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Perspective

Going Gently Into Flight: Analyzing Noncovalent Interactions by Mass Spectrometry

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Dedicated to Professor John Fenn, recipient of the 2002 Nobel Prize in Chemistry, for his extraordinary contributions to the development and implementation of electrospray mass spectrometry in the true spirit of scientific discovery and fun.

Historically, mass spectrometry came of age during the heydey of organic chemical analysis and structure determination, when the identification of molecular ions and their fragments complemented the fingerprinting of specific chemical functional groups by infrared and ultraviolet spectroscopies. As ever larger (i.e., >1000 Da) and more complex structures were characterized, increasingly by the use of NMR, mass spectrometry (MS) also rose to the challenge, with the development of newer ionization methods, including fast-atom bombardment[1] and matrix-assisted laser desorption ionization (MALDI)[2] techniques, as well as tandem[3] and secondary ion[4] mass spectrometry. Along the way, MS also made fundamental contributions to many other areas of chemistry, ranging from physical studies of the structures of gas-phase ions and their behavior,[5] to biological investigations of enzymatic reactions and other metabolic processes.[6]

A major breakthrough in the 'volatilization' of macromolecular and polymeric compounds (i.e., MW > 10,000 Da) propelled MS squarely into the interface of chemistry and biology. Using the process of electrospray ionization MS (ESIMS),[7,8] proteins, nucleic acids, and other substances that existed as charged species in aqueous solution could be transferred intact into the gas phase, usually as multiply-charged ions, by nebulization in the presence of an electric field (Fig. 1). By appropriate adjustment of the electric field, complete desolvation was achieved through collisions with inert gas molecules. The new ionization technique proved to be very robust and very gentle, and found numerous applications in liquid sampling methods[9] as well as in high-throughput chromatographic analysis.[10]

Just as it had done earlier with low-MW organic compounds, ESIMS soon revolutionized the identification and characterization of macromolecules. Since differently-charged protonated molecules gave different mass-to-charge ratios, all corresponding to the same parent structure, multiple independent MW determinations were possible in ESIMS. With precision at or below the single dalton, electrospray-based mass spectrometers became known as erstwhile truth machines for their ability to pinpoint structural assignments in natural, synthetic, or cloned macromolecules.[11] Equally impressive was the ability to analyze samples containing compounds in very low (femtomole) quantities.

During 1991, we published two reports describing the first mass spectra of noncovalent receptor-ligand, enzyme-substrate, and enzyme-product complexes using ESIMS.[12,13] Prior to this time such fragile biospecific complexes had not been observed to 'fly' as detectable, gas-phase ions in a mass spectrometer. Serendipity played a significant role in that work, which has since been cited over 400 times, according to the Institute for Scientific Information's ISI Web of Science. In studying the catalyzed hydrolysis of bacterial peptidoglycan, we had hoped to learn whether hen egg white lysozyme (HEWL) formed a transient, noncovalently bound glycopyranosyl cation or a glycosyl ester covalently attached to aspartate-52 of HEWL. By conventional thinking, any ions corresponding to the MW of glycosylated enzyme would have provided unequivocal evidence in support of the latter mechanism. The same rationale had earlier been used to identify covalently bound intermediates in the reactions catalyzed by nitrilase[14] and β-lactamase.[15]

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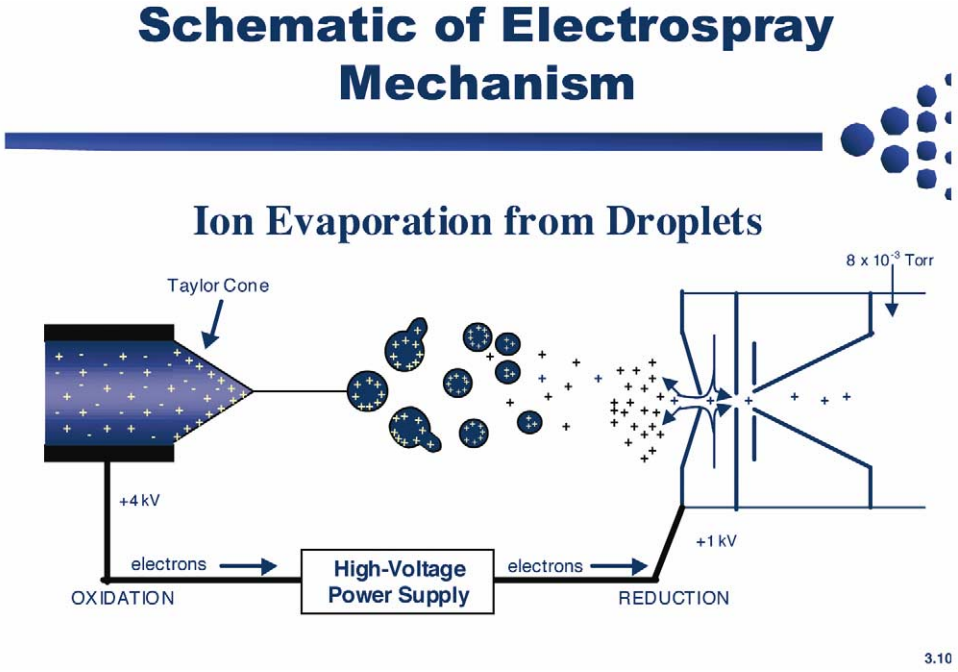

