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Electrospray ionization mass spectrometry: a technology for studying noncovalent macromolecular complexes

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Abstract

Electrospray ionization mass spectrometry (ESI-MS) has demonstrated utility for the detection and study of weakly bound, noncovalent complexes, including protein interactions with inhibitors, cofactors, metal ions, carbohydrates, other peptides and proteins, enzyme–substrate pairings, and nucleic acid complexes. From the measurement of molecular mass of the intact complex and the individual binding partners, the binding stoichiometry can be derived. In many examples, the relative and absolute binding affinities can be deduced by the MS-based method. A review of the experimental principles of the method for studying noncovalent complexes, with emphasis on proteins, and the early studies that aided in the development of ESI-MS for this application are presented. Examples of protein complexes, such as the calcium-bound calmodulin-melittin complex, streptavidin homotetramer, and the enolase protein dimer are used to illustrate important features of the technique. A discussion on current and future applications of ESI-MS, such as the determination of the topology of macromolecular complexes, is provided. (Int J Mass Spectrom 200 (2000) 175–186) © 2000 Elsevier Science B.V.

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1. Introduction

The field of structural biology is emerging as a prominent area of focus as the fruits of the human genome initiative begin to be harvested. The structural determination of proteins and protein complexes play an important role in the fundamental understanding of biochemical pathways. From structure (hopefully) derives function. Structural genomics and structural proteomics strive to uncover the functional role of protein components from their structure [1–5]. Proteins serve to interact with other biochemical entities, for example, with smaller molecular species such as metal ions, nucleotide cofactors or with other proteins. X-ray crystallography and nuclear magnetic resonance spectroscopy are the current methods of choice for obtaining high resolution structural information. However, the recent emergence of biological mass spectrometry has poised mass spectrometry as a technique that can provide important information to address the question of function.

From the initial pioneering development of electrospray ionization (ESI) by Fenn's group [6], the application of ESI mass spectrometry (ESI-MS) for studying noncovalent complexes has important utility in biology, biochemistry, and biomedical research [7–10]. ESI-MS has begun to be viewed as a useful

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tool for investigating the general area of molecular recognition. Proteins and other biomolecules interact to form functional molecular machines. They interact through electrostatic, hydrophobic, and hydrogen bonding interactions. The measurement of molecular mass can be used to study macromolecular assemblies and their individual components. The observation of complexes formed between targets for drug discovery, typically proteins but in some recent examples large oligonucleotides, and their potential inhibitors can be used as a drug screening method [11]. This report will review the early highlights that influenced the nature of ESI-MS and noncovalent complexes and the current and future prospects of the field. The application of ESI-MS to protein complexes will be the primary focus, although noncovalent complexes involving other macromolecules such as crown ethers, oligosaccharides, macrocyclic molecules, and oligonucleotides have been studied. This article is not intended to exhaustively review the literature, but will attempt to feature key studies and other reports that illustrate important concepts.

2. Early events that shaped the field

The early desorption/ionization methods, such as field ionization/desorption, fast atom bombardment, secondary ion mass spectrometry, thermospray, and Cf-252 plasma desorption were and still are viable techniques for the analysis of peptides and in some examples, small proteins. However, relative to ESI and matrix-assisted laser desorption/ionization (MALDI), they lack the sensitivity and molecular mass range necessary to analyze large proteins. Moreover, the "softness" of ESI, that is its ability to not only ionize macromolecules without disrupting covalent bonds, but also to maintain weak noncovalent interactions, has distinguished ESI for the study of biological complexes.

For electrospray ionization, one of the major distinctions from other desorption/ionization methods (other than thermospray, which may be related to ESI) is the relationship between biomolecule structure and its solution phase state. Does the solution composition affect protein structure and conformation, and is this translated into the gas phase? Much of the early work with electrospray ionization focused on the mechanistic aspects of desorption and ionization events. Although pH 3–4 solutions are most commonly used for basic peptides and proteins because of their optimal sensitivity, the effect of higher pH toward the neutral, physiological regime and to the more basic end of the pH scale was studied for their effects on the analysis of biological molecules [12–14]. One obvious difference observed from high pH solutions was the effect on the charge distribution, typically toward lower charging or higher mass-to-charge, *m/z*.

