What Does the Future Hold for Top Down Mass Spectrometry?

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Mass spectrometry (MS) research has revolutionized modern biological and biomedical fields. At the heart of the majority of mass spectrometry experiments is the use of Bottom Up mass spectrometry methods where proteins are first proteolyzed into smaller fragments before MS interrogation. The advent of electron capture dissociation and, more recently, electron-transfer dissociation, however, has allowed Top Down (analysis of intact proteins) or middle down (analysis of large polypeptides) mass spectrometry to both experience large increases in development, growth, and overall usage. Nevertheless, for high-throughput large-scale proteomic studies, Bottom Up mass spectrometry has easily dominated the field. As Top Down mass spectrometry methodology and technology continue to develop, will it genuinely be able to compete with Bottom Up mass spectrometry for whole proteome analysis? Discussed here are the current approaches, applications, issues, and future view of high-throughput Top Down mass spectrometry.   (J Am Soc Mass Spectrom 2010, 21, 193–202) © 2010 Published by Elsevier Inc. on behalf of American Society for Mass Spectrometry

Mass spectrometry-based proteomics research has led to a wealth of discoveries in biological fields ranging from the identification of proteins essential for fertility [1], biomarkers of human disease state [2], whole proteome identification [3], and signal transduction pathway analysis [4]. For about two decades, researchers have been utilizing Bottom Up mass spectrometry for protein sequence analysis (i.e., enzymatically or chemically digesting proteins into small peptides before MS analysis) due to the ease of separations at the peptide level, predictable fragmentation when threshold methods are used, and vast commercialization of both hardware and software options. These characteristics have allowed Bottom Up characterization of proteins to evolve into high-throughput, sensitive, and efficient analyses of very complex mixtures, and even allow investigations at the proteome-wide level. In very recent years, an astounding amount of both hypothesis driven and discovery high-throughput large-scale datasets using strictly Bottom Up methods have emerged. For example, Wolf and coworkers have reported the detection of over 4600 proteins in *Saccharomyces pombe*, equal to roughly 90% of the predicted proteome following extensive proteome fractionation and multiple MS analyses [5]. Mann and coworkers have also recently published one of the largest datasets known to date having identified slightly over 7000 proteins using a novel sample preparation technique termed filter-aided sample preparation, which is currently the largest reported proteome for a single MS experiment [6]. With regards to protein post-translational modifications (PTMs), Bottom Up mass spectrometry has also been used to generate some quite large datasets as well. Following strong cation-exchange and immobilized metal affinity chromatography enrichment of phosphopeptides, Gygi and coworkers localized a total of 13,720 phosphorylation sites on proteins extracted from *Drosophila* embryos with a false discovery rate of less than 1% [7]. This type of large scale experiment allows for such a wide amount of phosphorylated peptide sampling that both known and novel phosphorylation motifs can be determined, such as the potentially novel threonine phosphorylation motif (pT-P-X-P, X = P, E, T, or S preferred). Coon and coworkers have also characterized over ten thousand protein phosphorylation sites from human embryonic stem cells, including several that are found on the master regulators of pluripotency, transcription factors OCT4 and SOX2 [8]. These types of experiments highlight the tremendous power Bottom Up mass spectrometry currently allows mass spectrometrists, and has begun transitioning traditional biological studies into systems-wide quantitative analyses of the proteome at the molecular level.