Figure 1. Schematic of electrospray mechanism.

To standardize a widely used lysozyme assay that converted the hexasaccharide of *N*-acetylglucosamine (NAG_6) to NAG_4 and NAG_2, the electrospray mass spectrum of a mixture of HEWL with NAG_4 was obtained. It displayed an unexpected (and strong!) signal for the noncovalent enzyme-product complex HEWL-NAG_4 that was isobaric with the corresponding covalently modified enzyme. Thus, the original goal of differentiating the two reaction pathways became inaccessible; however, the remarkable robustness of this noncovalent enzyme-product complex also led the investigation in an entirely new direction.

The finding that biospecific noncovalently bound complexes can be detected and analyzed under physiologically relevant conditions by ESIMS has been reproduced and extended.[16] While the technique portends many new applications of MS at the interface of chemistry and biology, it must also address lingering questions about the structural specificity of the gas-phase complexes formed by electrospray and other soft ionization techniques.[17] It has been reported that under certain conditions, noncovalent ion clusters or aggregates can be detected that do not reflect solution-phase noncovalent complexes.[18]

Two central questions are whether nonspecific complexation can be minimized by appropriate choice of conditions, and whether quantitative binding information can be retrieved from relative ion abundances. If so, the ability to quantify enzyme-substrate or receptor-ligand noncovalent interactions would make possible high-throughput screening of small molecules and/or peptides, either in massive parallel fashion or in combinatorial mixtures, by ESIMS. Such a technique would be of significant interest in the pharmaceutical and biotech industries, especially if the process could be automated. However, biospecificity in complex detection needs to be both reliable and reproducible for the method to be broadly useful as a research or diagnostic tool.

Several important issues in experimental design have emerged over the past ten years. The design of ion sources, adverse experimental conditions (including denaturing solution conditions, and heat on the interface), adventitious collision-induced dissociation (CID) in the intermediate regions of the spectrometer, and varying ion-focusing devices can all complicate the detection and observation of biospecific noncovalent complexes. The design and type of the mass analyzer can also be important factors, particularly when detecting large complexes. Both time-of-flight (TOF) and Fourier transform mass spectrometry (FTMS) represent mass analyzers that are particularly well suited to the determination of very high molecular weight complexes. In addition, TOF and especially FTMS provide accurate mass measurement and high mass resolution capabilities. This latter feature can be very helpful in determining charge state as well as specific structural information on noncovalent complexes.[9]

Differences between mass spectrometers may contribute to variability in detecting specific complexes, underscoring the need for suitable calibration methods. Collette et al. have shown that the internal energy imparted to ions in the ESIMS experiment affects fragmentation.[20] That energy correlates closely with thermal distributions, which increase linearly with the cone voltage.[19]

Studies are needed to examine how noncovalent interactions are affected as solution-phase noncovalent complexes undergo desolvation and escape into the gas phase. Hydrophobic effects that play an important part

in aqueous solution might be expected to diminish in the gas phase. Likewise, hydrogen bonding, ion–dipole interactions, dipole–dipole interactions, electrostatic effects, and van der Waals forces may be affected differently, so that the stability of a gas-phase complex may vary significantly from its solution counterpart.

Such differences can have surprising consequences. In a recent study using CID to measure the intrinsic stabilities of noncovalent complexes between various drugs and their target DNA duplexes, some ternary complexes having the drug bound in the minor groove underwent base loss prior to breaking the weaker, noncovalent bonds between nucleotide strands.[21]

Such concerns notwithstanding, the detection and analysis of noncovalent macromolecular complexes has been particularly successful using ESIMS. Numerous early observations of DNA and RNA duplexes, both with and without ligands, have been extended to nucleic acid triplexes and quadruplexes. The fact that solution specificity is maintained in the gas phase may likely be attributed to the significant role in base pairing played by hydrogen bonding, which typically is preserved during ion evaporation and desolvation.

