate, pH adjusted to 1.6, black trace) and denaturing (20 mM ammonium acetate, pH adjusted to 9.5, 50% MeOH by volume, blue and red traces) conditions. The mass spectra are acquired in the positive (black and blue traces) and negative (red) ion modes. The inset shows the amino acid sequence and the crystal structure of pepsin, where acidic and basic residues are colored in red and blue, respectively.

of the unfolded polypeptide chain in the gas phase. Despite the small number of basic sites, pepsin ions accumulate up to 11 positive charges when desorbed from aqueous solutions at near-native pH ≤2 (the native environment of pepsin, gastric juice, is extremely acidic). A careful examination of masses (or, more precisely, mass distributions) of these ions suggests that the charges are actually a mix of protons $H^+$ and ammonium cations $NH_4^+$ [16]. The charge state distribution of pepsin ions (black trace in Figure 3) is remarkably narrow under the acidic conditions and does not change until pH is raised above 2.5 [24], a threshold of pepsin deactivation.

Large-scale unfolding of pepsin is known to occur in neutral and basic solutions. Therefore, it is not surprising to observe a dramatic change of pepsin ion charge state distributions in ESI MS acquired under these conditions (blue trace in Figure 3). What is rather surprising, however, is the high charge density of pepsin polycations representing the unfolded protein molecules. Indeed, the most abundant ion peak in the mass spectrum corresponds to a protein molecule accommodating 31 protons, more than six times the number of basic sites on the polypeptide chain! Furthermore, accurate measurements of masses of these highly charged pepsin ions indicate that they are multiply protonated species, not adducts of larger cations (such as $Na^+$ or $NH_4^+$). Average molecular weight of pepsin calculated based on the measured *m/z* values for charge states +26 through +35 is 34,570 ± 11 Da, which is within the experimental error range of the average mass of the protein calculated based on its sequence, 34,581 Da [12]. Should extensive $Na^+$ or $NH_4^+$ adduct formation occur, the measured mass would exceed the calculated one by a multiple of the cation mass less one (e.g., 16 for $NH_4^+$ adducts or 22 for $Na^+$).

It is quite remarkable that so many protons can be accommodated by a polypeptide chain, which contains only five basic sites. It is perhaps even more surprising that the extent of multiple charging of pepsin polyanions in negative ion ESI MS is very similar to that of multiply protonated species in positive ion ESI MS (compare the blue and red traces in Figure 3), despite more than a tenfold excess of acidic residues in this protein. It cannot be said with certainty what gives pepsin the ability to accommodate a number of protons far exceeding the number of the available basic sites; it may be argued that this phenomenon is a consequence of the great conformational flexibility of the polypeptide chain in the unfolded state. Such extreme flexibility may allow more than one functional group to participate in binding a single proton in a fashion similar to that observed in solvation of larger cations by polymer chains in the gas phase [25]. Such collective proton binding would certainly increase the apparent proton affinity of any particular binding site and, therefore, lift the restrictions on the extent of protonation imposed by thermodynamic considerations [22, 23]. This may allow the requisite amount of charges to be accumu-

lated by unfolded proteins even if they are deficient in basic sites.

Importantly, the fact that the polycationic charge state distribution of pepsin closely mirrors that of the polyanionic species (despite the abundance of acidic residues and the extreme deficiency of basic ones) strongly suggests that the extent of multiple charging is determined by the protein geometry in solution, not the number of available basic sites. The fact that it is the physical dimension of the unstructured protein species that ultimately dictates the ionic charge in ESI MS (regardless of the availability of the "requisite" number of basic or acidic residues in the polypeptide sequence) indicates that charge state distributions may in fact be used to evaluate the geometry of non-native protein conformations in solution.

