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REVIEW

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A mini-review of mass spectrometry using high-performance FTICR-MS methods

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Abstract Structural characterization of macromolecules is currently delivering new insights into the behavior of individual molecules or molecular ensembles. Technological advances have made it possible to examine smaller and smaller amounts (down to single molecules) of larger and larger molecular systems. Mass spectrometry in particular is capable of the detailed study of extremely small quantities (down to a single molecule) of very large (biological) molecules. The advent of new ionization techniques such as electrospray and matrix-assisted laser desorption are mainly responsible for these advances. As a result, mass spectrometry has evolved into an enabling discipline that plays an increasingly important role in combinatorial chemistry, polymer science, biochemistry, medicine, environmental and marine science, and archaeology and conservation science. This paper will review a selection of methodological developments in the field of high-performance Fourier transform ion cyclotron resonance mass spectrometry for structural analysis of these macromolecules.

Keywords FTICR-MS · Proteomics · High-resolution · Peptides · ESI · MALDI

Introduction

With the increased complexity of analytical biochemical problems a greater need is arising for high-performance molecular characterization techniques. Mass spectrometry can provide structural details on many levels for many different classes of biomolecule. Proteins can now be analyzed by MS to reveal elemental composition, complete or partial amino acid sequence, post-translational modifications, protein–protein interaction sites, and even provide insight into conformational aspects. With these pieces of information new insights into the molecular behavior of individual molecules or molecular ensembles can be obtained directly from mass spectrometric data. One key advantage of mass spectrometric analyses over other analytical techniques is its capability to study extremely small quantities of very large molecules. Modern ionization techniques such as electrospray [1, 2] and matrix-assisted laser desorption and ionization [3] are directly responsible for these advanced possibilities. As a result, mass spectrometry has evolved into an enabling discipline that plays an increasingly important role in many areas of science, particularly recently in characterization of the function of all expressed proteins in many different biological systems, the rapidly growing field of proteomics [4].

Of all mass spectrometric methods Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) offers a unique combination of analytical qualities. An FTICR mass spectrometer combines high resolution, high mass-accuracy, non-destructive multichannel detection, long ion-observation times, the possibility of performing gas-phase reactions on trapped ions, and, most importantly, tools for structural analysis of large biomolecules. The FTICR-MS is a complete ion laboratory by itself. Since the early days of FTICR-MS [5, 6], it has now developed into a technique that can delicately separate single species from extremely complex mixtures ranging for crude oil to protein digests, sequence large proteins and DNA [7, 8], and probe protein–protein [9, 10] and protein–oligosaccharide [11] interactions. These capabilities are a direct result of its unsurpassed resolution and mass accuracy and the panoply of fragmentation techniques available to the investigation.

In this mini-review we have tried to provide a concise insight into the different areas in mass spectrometry where FTICR-MS is employed, discuss some of the latest tech-

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nical developments in ultrahigh-resolution mass spectrometry, and highlight a few spectacular examples of its use. Note that this paper is not intended as a complete detailed review but to point the reader to the unique capabilities of FTICR-MS by highlighting its contribution in selected areas of biomolecular mass spectral research.

FTICR-MS Basics

What is FTMS and what lies at the basis of its unique capabilities? To understand the merits of FTMS it is necessary to reiterate some of the basic concepts of the methodology. Several reviews that detail the principles of FTICR-MS much more completely are available in the literature [12, 13, 14]. In this section the essential experimental concepts and physical principals will be discussed briefly.

The analyzer cell

The heart of each FTICR-MS instrument is the analyzer cell which resides in a strong, homogeneous magnetic field. The analyzer cell can take on different geometries [15, 16] but generally consists of a front and back trapping electrode, two opposite excitation electrodes and two opposite detection electrodes, as indicated in Fig. 1. The analyzer cell is in fact a low pressure (10^{-10} mbar) Penning trap in which ions can be stored for extended periods of time. The timescale of the experiment is one of the first distinctions of FTICR-MS, and is extensively used to study slow (and fast) ion–molecule reactions, slow conformational changes in biomolecules, the dissociation of very large molecules with a large number of degrees of freedom, and many more processes that require both gas-phase ions and time to complete. This type of study cannot be performed in beam-type experiments given the short residence time of the ions in the mass spectrometer. The residence time of ions in an FTICR-MS instrument can range from milliseconds to hours, compared with several tens of microseconds in most beam-type experiments.

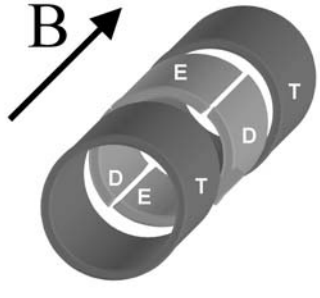


Fig. 1 The basic setup of an ICR analyzer cell, showing the two open trap electrodes (*T*), the excitation electrodes (*E*), and the detection electrodes (*D*). The direction of the magnetic field (*B*) is also indicated

Determination of *m*/*z*, excitation and detection

The purpose of the analyzer cell is to determine the mass-to-charge ratio of the ions stored in it. Each ion moving in a spatially uniform magnetic field will describe a circular, so-called cyclotron, motion as a result of the Lorentz force and the centrifugal force operating on it in opposite directions. The angular frequency of this motion is given by:

$$\omega_c = \frac{qB_0}{m} \tag{1}$$

where ω_c is the unperturbed cyclotron frequency and is solely dependent on the magnetic field B_0 and the mass-to-charge ratio m/q. Note that the cyclotron frequency is completely independent of kinetic energy and as such the translational energy of the ions is not relevant for the accurate determination of the m/q ratio of the ions trapped in the cell. Modern superconducting magnets with a field strength ranging between 3 and 15 Tesla usually drift only several ppm per year, so the cyclotron frequency can be an extremely accurate measure of m/q ratio.[1]