Chowdhury et al. first demonstrated that ESI-MS could be used to monitor a protein's solution conformation [15]. For the well-studied protein cytochrome *c*, they observed a change in its charge distribution as a function of solution pH that correlated well with the protein's known conformational states. Later, Loo et al. demonstrated the application of ESI-MS for studying the solution conformations of the small protein ubiquitin with changing organic solvent (i.e. acetonitrile) content [16]. They also noted a shift in multiple charging for disulfide-containing proteins, such as lysozyme and ribonuclease A [17,18]. Upon disulfide reduction with agents such as dithiothreitol, more charging was observed, which was related to the proteins' more "open" or "extended" conformation. Studies such as these suggested a unique link between the solution phase structure of a macromolecule and the ESI mass spectrum. The characteristics of the multiple charge distribution, i.e. *m/z* position, the absolute charge, and the relative width of the distribution, could be related to the structure or conformation of the solution phase protein. The nature of these reports also changed the mind-set of researchers engaged in the technique. Instead of "merely" measuring the molecular mass of the large macromolecules (which was not a routine endeavor during this time), higher order structural characteristics could be obtained by mass spectrometry. From an experimental point-of-view, ESI of solutions composed largely of aqueous media and near neutral pH was encouraged. Until then, suggested by the early work from Fenn et al. [6], organic modifiers such as methanol, acetoni-

trile, and isopropanol, and organic acids such as acetic acid and formic acid were used, largely because highly stable spray currents are easy to produce and maintain and many protein and peptide systems are quite soluble at acid pHs. Highly aqueous and neutral pH solutions are more difficult to electrospray, and many proteins tend to aggregate (which in its own right should have suggested the application of ESI-MS for studying protein-protein interactions) and precipitate out of solution. However, improvements in ESI spray techniques (e.g. nanoelectrospray [19]) and becoming accustomed to reduced ion currents compared to denaturing solutions encouraged the future application for studying noncovalently bound complexes.

Between 1991 and 1993, a series of reports appeared in the literature demonstrating the applicability of ESI-MS for studying noncovalent complexes, many of which appeared as Communications in the Journal of the American Chemical Society. The first reports were authored by the Cornell University collaboration between Ganem and Li and Henion. The intact receptor–ligand complex between FK binding protein and macrolides rapamycin and FK506 was observed by ESI-MS [20]. Shortly afterwards, the enzyme-substrate pairing between lysozyme and N-acetylglycosamine and its cleavage products was reported by the Cornell researchers [21]. These early reports established not only the feasibility of the ESI-MS method, but also the design of the experiment to ensure validation of the observations. The idea of control experiments was suggested to investigate the possibility that the ESI process produced gas phase adducts instead of complexes relating to the specific nature of the solution interaction. It is important to establish the validity of the results in order to assess a meaningful interpretation and link to the solution phase system. These papers also helped move biology, biochemistry, and medicinal chemistry to the forefront of applications for ESI-MS technology.

An article by Katta and Chait on the noncovalent binding of heme (protoporphyrin IX) to myoglobin was a landmark for ESI-MS also [22]. Myoglobin was a well-studied protein by ESI-MS, even at this early time, and was used as a reference and testing compound for ESI-MS practitioners. Heme adducts onto

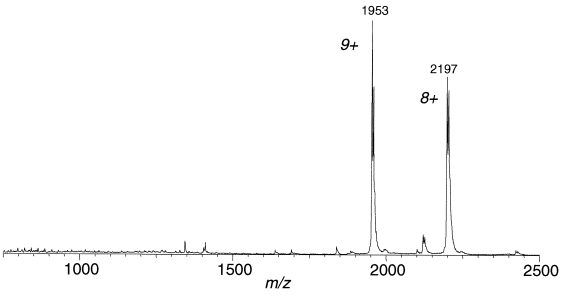

Fig. 1. Positive ion ESI-MS of horse heart myoglobin (10 μM, Sigma Chemical) in 10 mM ammonium acetate, pH 6.9. The spectrum was acquired on a magnetic sector mass spectrometer (Finnigan MAT 900Q, Bremen Germany) with a heated metal capillary ESI interface (capillary temperature 150 °C) and a solution flowrate of 1 μL min^{-1}. The labeled peaks represent the 8+ and 9+ charged molecules of the noncovalent holo-protein (myoglobin-heme-complex, M_r 17567).

the myoglobin protein were evident in some of the early ESI-MS mass spectra [23,24]. However, the solutions conditions typically used promoted the denaturation of the myoglobin complex, e.g. pH less than 3.5 and high organic solvent content. The Katta and Chait report showed dramatic differences in the myoglobin spectra from aqueous solutions between pH 3.35 and pH 3.90. Myoglobin is fully denatured at pH 3.35, and the mass spectrum shows only ions for the apo- or nonbinding form of the protein. Raising the pH to 3.90 allows the protein to fold properly to the more "native" or nondenatured form, and noncovalent binding of a heme molecule occurs. Because the protein is available commercially in large quantities (without straining the budget), myoglobin was and in some cases, is still being used as a "tuning" compound to optimize experimental conditions to maximize sensitivity for ESI of aqueous protein solutions and for observing noncovalent protein complexes. Such a typical ESI mass spectrum of myoglobin is shown in Fig. 1.