Ready access to such noncovalent complexes made it possible to probe their structures in unusual ways. For example, the effects of mismatched bases on duplex DNA stability in the gas phase could be studied using CID, which revealed a tendency for covalent fragmentation within duplexes at the site of defects induced by the mismatched base pairs.[17] The secondary and tertiary structures of such gas phase duplex ions could also be investigated using H/D exchange reactions, and provided insight into the accessible exchange sites within double-stranded oligonucleotides.

At present, the study of noncovalent duplexes is limited to fragments comprising several hundred base pairs, mostly on account of practical considerations that have recently been reviewed.[17] As these obstacles are overcome, ESIMS analysis of gene fragments will increasingly be used in genomic analysis to detect single nucleotide polymorphisms, base deletions, and other unique changes in oligonucleotide structure.

Mass spectrometry has also proven useful in characterizing physiologically relevant noncovalent complexes of small-molecules. For example, Siuzdak et al. investigated the structure of the sialyl Lewis X complex with calcium, using both computer modelling and MS evidence to pinpoint the binding site of the metal ion.[22] Applications have also been developed for the high-throughput binding assays of drugs and drug candidates,[23] as well as for affinity screens to identify the unknown ligand(s) of a macromolecule of interest.[24]

Beyond its many applications in biomedical research, ESIMS has been adapted in a more chemical context to problems in classical molecular recognition[25] and to the analysis of multi-subunit molecular assemblies. For example, solution host-guest complexation and the assembly of calixarene[26] and other self-complementary molecules, which had typically been studied by NMR, could now be investigated by mass spectrometry.[27] Porphyrins functionalized with uracil-type heterocycles were analyzed by ESIMS, and shown to self-assemble into a supramolecular cage.[28] Noncovalent organic coordination complexes have also been observed. For example, the addition of various metal ions to polycyclic 2,6-disubstituted pyridines resulted in self-assembling double and triple helical complexes that were readily detected by ESIMS.[29] Ashton et al. observed the intermolecular association of highly ordered catenanes having interlocking rings composed of either macrocyclic polythers or cyclophanes.[30] Even noncovalent complexes on the mesomolecular scale (MW >3 kDa) were shown to be amenable to detection and analysis. In a landmark study, the supramolecular structure of commelinin, a plant pigment consisting of six anthrocyanin and six flavone subunits noncovalently assembled around magnesium ions, was confirmed by ESIMS (MW 8849 Da).[31]

Several exciting recent developments promise to extend the reach of modern biomedical MS. As the physical and chemical mapping of genomes moves to its next stage, advances in MS-based proteomic analysis are already finding widespread use.[32,33] Mass spectrometry also shows promise as a diagnostic tool, and the recent report by Demirev et al. on the detection of heme within parasitic vacuoles as a molecular marker for malaria[34] suggests that MS may be particularly useful in identifying the early onset of disease. In addition, ESIMS and MALDI have been used to sample solution conformations of macromolecules. Both techniques represent promising analytical methods for monitoring protein folding, even in very large, dynamical systems.[35]

Where next for MS? The possibility of detecting complexes held together by weak intermolecular forces, which was unthinkable only a few years ago, should enable the analysis of intracellular assemblies that comprise the macromolecular machines and architectures within living systems. The ESIMS analysis of biospecific protein-protein and protein-nucleic acid complexes is now routinely performed on commercial electrospray instruments. Most recently, multi-subunit superstructures corresponding to viral protein assemblies,[36] antigen–antibody and other immune cell complexes,[37] and even an intact virus,[38] have been studied using MS. The field of neuroscience has relied heavily on MS as an analytical tool in drug partitioning and metabolism,[39] and will likely employ ESIMS to detect physiologically significant macromolecular complexes in the central nervous system. One may also expect that ESIMS, already a powerful tool in structural biology, will find numerous applications in the emerging field of systems biology,[40] where noncovalent assemblies of networked gene and protein superstructures within cells play central roles in transducing cellular signals involved in proliferation, differentiation, and apoptosis. As a gentle technique for coaxing large, fragile ions into flight without fragmentation or dissociation, ESIMS promises to play an important role in unveiling many elusive secrets of biology.

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