Now that the physical property that can deliver the m/q ratio accurately has been established we need to know how the cyclotron frequency of the ions trapped in the analyzer cell is determined. For that purpose the ions are exposed to an oscillating electric field that produces a net outward electric force on the ions for a limited period of time. This oscillating electric field is created by applying an RF potential on the two excitation electrodes and is referred to as the excitation pulse. The ions will only experience a net continuous outward force if the frequency of the oscillating electric field is resonant with the cyclotron frequency of the ions. To ensure excitation of all ions trapped in the ICR cell an RF pulse comprising multiple frequencies is employed such that all ions of different m/q ratios are exposed to a net outward electric force for the same amount of time. Many different excitation schemes have been used, some with better accuracy, some with more homogeneous amplitude, and some with different bandwidths, but all increase the radius of all ions (irrespective of their m/z ratio) to a significant percentage of the analyzer cell dimension. The radius after excitation is shown to be independent of m/q as long as the magnitude of the excitation signal is constant with frequency.

Not only is the radius of the ion cloud increased after excitation, all ions with the same m/z move coherently in a circular orbit. In the zero-pressure limit this coherent cyclotron motion can persist for infinite periods of time.

This coherently moving ensemble of charges at a radius close to the cell electrodes will induce an oscillating differential image current in two opposite detection electrodes. This image current is amplified and digitized

[1] Note that this is an idealized case. The actual motion of the ions also depends strongly on the radial forces which in Eq. (1) are assumed to be negligible. The radial field component introduces small but measurable effects on the accuracy of determination of m/q and is strongly dependant on ion density, geometry, and trapping voltage.

yielding a time domain signal or transient containing signal contributions, s, from all excited ions in the cell according to:

$$\sum_{i=1}^{k} s_i(t) \propto \sum_{i=1}^{k} N_i \exp(-t/\tau_i)\cos(\omega_i t)$$

where s_i is the signal contribution of ions with a cyclotron frequency ω_i, k is the different number of cyclotron frequencies related to the ions present in the cell, t is the time the signal has been digitized, and τ_i is the exponential damping constant. In the absence of ion–neutral collisions (i.e. the zero pressure limit) where $\tau_i >> t$, no significant damping will occur. The coherence length is directly related to the mass-resolving power that can be obtained. To obtain high resolution data it is imperative that the analysis takes place at the lowest possible pressure to ensure the $\tau_i >> t$ condition. After acquisition the time domain data are stored and subsequently Fourier-transformed to yield the cyclotron frequency spectrum, which in turn can be converted to a mass spectrum by simple calculation using Eq. (1).

The nature of an FTICR-MS experiment implies that the different analytical steps are separated in time. A typical sequence of events for a tandem mass FTICR-MS experiment is presented in Fig. 2. Before ion introduction the cell is emptied with a quench pulse. After the ions have been introduced into the cell a significant amount of time is required for the preparation/selection, fragmentation, excitation, detection, data storage, and Fourier-transformation events before the next experiment is started. The time involved in all of these events after ion introduction depends strongly on the type of instrumentation used and the analysis required. The total duration of a tandem FTICR-MS experiment in which no collision gas is used is approximately 1 s. Introduction of gas and pumping it away lengthens the experiments to several seconds.

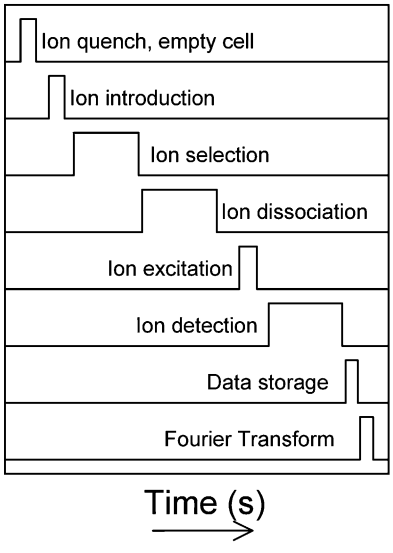


Fig. 2 An example of a tandem FTICR-MS sequence showing the order of the different time-separated process steps

External sources

Key to any mass spectrometric analysis is the production of ions from solid, liquid, or gas-phase samples in the ion source. Most if not all modern FTICR-MS instruments (especially those used for biomolecular mass spectrometry) use an external ion source in which the location of production of the ions is physically separated from the mass analysis. This separation enables not only the differential pumping between source and analyzer required for high resolution analysis, but also full flexibility in the type of ion source to be used for ion production and the use of different ion optical elements that can be used to store and/or purify the ion population before high-resolution analysis. Various ionization techniques have been coupled to FTICR-MS in this manner, ranging from electron-impact ionization (EI) in the early years to matrix-assisted laser desorption and ionization (MALDI) [3, 17] and electrospray ionization (ESI) [2, 18] at the onset of the proteomics era.

Internal energy, structural analysis, and dissociation techniques

Internal energy considerations

Will it still be possible to impart sufficient internal energy to these macromolecular systems such that structurally relevant fragments are produced? All molecules will eventually dissociate if a continuous net increase of internal energy can be realized. To achieve a net increase in internal energy, a method is needed in which loss of internal energy by emission of IR photons or cooling collisions [19] with the background gas is significantly lower than the energy input into the system. Time is of the essence given the low fragmentation rate and relatively high IR emission rates of macromolecules. Mass spectrometric methods that employ low-energy activation techniques combined with short analysis times will not induce sufficient fragmentation in macromolecular systems. Ion-trap mass spectrometry is the only MS technique that can accommodate these low reaction rates.