In contrast to Bottom Up mass spectrometry, Top Down mass spectrometry (analysis of intact proteins) [9] has not quite been as readily utilized due to many factors that seemingly keep it a specialized method. Most Top Down MS analyses seem to be performed on proteins where the sequences are known, and very few "large-scale proteomic" discovery reports have been published. Although the term Top Down mass spectrometry is a

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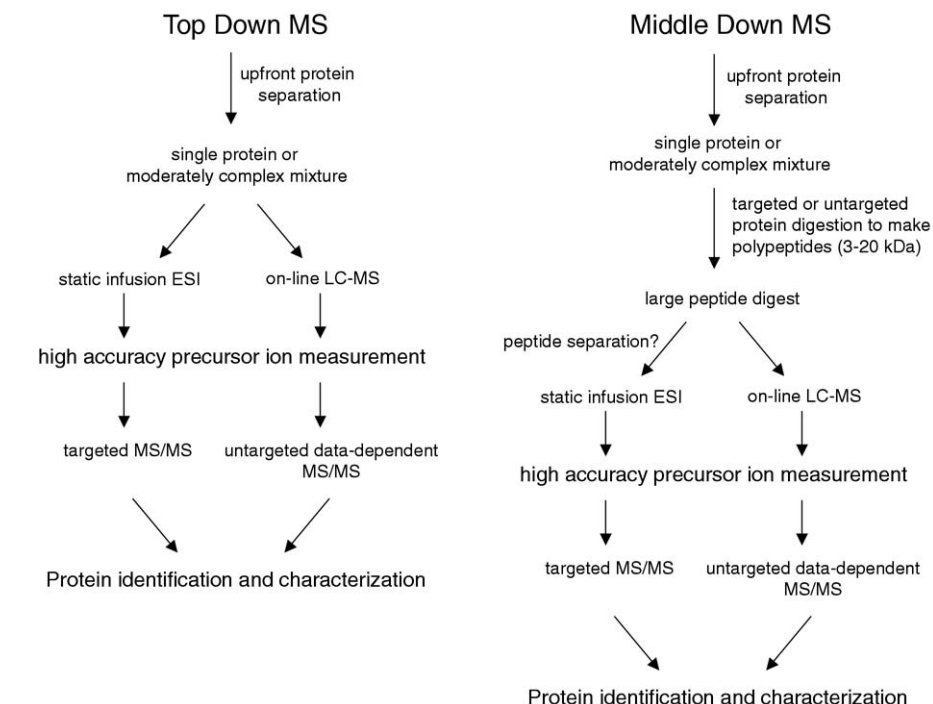
Top Down MS

upfront protein
separation

single protein or
moderately complex mixture

static infusion ESI   on-line LC-MS

high accuracy precursor ion measurement

targeted MS/MS   untargeted data-dependent
MS/MS

Protein identification and characterization

Middle Down MS

upfront protein
separation

single protein or
moderately complex mixture

targeted or untargeted
protein digestion to make
polypeptides (3-20 kDa)

large peptide digest

peptide separation?

static infusion ESI   on-line LC-MS

high accuracy precursor ion measurement

targeted MS/MS   untargeted data-dependent
MS/MS

Protein identification and characterization

Figure 1. Flowchart showing the possible routes to Top and Middle Down mass spectrometry analyses.

relatively recent term used to label the intact analysis of proteins, the analysis or profiling of intact proteins by MS, essentially Top Down MS experiments have been performed since the invention of matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI). For example, MS experiments on a variety of both low and high-resolution instruments with multiple threshold fragmentation methods have been used to examine noncovalent protein interactions, probe the secondary or even tertiary structures of proteins, or even to investigate protein PTM profiles [10–13]. Top Down mass spectrometry experiments generally follow the diagram shown in Figure 1. Here, Top Down MS usually begins with some type of up front protein separation to obtain a single protein or vastly reduce the complexity of the mixture. The protein sample is then either directly infused statically by ESI into the mass spectrometer, or further fractionated by on-line reversed-phase high-performance liquid chromatography (RP-HPLC) followed by ESI. From this point, a high mass accuracy measurement of the intact protein precursor ion mass is normally accomplished, usually on high-resolution instruments such as on a Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS). Tandem mass spectrometry is then performed in a targeted or data-dependent mode and data is then analyzed, often manually, or with specialized software.