The next few years following produced several reports of other biochemical noncovalently bound systems using ESI-MS detection, including the protein–nucleic acid complex between ras protein and GDP [25] and a ternary complex between the HIV protease dimer protein binding to a substrate-based inhibitor [26]. The latter example of a protein-protein

interaction would be a common theme for many studies in the future, as the study of protein quaternary structure is an important application in biological mass spectrometry. DNA–DNA interactions, or DNA duplexes, were studied by ESI-MS by the Ganem and co-workers collaboration [27] and a 20-mer DNA duplex was observed by the Smith group [28]. Much larger DNA duplexes have been measured using Fourier transform ion cyclotron resonance (FTICR) mass spectrometers [29,30], and double stranded DNA in excess of 1 MDa has been studied using a mass spectrometer with a special charge detection device [31]. Articles on the ribonuclease S system, composed from the noncovalent interaction between the 2 kDa S-peptide and the 11.5 kDa S-protein highlighted the importance of optimizing the experimental conditions [32,33]. The S-protein/S-peptide complex is highly sensitive to solution pH and temperature and also to gas phase dissociation. Energetics for collisional dissociation, such as the energy in the atmospheric pressure/vacuum interface, capillary interface temperature, and countercurrent gas temperature need to be minimized to promote observation of the complex. Many gas phase complexes studied to date are highly sensitive to dissociative processes. However, the requirement to desolvate the gas phase complex, i.e. strip solvent molecules from the molecular complex, prior to mass spectrometric detection needs to be balanced with the forces keeping the complex together. In many ways, these early reports firmly established the experimental foundation for subsequent ESI-MS studies of noncovalent complexes. Refinements in instrumentation and technique have allowed much larger macromolecular assemblies to be detected with higher performance (e.g. resolution, sensitivity).

Larger protein complexes were observed by ESI-MS by using larger *m/z* range analyzers. Quadrupole mass analyzers of limited *m/z* range, typically less than 3000, were the most commonly employed mass spectrometers for electrospray ionization in the early 1990's. Multiply charged ions for complexes such as protein–protein quaternary complexes exhibit relatively low charge at high *m/z*. The amount of charging that a biomolecule exhibits in an ESI mass

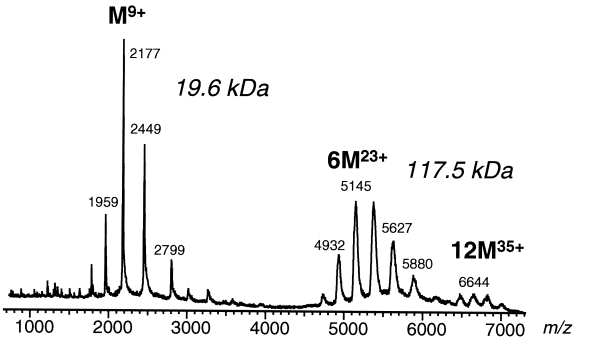

Fig. 2. ESI-MS of *E. coli* inorganic pyrophosphatase (Sigma Chemical) in 10 mM ammonium bicarbonate, pH 7.5. The spectrum was acquired on a magnetic sector mass spectrometer (Finnigan MAT 900T, Bremen Germany) with a heated metal capillary ESI interface (capillary temperature 200 °C) and a solution flowrate of 0.15 μL min^{-1}.

spectrum has been correlated to a global solution structure. The narrow charge distribution of a low charge state represents retention of the higher order structure of the native protein complex, presumably because either fewer charge sites are exposed, or the coulombic restraints restrict charging for a more compact structure [7].

To access the higher *m/z* range, Smith and co-workers used a quadrupole analyzer modified for high *m/z* (low frequency quadrupole) to study tetrameric proteins such as hemoglobin, avidin, and concanavalin A, with ions beyond *m/z* 10000 observed [7]. Loo et al. used a forward-geometry magnetic sector mass spectrometer equipped with an ESI source to study protein complexes from alcohol dehydrogenase and pyruvate kinase [34,35]. Fig. 2 shows a mass spectrum of homohexameric *Escherichia coli* inorganic pyrophosphatase with ions for the complex observed beyond *m/z* 5000. However, the Manitoba laboratory of Ken Standing first demonstrated the applicability of time-of-flight (TOF) analyzers for detecting ESI-generated ions from large protein complexes [36,37]. Although ion trapping instruments, such as the quadrupole ion trap [38] and the FTICR mass spectrometer, are used currently for such applications and have special performance advantages compared to other systems, the ESI-TOF mass spectrometers have become the predominant platform for studying nonco-