The strong relationship between internal energy and gas-phase structure or conformation is an additional reason for the growing interest in studies of the internal energy of protonated biomolecules. A thorough study is needed into the different pathways by which energy can be deposited into and extracted from a macromolecular system. It has already been shown that gas-phase activation and dissociation studies on isolated/trapped macromolecular ions potentially relate bond energies with structural/conformational information in solution [20]. The determination of activation and dissociation energies of high-molecular-weight molecules and their complexes will provide insight into the correlation between the structures/conformations and functionality of a wide scope of polymeric systems ranging from peptides, proteins, and/or their complexes to industrial synthetic polymers [21, 22, 23, 24, 25].

Many of these studies use collisional activation to increase the internal energy of the ion. Typically, after a collision gas has been introduced into the cell the kinetic energy of the ion of interest is increased by use of an RF pulse. On colliding with a neutral gas molecule, some of the kinetic energy can be converted into internal energy. The internal energy deposition function (EDF) during the collisional activation has been investigated in several studies including the "thermometer" ion method by Cooks and co-workers [25], the "deconvolution" method by Vekey [26, 27], the recursive internal energy distribution search (RIEDS) method by Futrell [28], the probability theoretical method [29, 30], and several other theoretical studies of the efficiency of the energy transfer on collisional activation [31, 32]. The effects of size and structure of the peptide, mass of the collisional activator, and the collisional energy have been addressed in these studies. Furthermore, the different effects on the energy-transfer efficiency of peptides with the α-helix and β-sheet structures have been described. Klassen and Kebarle have also obtained accurate activation energies from studies of the threshold energy [32, 33] for formation of fragment ions from protonated peptides [34]. In the meantime, the relative dissociation energies for the major fragments of some protonated peptides were reported by Hanley [35, 36]. In addition, the energy deposited during sustained off-resonance radiation collision-induced dissociation (SORI-CID) has also been reported recently by Futrell [37, 38]. In other studies, blackbody infrared dissociation [24, 39, 40, 41, 42, 43, 44] has been used to study the energetics of fragmentation of large molecules, to extract the activation energies for dissociation. Experimental determination of the effect of impact conditions during activation is very important in extending or examining the capability of application of collisional activation to the study of large biomolecules.

Tandem MS strategies

The high-resolution, high-mass capabilities (especially with ESI sources), and the possibility of performing MS^n on a given sample make FTICR-MS a valuable addition to the proteomics arsenal. In addition to unequivocal identification of proteolytic fragments by accurate or exact mass measurement, direct MS–MS analysis of intact protein ions has become routine. In this section we inspect several strategies for gleaning structural information from proteins, protein complexes, and protein mixtures.

The essence of the MS–MS experiment is selection of the ion for analysis from a mire of ions. Because even a simple sample of a single peptide or protein can result in many ion species and each of these is further complicated by the natural distribution of the component elemental isotopes, the monoisotopic species of the analyte must be temporarily preserved while ejection of the others is effected. These strategies are themselves the subjects of review articles; nevertheless, each takes advantage of the unique properties of the FTICR-MS for manipulation of ion motion.

By far the most facile and widely used method of ion isolation is stored-waveform inverse Fourier transform (SWIFT) [45] excitation. It should be noted that adequate isolation can also be achieved by use of correlated harmonic excitation waveforms (CHEF [46], also known as correlated chirp excitation), and quadrupolar excitation/axialization (QEA) [47, 48, 49]; SWIFT is described here merely as an example.

The SWIFT waveform is constructed on an acquired mass spectrum such that whole ranges of ions are chosen for excitation to a given radius and others are left with no net excitation. These ranges, specified in the mass-to-charge domain of the spectrum, are translated back to the frequency domain. Finally, this desired power spectrum is inverse Fourier-transformed into the time domain and the resulting waveform is piped to the cell via a digital-to-analog converter (DAC). If the radius of excitation exceeds the physical dimensions of the Penning trap the ions are neutralized on contact with the cell and pumped away, leaving only the target ions. Single-isotope species can be isolated in this manner from even high charge-state distributions (e.g. from ESI) as demonstrated in Fig. 3 on the 15+ charge state of cytochrome C. Isolation of the monoisotopic peak of an ion enables straightforward MS–MS interpretation, and MS–MS of an arbitrary isotopic peak can identify the ion's isotopic mass defect and, therefore, the monoisotopic mass of the isotopic envelope for that ion.

Before expanding the discussion on MS–MS strategies, it is useful to examine the various types of fragments expected from proteins and peptides. Along the amide backbone of any peptide there are typically three points of possible cleavage referred to as a, b, and c from the charge carrier if the N-terminus is intact and x, y, and z from the C-terminus charge. The broken bonds are identical except for the direction of the location of the charge so that a and x, b and y, and c and z ions are complementary to one another [50].

Collision-induced dissociation (CID)

Collision-induced dissociation (CID) is performed by exciting the isolated ion to a higher cyclotron radius (and, therefore, to higher kinetic energy) in the presence of an increased background pressure of a neutral gas. Collisions occur as a result of the reduced path length and increased ion velocity; this leads to a transfer of energy to the two collision partners with mostly kinetic energy transfer to the neutral and conversion to internal energy in the ion. This internal energy is rapidly redistributed about the ion's structure and, if it locally exceeds the energy required for dissociation, the ion breaks apart. CID largely produces b and y type ions.