Although not a completely new idea [14], an alternate version of a Top Down analysis coined Middle Down mass spectrometry (analysis of peptides with mass greater than ~3000 Da) has also been recently popularized. Middle Down mass spectrometry requires the proteolysis of proteins with enzymes that target less abundant amino

acid residues than trypsin, such as GluC or AspN, before a similar path as Top Down MS is taken (Figure 1). One advantage of a Top Down MS analysis is the ability to detect large polypeptides spanning long regions of a given protein as depicted in Figure 2. Therefore, if a protein is multiply modified at residues that are not very close in sequence, this combinatorial modification pattern can be deduced by Top Down MS or partially by Middle Down MS, but would mostly likely not be deciphered by Bottom Up mass spectrometry methods. Additionally, entire protein sequences are deduced by Top Down MS, this results in 100% protein sequence determination, which is near impossible to generate with Bottom Up approaches as well. As depicted in Figure 1, the main components for a successful Top Down analysis includes a high end protein separation platform, efficient ionization, and fragmentation of the proteins and mapping of the mass spectral data using appropriate database searching software. Here, Top Down and Middle Down mass spectrometry are outlined and discussed, although readers are also pointed to other recent reviews for complimentary views [15–17]. Strengths and weaknesses of the methods, current applications, along with projections for competing with Bottom Up mass spectrometry will also be discussed.

Up Front Intact Protein Separation

Off-line fractionation of proteins before MS interrogation has become a necessity, especially for whole proteome studies. Many diverse types of both gel free and gel-based methods have been utilized in an off-line mode to decrease the complexity of proteome samples
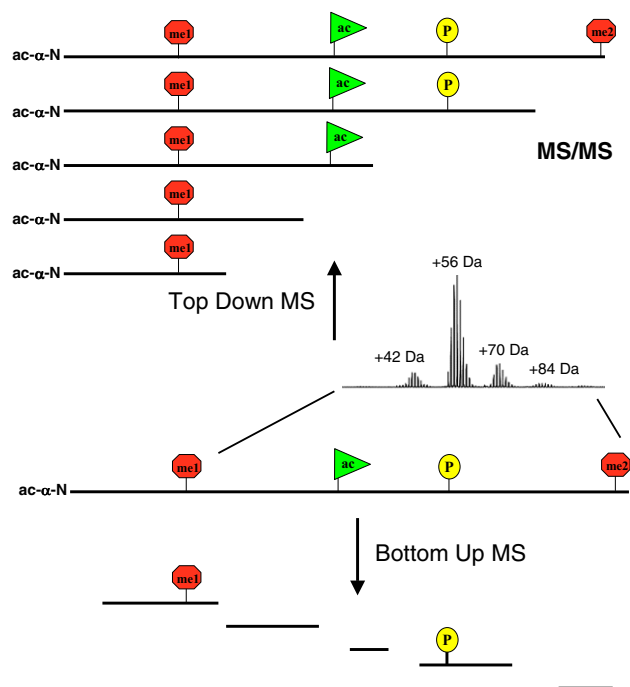
Figure 2. Schematic depicting the theoretical analysis of a modified protein (+42, +56, +70, and +84 Da shifts) by either Top Down or Bottom Up mass spectrometry. In the Bottom Up MS experiment, information concerning the combinatorial nature of the modifications is completely lost due to modifications usually not being on the same peptide. In addition, it is very easy to miss peptides that might contain modifications depending on the protein sequence and ability to make small peptides that can be detected. In contrast, in the Top Down MS experiment, after the protein isoforms are detected, Top Down MS/MS fragmentation on the intact protein forms will produce information concerning combinatorial PTMs or PTM hierarchy.