## Future Outlook: Charge State Distributions Beyond Proteins?

Analysis of ionic charge state distributions in ESI MS as a structural tool has been applied so far almost exclusively to probe structure and conformational transitions in proteins and their assemblies, where it has already earned a reputation of a very potent experimental tool in the studies targeting various aspects of protein interactions. For example, ionic charge state distribution analysis is indispensable in understanding the role of large-scale protein dynamics in directing ordered assembly of multiunit protein complexes [26]. Although the proteins are undoubtedly the most important constituents of interaction networks at the cellular level and beyond, a variety of other biopolymers participate in forming critical nodes within these sophisticated interactomes. In addition to protein-oligonucleotide binding events playing obviously important roles at the terminal points in such networks (e.g., gene expression), a variety of other interactions provide important mechanisms to transmit, suppress or modify the signals both inside and outside the cell. These include encounters involving noncoding RNA and glycosaminoglycans to name a few. Furthermore, the emergence and rapid progress of macromolecular therapeutics and nanomedicine brings to the fore the question of how biomolecules interact with abiotic macromolecules, such as polymers and functionalized nanoparticles. Although ESI MS is playing an increasingly visible role in structural studies targeting these systems, very few attempts have been made to explore the utility of ionic charge state as a structural probe for biopolymers other than polypeptides [27].

Because of the paucity of conformational space generally sampled by polynucleotides and the extreme flexibility common among polysaccharides, it remains to be seen whether analysis of ionic charge state distributions of these biopolymers in ESI MS may generate useful information (as it does for polypeptides). However, there is one area that will certainly benefit from expanding the scope of charge state distribution analysis. Protein-DNA and protein–polysaccharide interactions are certainly amenable to ESI MS analysis (although presently its role is limited to identifying the interacting partners and determining binding stoichiometry), while the large size usually places such systems outside of the reach of high-resolution NMR. Likewise, X-ray crystallography is often limited in its ability to provide structural information on such systems due to their frequently displayed structural heterogeneity and dynamic character.

Should ESI MS prove capable of providing estimates of SASA of protein-DNA complexes based on the appearance of their charge state distributions, it would become an extremely valuable tool in structural modeling of these assemblies. For example, one could envision using SASA estimates as a filter in modeling schemes based on random docking of the assembly subunits, which are amenable to high-resolution characterization (Figure 4). ESI MS is already playing an increasingly visible role in this field as a part of integrative computational methods [28], and its potential to provide an estimate of SASA for complex macromolecular assemblies would greatly increase the value in this field.

Synthetic polymers and polymer-protein conjugates (e.g., PEGylated proteins [29]) is another area where ESI MS is poised to make important and valuable contributions soon. Presently, a major technical difficulty associated with these studies is due to chemical heteroge-

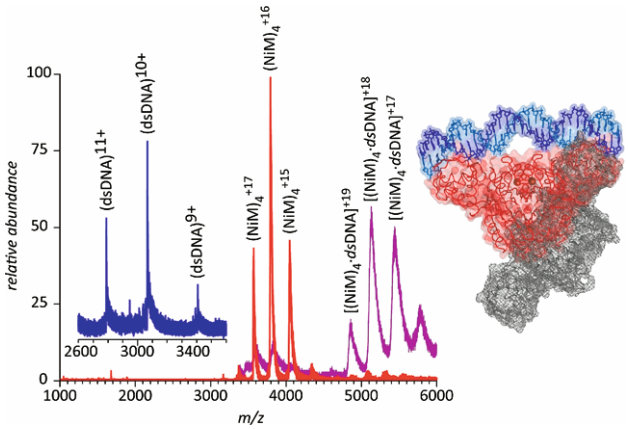


**Figure 4.** ESI mass spectra of nickel-bound NikR (red), a 50 bp operator DNA segment (blue), and their complex (purple). NikR, a regulatory metalloprotein from *E. coli*, controls expression of proteins facilitating nickel uptake by forming a tight complex with an operator DNA sequence. Only nickel-bound form of NikR (red structure in the inset) binds strongly to the DNA (blue) by forming a bidentate complex (note that the DNA segment in the crystal structure contains only 30 bp; PDB id 2HZV). Nickel-free form of the protein (gray) fails to adopt the characteristic "bent" conformation required for making a bidentate contact with the DNA, although weak binding can still be detected by ESI MS (data not shown). Charge state distributions of NikR–DNA complexes may provide an opportunity to distinguish between the canonical structure formed by holoprotein and DNA and a putative monodentate complex formed by apo-form of NikR and the DNA.

neity of PEGylated proteins. Multiple charging in ESI MS makes the analysis significantly more challenging. Even a limited extent of protein conjugation with relatively short PEG chains produces an extremely convoluted spectral pattern in ESI MS (Figure 5), while higher molecular weight or multiple conjugates will inevitably result in unresolved or poorly resolved ionic signal. Therefore, new data processing methods will have to be developed to enable extraction of structural information from ESI MS of protein-polymer conjugates, including the geometry of PEGylated protein molecules in various environments, details of their large-scale conformational dynamics and interaction with other biopolymers.