There are multiple strategies for increasing the kinetic energy of the ions but the most commonly used are resonant excitation and its gentler cousin sustained off-resonance ion (SORI) excitation. In resonant excitation the exact cyclotron frequency of the ion is used to increase the ion radius rapidly to a pre-calculated kinetic energy and

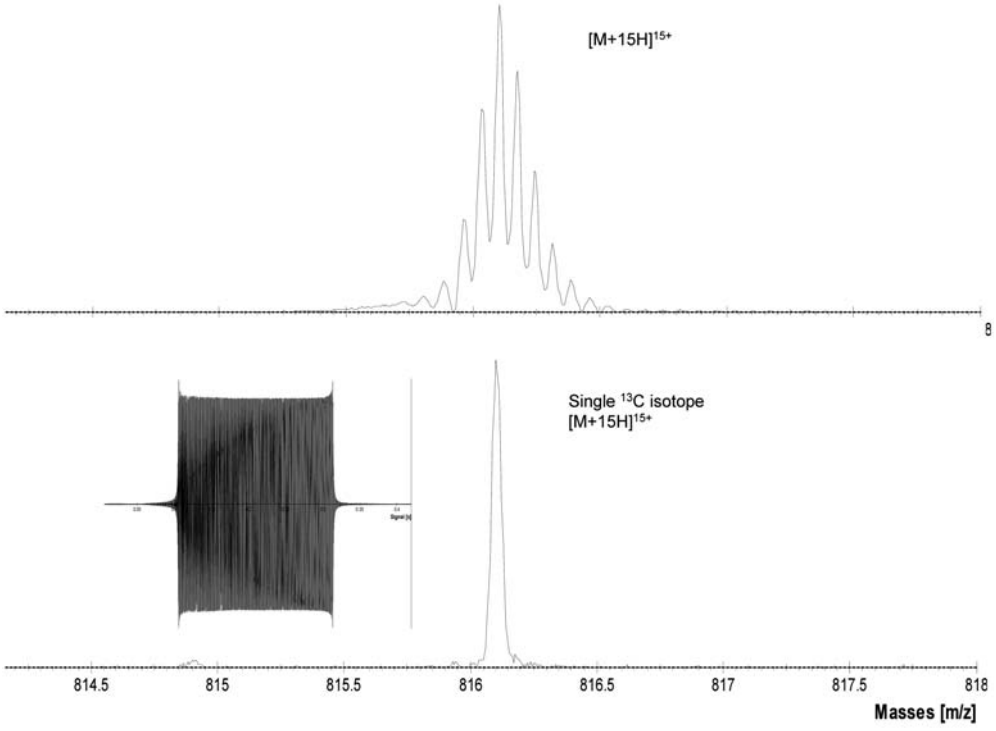


Fig. 3 High-resolution FTICR-MS isolation of a single isotope from a 12 kDa protein using a SWIFT ion isolation pulse. This figure illustrates the ease of generating isotopically pure species for further experiments, be it structural analysis by fragmentation or H/D exchange

the product ions subsequently measured. Although this is the most straightforward approach, an excess of internal energy is necessarily imposed, resulting in a more complicated product spectrum because of side-chain losses and possible ion rearrangements.

SORI is much softer, enabling the operator to focus on the lowest energy fragmentation pathways. In SORI, the precursor ion is subjected to dipolar radiation at a frequency slightly offset from its cyclotron radius. This results in the ion alternately increasing and decreasing in radius (and, therefore, kinetic energy) over the course of the SORI excitation so that collisions deposit lower internal energies per collision (typically ≤0.3 V), but many more collisions occur (hundreds per second, typically). As the internal energy accumulates (assuming that cooling mechanisms such as infrared radiative cooling [51] are slower than the internal energy build-up) it is rapidly randomized throughout the ion and the lowest dissociation pathways are sampled. The product ions formed in this way have cyclotron frequencies separated far enough from the SORI frequency that their continued excitation is minimal and any subsequent collisions only serve to cool their residual kinetic energy.

Slow, low-energy deposition into a precursor ion can also be achieved by infrared irradiation. This can be achieved as simply as thermally heating the local environment of the ICR cell, as in blackbody infrared radiative dissociation (BIRD); however, use of single line infrared laser irradiation to pump energy into the ions is now more common. This second technique, known as infrared multiphoton dissociation (IRMPD) involves firing a relatively high-powered IR laser through the center of the cell and, as in SORI, slowly climbing the dissociation ladder. For a typically applied CO_2 laser with a fundamental line at 10.6 μm, the steps in energy are 0.12 eV. Once more, the lower dissociation pathways are sampled but the product ions are not removed from further activation as in SORI; they continue to absorb photons and can continue to fragment, leading to more complicated spectra. This continued activation has been avoided in some experiments by using SORI-style excitation to drive the target ions into and out of a laser beam positioned parallel to the trap axis but offset from the center by a few millimeters.

Electron-capture dissociation (ECD)

Electron-capture dissociation (ECD) was introduced in 1998 by Zubarev et al. [52] as a new tandem mass spectrometric technique for study of polypeptides and proteins. The most important difference between ECD and other, more conventional, techniques, for example low-energy CID and infrared multiphoton dissociation (IRMPD) is that the last two methods generally increase the internal energy of an ion in small steps until the weakest chemical bonds are cleaved to yield mainly b and y″ ions [50, 53]. With ECD, instead, it is believed that the 5–7 eV energy released by neutralization during the electron-capture event can cause cleavages before the energy is randomized [54]. Compared with CID, ECD results in extensive fragmentation of the backbone of small proteins [55, 56]. ECD does not seem to generate many internal fragment ions, in contrast with CID [57]. ECD involves the trapping of multiply charged cations and a subsequent exposure to low-energy (thermal) electrons. The capture of an electron by a multiply protonated peptide or protein ion leads to the formation