before Bottom Up mass spectrometry. Two of the most widely used intact protein separation techniques used ahead of Bottom Up proteomics are SDS-PAGE and ion-exchange chromatography. 1D-SDS-PAGE separation of proteins followed by in-gel trypsin digestion is a common approach used by many research laboratories world-wide, but is relatively incompatible with Top Down MS due to the difficulty in eluting proteins out of the gel in contaminantless buffers suitable for intact protein MS analysis. Nevertheless, the Kelleher research group has used gel-based methods for protein separation for eventual Top Down MS characterization. One approach using acid labile detergents in combination with gel-based fractionation resulted in a continuous elution of the yeast proteins from the gel in well separated protein fractions (separated in roughly 5 Da increments) that were then analyzed using static infusion ESI coupled to a quadrupole-FT-ICR-MS hybrid instrument [18]. Different types of ion-exchange-based methods have also been reported with success for Top Down proteomics [19–22]. These methods usually involve an orthogonal workflow coupling of anion-exchange or cation-exchange separation of proteins followed by RP-HPLC. For example, Roth et al. used both continuously

eluting gel electrophoresis and an two-dimensional chromatography instrument (fractionates proteins by p*I* and then by RP-HPLC) to fractionate human cell lysates for Top Down MS analysis [19]. Among the findings were the characterizations of many proteins possessing single nucleotide polymorphisms or alternative splicing forms as well as identification of PTMs, some of which were previously not known. Pasa-Tolic and coworkers have published a protein profiling method that is centered on using a first dimension weak-anion exchange separation of proteins followed by a more traditional on-line RP-HPLC separation with detection afforded by a 12T FI-ICR mass spectrometer [20]. The samples originated from a *Shewanella oneidensis* MR-1 cell lysate, and this technique resulted in the detection of 715 intact protein forms from which about 10% were fully characterized by the Top Down profiling and subsequent Bottom Up experiments.

As steady progress in the area of separations for Top Down proteomics has been made, one key observation has been made in that RP-HPLC or ion-exchange separations at the protein level are very difficult and essentially barely adequate for Top Down MS. Therefore, the need to search for new types of protein fractionation methods besides RP-HPLC and ion-exchange modes has been an increasing focus of many proteomic research groups, as the ability to separate slightly differing isoforms of the same protein (either amino acid sequence variants or PTM modified forms) is not a trivial task. There are some new separation methods that have been recently applied in proteomics research that are beginning to gain some momentum. One such method is isoelectric focusing by free-flow electrophoresis (FFE) [23], which has been more recently used to revolve peptide mixtures before Bottom Up mass spectrometry [24, 25]. FFE separation is primarily based on fractionation of peptide/protein according to their isoelectric points (p*I*s) with much higher resolution and accuracy than other related approaches. FFE is an electrophoresis method which, unlike capillary electrophoresis methods, does not utilize a matrix and can be coupled to a variety of sample preparation methods, including varying sample concentration, salts, and even detergents. Although peptide fractionation using FEE has been accomplished and used in conjunction with Bottom Up MS [24, 25], there are some attractive characteristics of FFE that seem to make it a potentially ideal method for protein separations before Top Down MS. First, FFE is a continuous electrophoretic separation technique, which means that large amounts of samples (almost unlimited) can be used. This of course bodes well with Top Down mass spectrometry, as the MS analysis of intact proteins often require much larger amounts of starting material than their peptide digest counterparts. Additionally, FFE is non-gel-based (easing sample preparations) and, as such, is directly compatible with other liquid chromatographic methods like RP-HPLC. The high-resolution of FFE also may allow for high-resolution separation of protein isoforms. One of the first attempts to marry FFE with Top Down MS was reported by Borchers and coworkers [26]. A multitude of experiments using different

conditions were used to separate test protein mixtures and were found to be highly reproducible across analyses, even on different instruments in different research laboratories. In addition, the authors used this FFE approach to resolve the major isoforms of chicken histone H2A-IV, lending support that FFE may be a solid up front separation platform for the fractionation of closely related protein forms before Top Down MS.