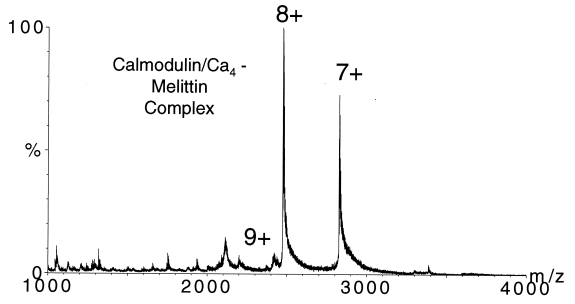

Fig. 3. Positive ion ESI-MS of the complex between bovine calmodulin (M_r 16952) bound to 4 calcium ions with melittin (M_r 2845). The spectrum was acquired with a Q-TOF mass spectrometer (Micromass, Beverly, MA) and a nanoelectrospray ionization source.

valent protein complexes. The sensitivity and resolution at very high *m/z* make the TOF analyzer an ideal system for large noncovalent complexes.

3. Recent applications

The types of biochemical systems studied by ESI-MS span a wide range and have been reviewed in previous articles [8,10]. In the area of proteins, mass spectrometry has probed their interactions with metal ions, cofactors, inhibitors, other proteins, polysaccharides, and oligonucleotides. Amster and co-workers reported an elegant study on the metal binding properties of rubrerythrin-type proteins, their effect on protein-protein interactions, and even the metal oxidation state [39]. The calcium binding properties of EF-hand proteins such as calmodulin and with their target peptides have been the subject of ESI-MS studies [40–42]. A positive ion mass spectrum of the calcium-bound calmodulin–melittin complex is shown in Fig. 3. Its specificity for binding four calcium ions is demonstrated by the molecular mass measurement. Protein–nucleic acid interactions are involved in many cellular processes including transcription and translation. The ESI mass spectrum of the *trp* repressor protein and its specific DNA operator was measured, demonstrating the value of the method for discerning specific interactions [43]. Only the DNA sequence with the correct spacing between binding sites formed a complex with the protein. Naylor's group has examined ligand-dependent transcription factors that bind to DNA to enhance or inhibit gene transcription [44]. Complexation of the human vitamin D receptor and the retinoic X receptor-α with the osteopontin vitamin D response element and the influence of ligands on transcription complex formation were observed.

4. Important experimental variables

From an experimental point of view, the keys for a "successful" ESI-MS analysis include maintaining proper solution conditions for keeping the protein complex in its folded, native state and efficient and effective desolvation of the ESI-generated droplets. Using the proper solvents (almost exclusively water for protein and oligonucleotide complexes), pH (again, almost exclusively near neutral physiological pH, but for the case of HIV protease, acidic pH was preferred [45]), and ionic strength buffer systems are necessary to maintain complexation. Deviation from these optimal conditions may reduce the observed relative proportion of complex formation for many systems. To date, volatile buffers such as ammonium acetate and ammonium bicarbonate are the most popular choices for ESI-MS experiments because they do not often form extensive gas phase adducts with the macromolecules (as do phosphate- and sulfate-based buffers) and background ion formation is reduced without significant reduction in protein ion formation. Buffer concentrations are typically at the 5–50 mM concentration levels. However, the work of Robinson and co-workers with the DNA binding protein HU required ammonium acetate concentrations up to 500 mM to maintain protein dimerization [46]. Also critical to the success of the analysis is the purity and quality of the sample. Because of the limited choice of buffers found compatible with ESI-MS, experimentalists have become quite proficient with exchanging buffers and removing other extraneous salts and additives prior to mass spectrometry. Dialysis and the use of centrifugal membrane filtration are popular methods for salt removal and

sample concentrators. On-line microdialysis [47] and a recent report from Naylor and co-worker on on-line size exclusion chromatography [48] may provide a rapid means to introduce the sample to the ESI source with sufficient sample clean-up.