New approaches to ESI MS data processing will also be required to expand the scope of this technique to include functionalized nanoparticles, which show great promise in diverse fields ranging from clinical diagnostics to drug delivery to fine tuning and modulation of biological interactions in vivo [30]. While the great chemical diversity and multivalence of functionalized nanoparticles are certainly key features allowing the desired properties to be precisely engineered, they also present a grand challenge for analytical characterization, including ESI MS (Figure 6). However, it is not inconceivable that the continuous progress in both hardware design and methodology of ESI MS may soon allow reliable analytical procedures to be developed, aiming at characterization of architecture of complex nanostructures assembled by binding biopolymer chains to functionalized nanoparticle templates.

The ability to use ESI MS to characterize behavior of highly heterogeneous systems, including their architecture, dynamics, and interaction with biological partners and therapeutic targets, will undoubtedly provide an enormous benefit to biotechnology and nanomedicine,

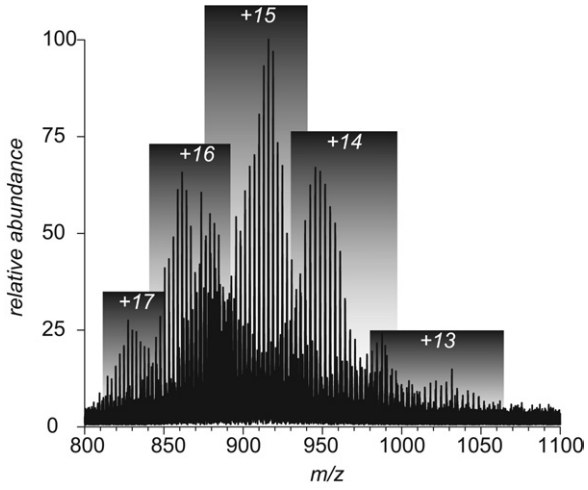


**Figure 5.** ESI mass spectrum of ubiquitin conjugated to a 5 kDa PEG chain (measurements were carried out in 10 mM ammonium acetate solution). A dramatic increase of the extent of multiple charging (more than twofold) of the protein following conjugation to a relative short polymer chain is consistent with the notion of a compact protein core and a highly flexible PEG chain.

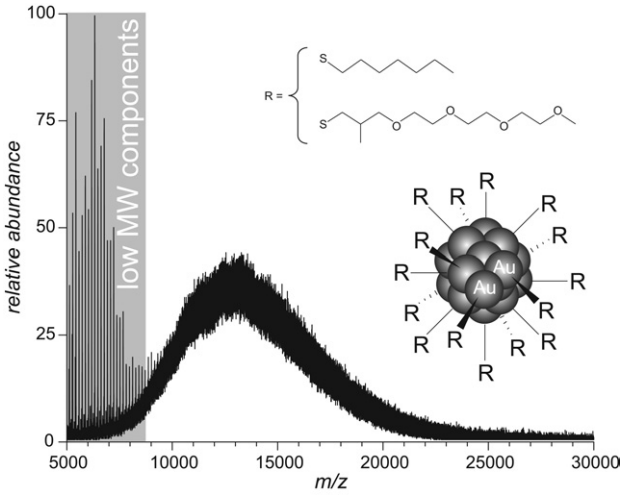


**Figure 6.** ESI mass spectra of a functionalized gold nanoparticle produced at the Center for Hierarchical Manufacturing at UMass-Amherst (PEGylated nanoparticles were generated by modifying thiolate monolayer-protected gold cluster of ca. 2.5 nm diameter through place exchange reactions to yield functionalized nanoparticles with total mass of ca. 75 kDa). Intriguingly, the ESI MS data indicate that the average charge of the corresponding ions is very low (ca. +6) compared with other macromolecules of a similar mass. The anomalously low extent of multiple charging most likely reflects the small physical dimensions of the nanoparticle, whose relatively high mass is mostly due to a very dense (and compact) metal core.

where the explosive growth in the use of polymer-conjugated biomaterials and nanoparticles places a premium on the ability to characterize their behavior, a challenge that is yet to be addressed by analytical chemists.

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