Another orthogonal separation approach to RP-HPLC with the potential to supplant standard liquid chromatography methods is hydrophilic interaction liquid chromatography (HILIC) [27]. HILIC-type separations have been around for decades and have most often been used to resolve small molecules in an on-line separation coupled to MS. In most HILIC separations, a hydrophilic stationary phase/resin and hydrophobic organic mobile phase with or without salts are used. HILIC is essentially similar to a normal phase separation (except that nonaqueous buffer solvents are used) where the order of elution is opposite that of RP-HPLC with hydrophilic analytes being retained longer than hydrophobic ones. An incredible number of HILIC stationary phases have been reported for diverse selective and specialized applications to a range of analytes. Accordingly, in recent years, HILIC has experienced a dramatic growth for proteomic applications, ranging from the separation of complex peptide mixtures [28, 29], to the enrichment of subproteomes, such as phosphorylated [30] or glycosylated peptides [31], or N-terminally acetylated peptides [29], usually before Bottom Up mass spectrometry is applied.

With regards to intact protein separations for Top or Middle Down mass spectrometry, HILIC methods have also currently started to make their mark. This has been most evidently demonstrated with the application of a slight variation of HILIC to the separation of highly modified histone forms [32–34]. Histones are small basic proteins (>22 kDa) that complex with DNA to form the nucleosome, the fundamental repeating unit of chromatin [35]. Interestingly, histones are highly posttranslationally modified with many types of covalent modifications, the most common being acetylation and methylation of lysine residues mostly on the N-terminal tails, and these PTMs have been linked to distinct transcriptional states influencing gene expression by repression or activation of genes. Mass spectrometry (mainly Bottom Up MS) has played a key role in histone PTM biology being used to discover novel PTM sites and quantify PTM expression from unique cellular states [36]. Nevertheless, when histone proteins are digested into small peptides, the connectivity of these PTM sites is lost and information concerning the combinatorial nature of these modifications (histone codes) cannot be determined. Thus, Top Down MS is ideal for characterizing intact histone codes, and several papers have indeed shown that Top Down MS hold great promise for these types of analyses. However, as histones are highly modified in very complex combinatorial patterns, standard RP-HPLC separation of modified
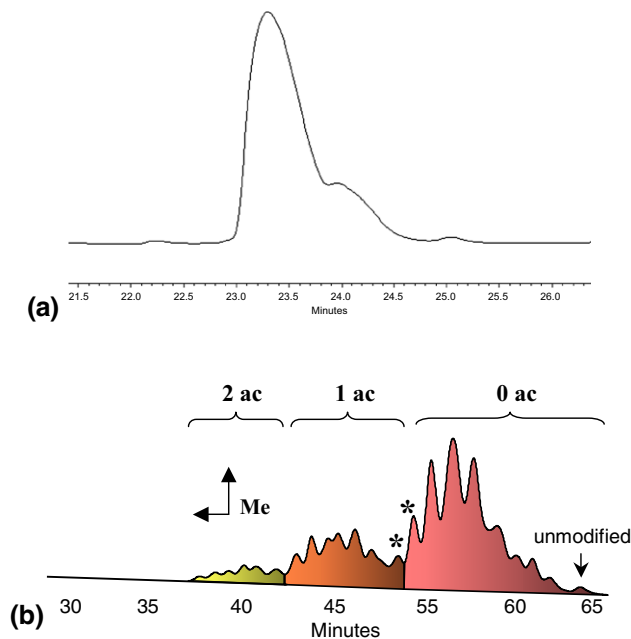

Figure 3. Separation of the 1-50 AA fragment of histone H3.2 using (**a**) C18-based RP-HPLC and (**b**) weak-cation-exchange HILIC. Note that more resolved peaks spanning a much longer elution period are observed in the HILIC separation.

histone isoforms has proven to be only a preparative method for separating histone family members from one another, but not for fractionating differently PTM modified single forms of the same histone protein.