Desolvation of ESI-generated droplets is a critical parameter for direct observation of the noncovalent complexes. Although it is an important factor that affects the overall sensitivity of experiments using the typical denaturing solution conditions (composed of a mixture of aqueous and organic co-solvent with a low concentration of organic acid), desolvation has as much or even more of an impact on noncovalent complex studies. Solvent adducts to the multiply charged molecules are often observed from near 100% aqueous, neutral pH solutions. This reduces the overall sensitivity because the observable ion current is distributed over heterogeneously solvated molecular species. Broader peaks in the mass spectrum complicate the measurement of molecular mass. Physical methods used to remove solvation include gas flow countercurrent to the spray of multiply charged droplets, application of heat in the form of a heated countercurrent gas flow or the application of a heated glass or metal capillary inlet, and gas phase collisions in the interface region downstream from atmospheric pressure. Fig. 4 shows the effect of temperature of a heated metal capillary interface on the mass spectra. At a capillary temperature of 100 °C, ions for the streptavidin homotetramer complex are poorly desolvated. Increasing the metal capillary temperature greatly improves the desolvation process, and the resulting peaks are sharpened. Yet, despite the plethora of methods to remove solvent from the gas phase complex, it is critical to maintain a balance between removing solvent molecules and possibly disrupting the macromolecular complex of interest. In general, the noncovalent complex is extremely fragile in the gas phase state. It is easily dissociated from application of excess heat or too high collision energy in the atmospheric pressure/vacuum interface. A complete understanding of the characteristics of a given ESI interface and overall system is necessary to optimize the performance for observation of noncovalent complexes, and also to

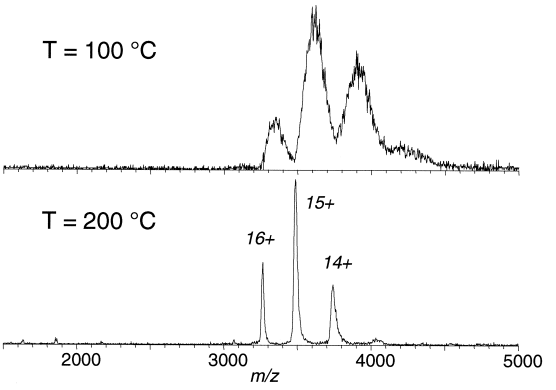

Fig. 4. ESI-MS of streptavidin (Boehringer Mannheim) in 10 mM ammonium acetate, pH 6.9, and a protein monomer concentration of 5 μM. The spectrum was acquired on a magnetic sector mass spectrometer (Finnigan MAT 900Q, Bremen Germany) with a heated metal capillary ESI interface (capillary temperature 100 °C, top and 200 °C, bottom) and a solution flowrate of 0.25 μL min^{-1}. At lower metal capillary temperatures, the multiply charged molecules of the 52 kDa tetramer are not well desolvated.

interpret the obtained data in the proper context. For example, one could use myoglobin as a test system or "calibrant" to optimize the instrumental parameters necessary to observe 100% complex formation between the polypeptide chain and the heme molecule (i.e. minimal observation of the apo-protein and free heme). Every instrument and every biochemical system of study has its own unique characteristics. Although this does not guarantee observation of the noncovalent complex at hand, it does provide a reference for one to interpret the results.

The recent application of the ESI-TOF and the quadrupole time-of-flight (Q-TOF) mass spectrometers has highlighted the differences in tuning behavior between the various analyzers. Researchers have found that increasing the pressure in the ESI interface and downstream prior to the detector (e.g. in the collision quadrupole in the case of the Q-TOF) greatly aids in the detection of very large noncovalent protein complexes. This is illustrated by the mass spectrum shown in Fig. 5 for the homodimer, enolase, acquired with a Q-TOF mass spectrometer. Although the increase of pressure enhances the performance for detection of most complexes, the enhancement is most noticeable for very large assemblies observed at

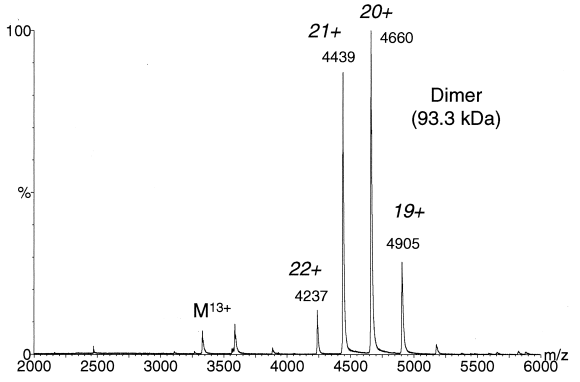

Fig. 5. ESI-MS of yeast enolase (Sigma Chemical) in 10 mM ammonium acetate, pH 6.9 with a Q-TOF mass spectrometer (Micromass, Beverly, MA) and a nanoelectrospray ionization source. The predominant ions are consistent for the 93.3 homodimer form of enolase. Vacuum pressure in the atmosphere/vacuum ESI interface and in the collision quadrupole region was increased to acquire the spectrum.

high *m/z*. Whether the effect of high pressure results in a collisional focusing effect [49,50] or efficient desolvation or both remain to be seen. However, the results are difficult to dispute, as demonstrated by the recent reports on protein complexes in excess of 1 MDa [50–52].