Table 1 Comparison of the amount of sequence coverage for peptide-sequence analysis directly in the Penning trap of the FT-ICR-MS, using CID, ECD, and CID/ECD combined. Combined CID/ECD numbers are obtained using all data presented in the references cited between the brackets

Peptide	CID	ECD	Combined
Insulin B	76% [107]	45% [108]	86% [107, 108]
Mellitin	80 [109], 90% [110]	68% [108]	84 [108, 109], 96% [108, 110]
Substance P		70 [108], 80% [111, 112]	
Neurotensin		87% [112]	

of an odd-electron $[M+nH]^{(n-1)+\cdot}$ reduced molecular ion. Electron capture is believed to occur at a protonated site, thereby releasing an energetic $H^{\cdot}$ atom [56]. This $H^{\cdot}$ atom can initiate a radical site reaction [58]. In peptides common high $H^{\cdot}$ affinity sites are the carbonyl groups [54]. This is reflected by the preferred electron capture-induced formation of c and z· ions [54, 59, 60].

It has been shown that ECD is useful for identification and location of post-translational modifications. Tandem mass spectrometry of 28-residue peptides containing *gamma*-carboxylated glutamic acid residues using either collisions or infrared photons resulted in complete ejection of the *gamma*-CO_2 moieties before cleavage of peptide backbone bonds. ECD, on the other hand, cleaved backbone bonds without ejecting CO_2, enabling direct location of this labile modification [61]. Sulfated side-chains were retained in ECD backbone fragmentation of a 21-mer peptide whereas CID caused extensive SO_3 loss [61].

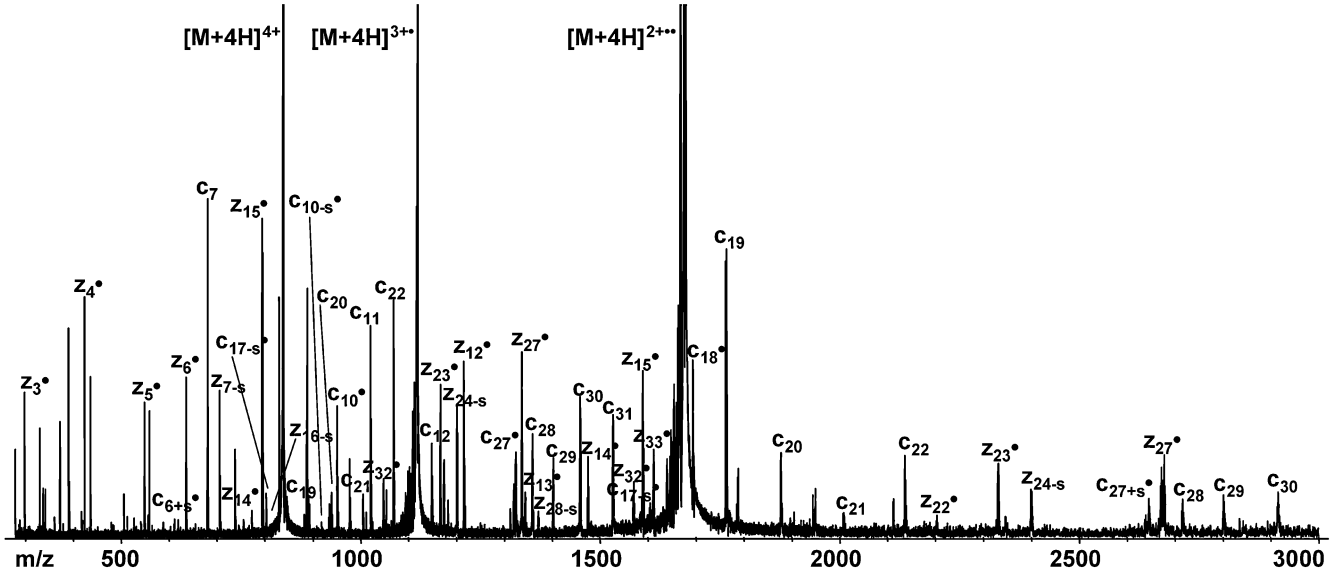
Mirgorodskaya et al. [62] investigated O-glycosylated peptides using ECD. The observed c series provided direct evidence of the glycosylation sites, with no glycan (GalNAc and dimannose) losses observed from these ions. Fragmentation of N-glycosylated peptides, using CID, typically yielded product ions that result from dissociation at glycosidic bonds, with few peptide backbone cleavages. ECD provided c and z· ions derived from the peptide backbone, with no observed sugar losses.

ECD spectra of phosphopeptides appeared less complex than CID mass spectra of these species. ECD of multiply protonated phosphopeptide ions generated mainly c and z· ion series. No loss of phosphate groups or phosphoric acid from intact phosphopeptide ions nor from the c and z· fragment ion products was observed in the ECD spectra [55]. Shi et al. performed the first direct site-specific characterization of this key post-translational modification on a protein without its prior degradation [63].

ECD has also been shown to be an effective fragmentation technique for characterizing the site and structure of the fatty acid modification in ghrelin, a 28-residue peptide that releases growth hormone with an unusual ester-linked *n*-octanoyl (C8:0) modification at Ser-3. ECD cleaved 21 of 23 possible backbone amine bonds, resulting in a higher sequence coverage than obtained by CID [64]. In Table 1 we compare peptide sequence coverage for three different peptides studied with different dissociation techniques, including the combination of ECD and CID.