To this end, a weak-cation exchange HILIC method has been applied to reduce the complexity of all histones, with some very nice separations at the intact level shown for the multiply modified forms of histone H4, allowing quantification of individually modified isoforms [37]. Intact histone H3, arguably the most highly modified histone, however, has shown to be difficult to fractionate by any means. However, recently Middle Down MS approaches using the 1-50 amino terminus of histone H3 (>5 Da) has revealed a great modification pattern complexity and will be pivotal for deciphering the meaning of the combinatorial histone H3 code [38, 39]. In fact, Mizzen, Kelleher and coworkers used cation-exchange HILIC and FT-ICR-MS to identify over 170 unique forms of histone H3.2 alone [38]. Figure 3 shows the resolving power of a weak-cation exchange HILIC over standard RP-HPLC separation. The RP-HPLC separation of the 1-50 residue fragment of histone H3.2 is shown in Figure 3a, and only one main peak containing a small shoulder can be resolved within a 2 min window. The same histone H3.2 (1-50 residue fragment) sample, however, can be fractionated into several peaks, all containing different histone H3 modified forms eluting in nearly a 30 min timeframe. This particular type of HILIC separation is driven primarily by fractionating the histone by charge (or acetylation status) imparted by the weak-cation exchange and secondly by the methylation content of the
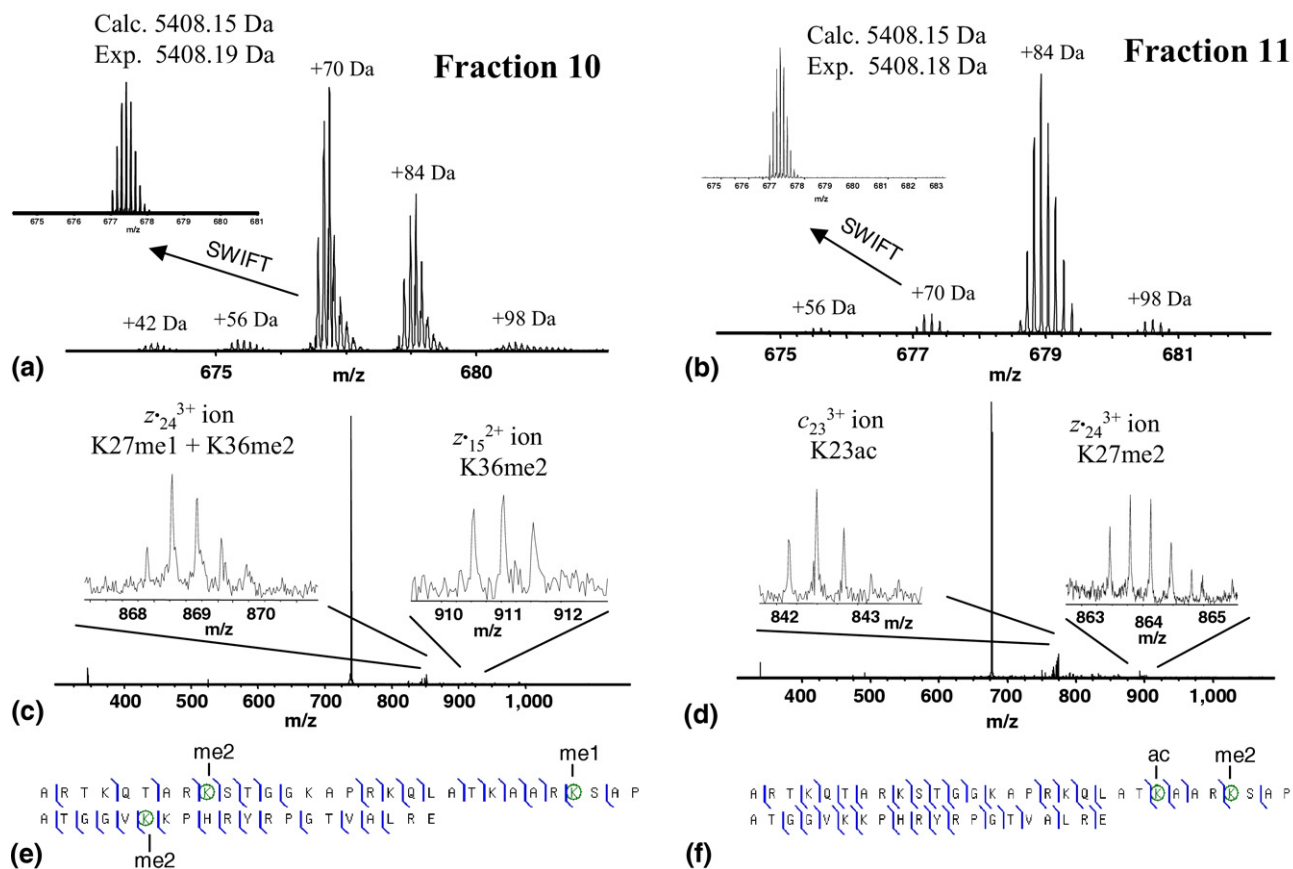
Figure 4. Full mass spectrum taken on an FTMS instrument of different modified forms of the 1-50 AA polypeptide of histone H3.2 from fractions (**a**) 10 and (**b**) 11 of a HILIC separation. Isolation of a species containing a +70 Da shift at 5408 Da for each fraction is shown as inset mass spectra. (**c**) and (**d**) show the ECD tandem mass spectra of each species isolated in (**a**) and (**b**), respectively. As can be seen by inspection of the ECD fragments, distinctly modified H3 forms are present in the isomeric species separated by HILIC chromatography. (**e**) and (**f**) show ECD fragment maps generated from the spectra in (**c**) and (**d**), respectively, showing that the species in (**e**) is a methylated form of histone H3.2, while the species in (**f**) contains both acetylation and methylation ate different residues, adapted from [38].