The development of low-flow micro- and nanoelectrospray sources by Mann and coworker [19] has played a significant role in not only ESI-MS of biomolecules in general, but also in the study of noncovalent complexes. The advantage of nanoliter per minute analyte flow not only reduces overall consumption of precious sample without compromising signal intensity, but also generates smaller droplets. This may help in the requirement for desolvation for noncovalent complex studies, but it remains in the "anecdotal" stage. The definitive study has yet to be performed to test this hypothesis. However, it is clear that the advent of nanoelectrospray has been a major aid in such studies.

5. Correlation between the solution phase and the gas phase ESI-MS measurement

Certainly, the most important factor for the popularity of the application of ESI-MS toward noncovalent complexes is its link to the solution phase. Living creatures are composed largely of water. For ESI-MS analysis, a biochemical sample is delivered initially to the mass spectrometer as a solution and is transformed to the gaseous state. Although some biophysical studies find utility to study the solventless environment, the majority of the questions posed relate to the solution characteristics. Can the measurement of gas phase molecules be related to the original solution phase? What is the fidelity between the gas phase and solution structure? In general, researchers endeavored in this field have found a high correlation between the ESI-MS data and expectations from the solution state world. It may not be a perfect correlation, as the precise three-dimensional structure of the gas phase molecule or complex may or may not be the same as the solvated species, as McLafferty and co-workers [53], Jarrold [54], Clemmer and co-workers [55], Williams and co-wokers [56], and several others have suggested. However, there may be some structural elements that are preserved upon lifting a biomolecule to the gaseous state. For example, the collisionally activated dissociation behavior of a small, cyclic polypeptide was found to correlate to its solution structure [57]. The naturally isolated peptide is composed of a peptide "tail" region that inserts into a 7 residue "loop" section, and the tail is held inside by noncovalent forces. A synthetic version of the same sequence peptide was found to have the tail region external to the loop section. The MS/MS spectra for the two peptide forms show dramatically different fragmentation patterns, which are consistent with their respective solution structures. This system represents an example in which the peptide solution structure is consistent with the gas phase and the results suggest that probing the gas phase structure of biomolecules can be used to elucidate their solution phase geometry. Others have found a correlation between the relative dissociative behavior of noncovalent complexes and the solution phase stabilities. Podjarny and co-workers presented an interesting study comparing x-ray crystallographic data and ESI-MS analysis of the binding of aldose reductase inhibitors [58]. The energy of the electrostatic and hydrogen bond interactions involved in contacts be-

tween aldose reductase and the various inhibitors correlated with the energy required for dissociation of the 1:1 enzyme-inhibitor gas phase complex.

In general, the strongest correlations to the solution phase are found in the stoichiometry measurements and the observed relative abundances from the ESI-MS experiments. The early reports, such as the myoglobin study of Katta and Chait [22] and the experiments of Loo and co-workers with ribonuclease S protein [32,33], reflected the consistency between observation and expectation. Many more similar ESI-MS reports too numerous to describe have followed. The observation of complex formation usually is not simply the result of gas phase aggregation. The specificity of complexation and all the factors that drive two or more macromolecules to interact in solution are preserved in some form as solvent molecules are removed. Subtle differences in the composition of the interacting partners can impart a large effect in their interaction behavior. Alcohol dehydrogenase (ADH) proteins from equine and yeast show a high degree of homology and have very similar primary structures, yet equine ADH is an 80 kDa homodimeric complex and ADH from yeast is a 160 kDa homotetramer in solution. ESI-MS mass spectra reflected this difference in aggregation for the two species of ADH [35]. SH2 structural motifs in proteins are important in many signal transduction pathways of growth factor receptors. Our laboratory used ESI-MS to differentiate the interactions between src SH2 domain proteins and various phosphotyrosine-containing polypeptides [59]. The nonphosphorylated version of the high affinity phosphopeptide showed very low binding affinity towards the SH2 domain protein, consistent with previous solution phase biophysical studies. Moreover, relative affinities for a range of stereoisomer peptides were determined.

The positive correlation between the mass spectrometry observations and the solution phase behavior has suggested the application of ESI-MS for the determination of solution equilibrium binding constants. Several reports of this application have been reported. Henion's group demonstrated that data from ESI-MS experiments could be used to construct Scatchard plots for measuring the binding constants of vancomycin antibiotics with peptide ligands [60], and others have used similar ESI-MS methods for binding affinity measurements for the vancomycin system. We built upon the Henion experiments and applied the technique to the src SH2-phosphopeptide system [59]. The equilibrium dissociation constants of the oligomeric forms of citrate synthese binding to NADH, an allosteric inhibitor of the enzyme, was determined by Standing and coworkers [61]. Griffey and colleagues measured the dissociation constants for oligonucleotide binding to albumin protein [62], and their group have recently demonstrated the applicability towards small molecule binding to RNA targets using a high performance FTICR mass spectrometer [63].