ECD of polypeptides containing disulfide bonds has been studied. A polypeptide containing a disulfide bond resulted in far more extensive fragmentation than polypeptides containing no disulfide bond. This was rationalized by the fact that disulfide bridges have even higher $H^{\cdot}$ affinity than carbonyl groups. Usually one of the major product ions in ECD spectra is the reduced $[M+nH]^{(n-1)+\cdot}$ ion. In the ECD spectrum of a 10-kDa protein containing an S–S bond little of this product ion remained. Dominating fragment ions were ions resulting from cleavage of the disulfide bridge. In comparative CID experiments no such fragment ions were observed [60], which indicated that

Fig. 4 ECD FTICR-MS tandem mass spectrum of the $[M+4H]^{4+}$ ion of nisin, showing localization of intramolecular monosulfide bridges



ECD is far more effective than CID at cleaving disulfide bridges [65]. In proteins cyclized by disulfide bridges capture of a single electron can cleave both a disulfide bridge and a backbone bond in the same ring or even both disulfide bonds holding the two peptide chains of insulin together [60].

Four thioether bridge-containing lantibiotics have been studied by use of ECD. Most importantly, c· and z ions were observed in the ECD spectrum presented in Fig. 4. Those fragments resulted from the cleavage of both a backbone amide bond and the thioether bond in a lanthionine bridge. This specific fragmentation could be used in the future as a tool for identification of the C-terminal attachment site of lanthionine bridges in newly discovered lantibiotics. Comparative low-energy CID showed no such specificity [66].

ECD has been combined with conventional fragmentation methods. In comparison with CID this "activated ion" (AI) ECD provides more extensive, and complementary, sequence information [59].

Breuker et al. showed that ECD can be used to study tertiary structures of protein ions in the gas phase. The unfolding enthalpy of the native state of ubiquitin in solution is 5 to 8 times that of its gaseous ions, as determined by ECD. In solution two-state folding occurs; ECD pointed to three-state folding in the gas phase. ECD data on non-covalent bonding in the 5+ to 13+ ions, determined overall in 69 of the 75 inter-residue sites, showed that thermal unfolding proceeds via a diversity of intermediates whose conformational characteristics also depend strongly on charge site locations. As occurs with increased acidity in solution, adding 6 protons to the 5+ ions completely destroys their tertiary non-covalent bonding [67].

FTICR-MS and proteomics

The attraction of using FTICR-MS for proteomics [4, 68, 69] is that the high mass-accuracy can be used as an additional constraint in searches of genome sequence data bases and the high resolution inherent to the technique enables many components to be analyzed simultaneously. FTICR-MS proteomics research can be separated into several regimes:

1. intact protein analysis (top-down analysis)
2. analysis of proteolytic digestions (bottom-up analysis)
3. mapping three-dimensional structure (conformational analysis)

Armed with the ion manipulation and dissociation techniques described in the previous section, and a variety of wet chemical methods beyond the scope of this review, the analyst is faced with the daunting task of accurately sequencing proteins. The choice between fragmentation of the protein by an enzymatic or other chemical method before mass analysis (bottom up) versus examination of whole (or largely so) proteins followed by MS*n* techniques (top down) is made on the basis of factors outlined in the next few paragraphs.

Top-down analysis; intact protein analysis

One of the most direct techniques for protein analysis is the top-down method in which protein ions or large protein fragments are analyzed intact rather than being fully digested before analysis. Electrospray ionization is usually used with MS–MS experiments following. Most top-down experiments on protein ions have been carried out on FTICR-MS instruments, because of the mass-resolution requirements and the ion-storage capabilities.

The protein ion (or large peptide) is presented to the mass spectrometer and interrogated with one of the various MS–MS techniques. The mass of the intact protein is especially useful, because it can point to post-translational modifications not indicated by the DNA sequence information. A further advantage is that a proteolytic digest, as in the bottom-up approach, can result in a crowded (or "rich") spectrum with many peaks in a narrow mass range; the technique is also sensitive to autodigestion of the proteolytic enzyme and digestion of any other protein present in the initial reactor (keratins from skin flakes are often found). The top-down approach also results in many peaks, but because they are derived from a multiply charged ion these products are spread out over a larger number of mass-to-charge channels (i.e. the product ions can have any charge state from 1 to n where n is the parent ion charge number) and because purification occurs in the gas phase, only product ions from the parent species are inspected.

Considerable effort has been expended in the FTICR-MS community to identify intact proteins using accurate mass measurements combined with other constraints.

Smith and co-workers used a combination of capillary isoelectric focusing (CIEF) and FTICR-MS to obtain 900 individual protein signals from *Escherichia coli* from only 300 ng proteinaceous matter obtained from cell lysates [70, 71]. By culturing the cells in isotope-depleted media the mass accuracy of the measurements was improved and enabled some proteins to be identified solely on the basis of mass accuracy and isoelectric focusing [72]. More recent work used isotopically labeled amino acids to determine the number of these amino acids in a protein [73]. When combined with accurate-mass mass spectra this additional constraint enhanced protein identification confidence to the extent that post-translational modifications could be identified.

Wilkins and co-workers have recently shown how bacterial proteins can be observed and identified directly from whole-cells (i.e. without cell lysis and purification) by use of MALDI-FTICR-MS [74]. High-accuracy mass measurements and high-resolution isotope profile data were used in that work to confirm post-translational modifications proposed previously on the basis of low-resolution mass measurements, clearly illustrating the added value of FTICR-MS in proteomics.