histone governed hydrophilic interactions. This improvement in resolving power can be better visually observed by Middle Down MS analysis of adjacent HILIC fractions from the above separation shown in Figure 3b. Figure 4 shows Middle Down MS experiments characterizing two peaks from adjacent HILIC fractions (asterisk labeled peaks in Figure 3b) possessing the same nominal mass at 5408 Da corresponding to the sequence of histone H3.2 (1-50 residue fragment) with the addition of 4 methylation equivalents (Figure 4a and b). Experiments were carried out by statically infusing the fractions by ESI into a quadrupole FT-ICR-MS. After isolation of the +70 Da species (5408 Da), each species was subjected to fragmentation using electron capture dissociation (ECD) (Figure 4c and d), and the ECD fragment maps of each species are shown in Figure 4e and f. As can be deduced by the ECD fragmentation, although the peaks have the same nominal mass, they clearly are distinct modified H3 forms harboring different PTM types on different residues. Clearly, improved separation of proteins will be a vital component will continue to benefit Top or Middle Down MS analyses.

Top Down Mass Spectrometry Instrumentation and Techniques

Traditionally, Top Down MS has been performed on high-resolution FT-ICR mass spectrometers [9]. One of the main advantages of using these instruments is that high-resolution measurements of the MS/MS ions, which are frequently of very high charge state, can be resolved. Nevertheless, since these types of mass spectrometers are somewhat expensive and less user friendly than other bench-top instruments, Top Down MS on FT-ICR mass spectrometers has not become as widespread as Bottom Up MS. However, Top Down MS has been performed to some extents on many other types of mass spectrometry instrumentation [11, 40–44]. For example, Loo et al. performed perhaps the first Top Down MS/MS analysis of the small 14 kDa ribonuclease A protein on a triple quadrupole mass spectrometer [11]. Top Down MS has also been performed on quadrupole time-of-flight instruments using CID fragmentation. Ginter et al. showed that proteins up to 66 kDa in molecular