The types of interactions that govern noncovalent binding in solution not only can play a role in the observed MS results but also can be distinguished by the ESI-MS gas phase measurements. Electrostatic forces are greatly strengthened in a solventless environment, and thus complexes held together by electrostatic interactions are extremely stable in the gas phase. Electrostatic interactions in solution are decreased by its dielectric constant [11,64]. We had observed that ions for protein-RNA complexes, noncovalent complexes between a highly positively charged molecule and a negatively charged macromolecule such as Tat peptide-TAR RNA complex [65] and the zinc finger human immunodeficiency virus (HIV) nucleocapsid protein NCp7-ψ-RNA complex [45], were extremely stable, as the complex was not observed to dissociate at very high collision energies. Inhibitor binding studies to HIV-1 TAR RNA highlighted differential binding modes [11]. Aminoglycosides such as neomycin are known to bind to RNAs through charge–charge interactions. The neomycin-TAR RNA complexes were not observed to dissociate in the gas phase. However, inhibitors with similar solution binding affinities that bind through hydrophobic-type means are extremely labile in the gas phase [66]. Interactions that are largely governed by hydrophobic interactions in solution appear to be weakened in vacuum. Robinson's group had noted that the apparent relative affinities measured by ESI-MS for small molecule hydrophobic

binding to acyl CoA-binding protein did not correlate with their solution affinities [67]. The different relative stabilities of gas phase interactions have implications for using ESI-MS to determine solution phase absolute and relative binding affinities. For compounds which bind to a target molecule with similar-type binding mechanisms, and thus may have similar gas phase stabilities, determining their relative binding affinities by ESI-MS should not be problematic. However, if hydrophobic interactions are in play, the lability of the gas phase complex may conspire to reduce the confidence of the mass spectrometry data.

Although the ESI-MS experiment utilizes a variety of means to remove water from the macromolecular complex, water is an important component that mediates many biochemical interactions. Can critical water molecules be observed by mass spectrometry? To differentiate the few essential water molecules from the bulk water comprising the solvation sphere (and beyond) is a difficult task because of the liability of the interaction between water and macromolecule. A few examples of water binding to protein have been observed, for example the mass spectrum of water binding to a small peptide, gramicidin S [68] and other larger proteins [69,70]. The mass spectrum of streptavidin shown in Fig. 4 shows broad, solvated peaks at lower metal capillary temperatures. Fabris and Fenselau observed water binding to the zinc-bound insulin hexamer complex [71]. Water molecules are coordinated to the zinc metal ions. A study on protein complexes important in HIV included the interaction between HIV protease dimer and an inhibitor [45,70]. Previous x-ray crystal structures of HIV protease have detected a high affinity water molecule, "water-301," critical for inhibitor binding. However, despite attempts to detect the water molecule bound to the protein complex using ESI-MS by reducing the temperature of the metal capillary interface and minimizing the collision energy used for desolvation, water binding was not observed. The liability of the interactions and/or short lifetime of this water molecule, which rapidly exchanges in solution, may preclude its observation by mass spectrometry. However, a recent report by Robinson's group suggested that in some examples, water may be observed [72]. Several discrete water adducts to an SH2 domain protein were observed in the ESI mass spectra, and the number of adducts appeared to correlate to that expected from crystallographic analysis.

The vast number of publications in the literature, review articles, and presentations at scientific conferences highlighting the positive correlation between the gas phase ESI-MS measurement and the solution phase system would seem sufficient validation of the technique. (Literature reports of negative results are fairly rare and are not particularly encouraged in the scientific venue. In some sense, this is unfortunate for the scientific community because one can often learn much from such instances as much as the successful experiment.) However, it is still important to be cautious when interpreting the results from such experiments. One cannot perform enough control experiments to be completely certain of the interpretation. At best, the ESI-MS results may be classified as "suggestive" or even "highly suggestive or supportive" of a given hypothesis.