Bottom-up analysis; analysis of digestion products

In the bottom-up (or shotgun, or protein mass fingerprinting) approach to determine a protein's amino acid se-

quence, the target protein is proteolytically digested to produce a mixture of small fragments (typically under 5000 Da), and the digest mixture analyzed by mass spectrometry. Because the number of amino acids is limited, the accurate masses of the digest ions can be readily matched to their component residues. Sequencing of these peptide ions can be achieved by fragmentation methods (MS–MS) or by parallel digestion with different proteolytic enzymes; overlapping sequences can be matched to show the full protein sequence and to identify local variations (deamidation, crosslinking, post-translational modifications, etc.).

As an alternative to deriving the protein sequence from a single known DNA sequence, database searching and fitting of these peptide masses can be used to probe the entire suite of known proteins. In this technique the resulting mix of peptide ion masses is fitted to multiple known protein sequences by a database-searching algorithm, thereby greatly reducing the total dataset. Redundant masses are then characterized by MS–MS further reducing the probable hits. The bottom-up approach is well established and several instrument companies have developed automated methods. Although peptide fingerprinting techniques are capable of identifying suspect proteins from a database without a priori knowledge of protein identity, it is susceptible to data skewing, because many of the peptides from proteolytic digestion might have low ionization efficiencies resulting in lower levels of sequence coverage.

Proteolytic digestion of a protein followed by mass analysis of the resulting peptides and/or tandem mass spectrometry of one or more of these peptides is the most common method of performing mass spectrometry-based proteomics. The accurate-mass capabilities of FTICR-MS translate as more specificity. Figure 5 shows the percentage of unique tryptic peptide masses predicted for yeast and for *Caenorhabditis elegans* for different mass accuracy [75]. It is readily apparent that more accurate measurement can provide a more specific match. Indeed, with sub-ppm mass accuracy Smith and workers have shown that for small proteomes a significant proportion of tryptic peptides (85%) are protein-specific [76]. Such direct identification permits fast, high-throughput analysis.

In addition to accurate-mass measurements FTICR-MS offers unparalleled mass-resolution capabilities. For example, Bossio and Marshall have demonstrated how FTICR-MS can be used to resolve a phosphorylated peptide from a sulfated peptide, a mass difference of just 9.5 mDa [77]. These high-resolution accurate-mass measurements were, moreover, made in the presence of other compounds. It is this ability to record high-resolution accurate-mass mass measurements of multiple compounds simultaneously that has driven the interest in FTICR-MS-based proteomics [78, 79, 80] (and natural product [81] and synthetic polymer analysis [82]).

Owing to ionization biases when analyzing multi-component mixtures [83, 84] FTICR-MS-based proteomics experiments are routinely preceded by a separation step, frequently liquid chromatography [78, 79, 80, 85]. Although this increases the number of peptides detected, it also results in a variable number of macromolecular ions

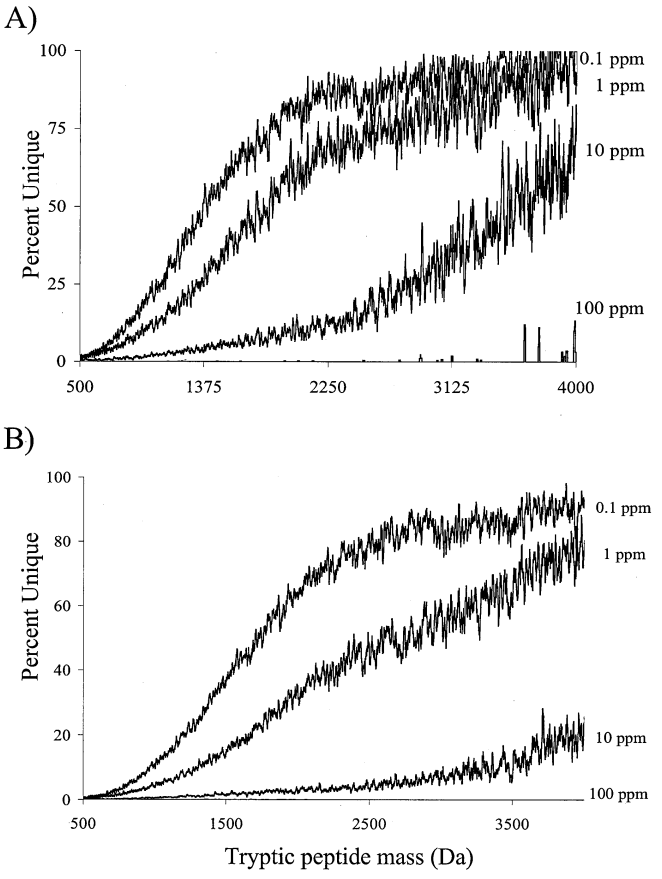


Fig. 5 Mass accuracy and unique peptide identification capabilities (reprinted with permission from reference 75)

being detected in each FTICR-MS scan. This adversely affects the mass accuracy of the measurement [86]. To improve the probability that the correct protein has been identified, methods using additional constraints [87], error and connectivity analysis [80] and instrumental improvements to optimize mass accuracy have been implemented [88]. The improved mass accuracy throughout the LC run resulted in a tenfold increase in the number of tryptic peptides identified. Finally, sensitive and high-speed tandem mass spectrometry techniques capable of performing tandem mass spectrometry on a time-scale suited to LC separations have been developed [89, 90, 91].

The two approaches (top-down and bottom-up) can be used in concert, in that the bottom-up approach generates a large pool of species that have in common their mode of derivation from the intact protein whereas the top-down approach starts with the protein mass (or that of a high-m/z species from incomplete digestion or MS–MS) and produces fragment ions by one of several ion–molecule or ion–ion reaction schemes. Both have shown near 100% sequence coverage for proteins up to 66 kDa.

Mapping three-dimensional structure

By use of chemical cross-linking, proteolysis, and mass spectrometry it has proven possible to obtain information

Table 2 An overview of the amount of data produced by different mass spectrometric techniques. Clearly the evolution in instrumentation, computational possibilities and resulting quality of mass spectrometric information has led to the exponential increase of dataset sizes originating from a single experiment

MS Technique	Time per spectrum	Number of spectra	Single dataset size	Remarks
Quadrupole MS	~1 s	1	16 kB	Single spectrum
TOF-MS	$1–10^{-4}$ s	1	64 kB	Single MALDI spectrum
GC–MS	~1 s	3600	20 Mb	Sector MS, 1 h measurement
MALDI-Imaging MS	~1 s	10^4	650 Mb	Array of 100×100 MALDI spots analysed
Cap-LC–FTMS	~1 s	800	1.2 Gb	Continuous HR acquisition, all data stored

on protein folding, although the complexity of the reaction mixture can hamper the analysis. The high resolution and mass accuracy of FTICR-MS is clearly advantageous for such complex samples [92]. FTICR-MS mass spectrometry folding studies have also been performed top-down, by sequentially fragmenting the cross-linked protein to identify cross-link positions [93].

FTICR-MS data processing and informatics

Developments in high-resolution proteomics have narrowed an existing bottleneck in bioinformatics – high-quality interpretation of acquired high-quality MS data. The ability to generate thousands of high resolution MS spectra per day, and the growing availability of this approach, requires new tools moving away from manual methods for data analysis and interpretation. It also emphasizes the need to transfer the advanced expertise and knowledge of an expert human experimentalist and/or user through easily accessible software tools.

An FTICR-MS analysis provides extremely detailed mass spectrometric information from complex mixtures. It has been demonstrated that it is possible to analyze complex mixtures, for example synthetic polymers, tryptic peptide mixtures, naturals products such as crude oil [81, 94], or humic substances [95] with FTICR-MS. For these samples the resolution is high enough to resolve and identify the individual components in the mixture. The resulting high-resolution mass spectrum can contain several thousand individual peaks. The result of this high information density is that individual datasets are becoming very large and the information density in them very high.

In these dense spectra some peaks provide relevant information and some peaks are redundant. Analysis of a single multicompound high-resolution spectrum is a daunting task for an individual researcher. Various new algorithms [96, 97] and software packages have been developed to provide some form of data reduction, automated spectral interpretation, or structural analysis. Comparing results to existing external databases is now more rule than exception in modern proteomics [98, 99, 100]. Protein identification by mass spectrometry (MS) and sequence-database searching is well established. The proteomics community has witnessed a proliferation of analytical strategies for protein identification. MS-based analytical strategies comprise three components – MS analysis, spectra–data base sequence correlation methods, and sequence databases. Multiple strategies are now applied simultaneously to increase sensitivity, throughput, and reliability of the characterization of proteomes.

If the complexity of the mixture to be analyzed increases, chromatographic separation is often employed to reduce the amount of information in a single mass spectrum. This capability for dealing with increasingly complex mixtures is exploited by many research groups and facilities. Table 2 illustrates how the amount of data produced by mass spectrometric techniques has increased over the years.

A typical 20-minute high-resolution LC-FTICR-MS experiment produces a dataset of 1.2 Gbyte if all raw data is stored. Increasing numbers of these larger datasets are being produced in various high-throughput approaches. The question arises how to deal with storage space, automatic data processing, and extracting the relevant information out of these data sets that will answer the scientific questions posed to the system? Automatic high resolution data analysis techniques are already developed, but how to make good use of them? So far there is no clear answer yet, and here lies the challenge of the future. One possible contribution might come from current GRID and virtual laboratory developments [101, 102]. These approaches build on shared mass spectrometric resources in an attempt to make mass spectrometric expertise available to the non-expert user and to provide the computational resources for data processing, database searching, and meta-data generation. Descriptive meta-data, in particular, will become important in the future for rapid searching of large data sets.

Summary and outlook

FTICR-MS has matured to become an indispensable tool in bioanalytical studies for analysis of complex mixtures, such as encountered in proteomics or natural product studies. We have described a selected set of technological and application developments that have strongly contributed to this maturation process. The examples selected indicate that it remains important to address high-end analytical methods from a fundamental, instrumentation, and application point-of-view to obtain analytical information of the highest quality. In recent years we have seen how FTICR-MS has become available as a "routine" high-resolution

detector on a linear ion-trap mass spectrometer. The success of this approach is indicative of the need for easy-to-use high-resolution mass spectral studies. This is an area that is still developing strongly.

In the future we will see other high-end applications and methods developed into research grade instruments, for example the coupling of microscale separation and ionization systems [103]. New smaller introduction systems will lead to new multiplexing capabilities between different sample streams in pharmaceutical laboratories. We expect to see the area of imaging mass spectrometry [104, 105] for study of biological surfaces extend into the realm of high-resolution FTICR-MS. This will combine high spatial resolution with high mass resolution, leading to a unique screening tool for biological tissue. Also, imaging as a parallel detection technique has the potential to become instrumental in the read-out of biomolecular arrays or other screening devices. Conformational detail obtained by mass spectrometric studies will be greatly enhanced by coupling of techniques such as FAIMS [106] with high-resolution trap-based mass spectrometers. This will enable a concerted study of biomolecular conformation through ion-mobility separation and gas-phase H/D exchange. These developments will all enhance sample throughput and data production even further. New methods of dealing with larger and larger datasets will need to be developed. New tools for combining analytical information from different instrumental or data resources, not necessarily located in a single building, will be essential to the future success of high-resolution mass spectral technology.

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