6. Future prospects

The recent applications of ESI-MS for studying noncovalent complexes suggest the future picture of the field: bigger, faster, and more. Massive protein complexes can be measured with higher *m/z* range mass analyzers. Siuzdak et al. first suggested that extremely large complexes could be detected intact and the electrospray process was a nondestructive technique [73]. Rice yellow mottle virus and tobacco mosaic virus were "electrosprayed" into a triple quadrupole mass spectrometer. A glycerol-coated metal plate inserted in front of the electron multiplier detector collected the ions. Electron microscopy of the collected viral particles confirmed that the virus retained their structure, and further experiments demonstrated that the collected material was still viable. Later, Standing and co-workers attempted to measure mass spectra of an intact virus with a TOF analyzer [37]. Although the spectra showed an unresolved "hump" at high *m/z*, the estimated maximum charge was consistent with the extrapolated value from the

measurement of over 30 noncovalent protein complexes. The last several years have observed several reports of complexes in excess of 500 kDa [50]. The 800 kDa chaperonin GroEL 14-mer assembly was observed with ions at *m/z* 10000 by Rostom and Robinson [74]. The same group reported on the intact 850 kDa 30S subunit of the *E. coli* ribosome, composed of 21 protein components and the 16S RNA molecule [75]. Heck and coworkers showed baseline resolved (for the individual charge states) for the 0.5 MDa octamer, 1.0 MDa octamer-dimer, and 1.5 MDa octamer-trimer complexes of the vanillyl-alcohol oxidase enzyme [52]. The largest complex to date observed by ESI-MS is the 2.5 MDa bacteriophage MS2 virus capsid composed of 180 copies of the viral coat protein [51].

More emphasis on high-throughput ESI-MS analysis for the detection of noncovalent complexes may be a future trend, especially for the analysis of macromolecule—inhibitor complexes. The screening of potential inhibitors is an important aspect in the process of discovering more potent drugs for the pharmaceutical and biotechnology industries. Mass spectrometry may provide a relatively unique tool to achieve this end. Although mass spectrometry may not compete against the ultra-high throughput methods large pharmaceutical companies are using to screen tens of thousands of compounds per day, the ESI-MS technique can fill a role in more targeted analysis. Smaller compound libraries derived from combinatorial chemistry can be screened by masss spectrometry to aid in the optimization of structure-activity-relationships for small molecule drug design. Cheng and co-workers applied the FTICR mass spectrometer for the binding analysis of a library of over 200 components to the protein carbonic anhydrase [76,77]. The high resolution of FTICR mass spectrometry could resolve the simultaneous binding of the high affinity compounds. Ibis Therapeutics (Carlsbad, CA) is using ESI with FTICR mass spectrometry as a front-end screening tool for the discovery of novel inhibitors against RNA targets [78]. They estimate their throughput to be over 40,000 molecular interactions evaluated in less than 10 h [79].

Although ESI has been the focus of this review, the application of MALDI-MS for the detection of noncovalent complex has received some attention also [80–82]. The relative experimental simplicity and the speed of data acquisition would be a marvelous advantage for the analysis of protein complexes. In several instances, the observed stoichiometry and specificity of complex formation suggested by the MALDI-MS is consistent with expectations from the solution phase behavior. However, despite the reports of the positive correlation between MALDI-MS and the solution structure, much more work is necessary to assess the general applicability of MALDI-MS for noncovalent complexes. In the MALDI mass spectrum, the relative abundance of the free, unbound components is in some cases (but not in all cases) much greater than the complexed species, which does not correlate to the solution phase. The experimental variables that control the results obtained need to be mastered and understood before the application of MALDI-MS for the study of noncovalent complexes is generally accepted.

Confirmation of the stoichiometry of the binding partners is an important aspect of the mass spectrometry method, but obtaining the structural features of the noncovalent complexes will be the subject of more studies in the future. Dissociation of the gas phase complex can reveal insights of the assembly, as demonstrated by Robinson in their study of the intact ribosome [75]. The combination of limited proteolysis and on-line ESI-MS can be used to determine the topology of complexes [83]. HIV nucleocapsid protein, NCp7, contains two zinc finger structures [45]. Limited trypsinolysis in conjunction with ESI-MS monitoring was used to determine the binding site of an initial zinc ion exposed to NCp7. The noncovalent interactions are monitored by mass spectrometry during the time course of the proteolysis. The N-terminal zinc finger was found to be the primary binding site of the first zinc ion. Similar limited proteolysis experiments on the binding of NCp7 with a pentanucleotide suggest the participation of both zinc fingers, consistent with solution phase binding studies. These types of studies can augment the existing body of knowledge for a given biochemical complex and they can provide key preliminary insight on the structure of

supramolecular assemblies not amenable to x-ray crystallography or nuclear magnetic resonance.

It is clear that mass spectrometry has tremendous potential for the study of noncovalent complexes. A recent review on methods for monitoring protein interactions by Mendelsohn and Brent made the following conclusion: "As their sensitivity and ease of use improves, mass spectrometry will come to complement biological methods for detecting and analyzing protein interactions, and may eventually supplant them" [84]. This prediction can come to fruition in an accelerated fashion if the "noncovalent" (i.e. weak) interactions between the biological scientists and the mass spectrometrists themselves are strengthened and become "covalent" in nature.

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