weight could be dissociated in a two step process involving in-source fragmentation and subsequent CID-based MS/MS of the in-source dissociated fragment ions, resulting in sequence tags that could be interpreted manually and by database searching using software provided by the instrument manufacturer [43]. For several years now, ion–ion and ion–molecule methods on quadrupole ion trap and quadrupole ion trap-hybrid instruments have been developed for the Top Down analysis of proteins and other biological molecules [45, 46]. In very recent times, the LTQ-Orbitrap mass spectrometer has become an important tool for proteomics research [47, 48]. Like an FT-ICR-MS, the Orbitrap also measures ion frequencies to obtain high-resolution mass spectra (up to 100,000 in commercial instruments) with less than 2 ppm mass accuracy of the detected ions. Although having only been commercially available for the last few years, there are already signs that the Orbitrap will have a role in future developments of Top Down mass spectrometry. Mann and coworkers have demonstrated Top Down sequencing of intact proteins up to 28 kDa [49]. A "lock mass" approach [50] was used to obtain 1–3 ppm mass accuracies of the proteins and sequencing was performed via MS^3 in the linear trap with fragments detected in the Orbitrap. This group has further shown that quantification of proteins labeled by stable isotope labeling by amino acids in cell culture can also be achieved in the Orbitrap [51]. The SILAC labeled proteins also improved protein identification from CID fragmentation by providing information about the number of labeled residues in the fragments. Top Down MS analysis of proteins > 100 kDa detected by the Orbitrap mass analyzer have also been reported [52].

Initial Top Down MS research utilized low-energy CID methods to induce protein fragmentation. However, for protein work, CID does not produce comprehensive fragmentation into order to fully characterize proteins, but rather produces enough fragments or sequence tags to identify the protein. Additionally, if the protein contains PTMs, low-energy CID most likely will not be sufficient to localize the modified site or the PTM may be the preferred site of cleavage. McLafferty and coworkers developed an alternative to CID methods, called electron capture dissociation, which involves the capture of thermal electrons by multiply charged cations to generate odd-electron products having the energy required for breakage of bonds creating c- and z-type fragment ions [53]. This type of fragmentation process is nonergodic and fragmentation loss of labile modifications as seen in low-energy CID processes is not observed, therefore keeping PTMs intact on protein and peptide sequences. ECD has been heavily used for Top Down MS, being able to fragment proteins with very high molecular weights [54]. However, due to having to average many spectra to obtain favorable signal to noise, ECD has been historically noncompatible with on-line LC-MS/MS experiments,

but that may be changing with new generation FT-ICR-MS instruments [55]. The Hunt laboratory developed an analog of ECD named electron-transfer dissociation (ETD) transfers an electron from anion of low electron affinity to a multiply charged cation, again resulting in fragmentation of the c- and z-ion types similar to ECD, but this all occurs in a ion trap mass spectrometer [56]. ETD has been shown to be compatible with LC-MS/MS timescales, allows for sequencing of labile PTMs, and can also be used to fragment large polypeptides or intact proteins, especially when coupled with proton transfer reactions (PTR) to reduce the charge state of the resulting fragmenting ions [57]. The coupling of ETD with the Orbitrap mass spectrometer has created a powerful instrument capable of producing high-resolution Top Down information with Bottom Up like speed, sensitivity, and dynamic range [58–60]. Figure 5 shows the ETD mass spectra of the histone H3 (1-50 AA) fragment fractionated by HILIC and detected in the ion trap (Figure 5a) or in the Orbitrap (Figure 5b). The high-resolution of the Orbitrap is needed for the important unambiguous assignment of a trimethylated versus acetylated ion (Δm = 0.036 Da), as comparison of the observed c_9^{2+} ion to the potential calculated masses of the ion with either acetylation or trimethylation determines this ion as being the trimethylated K9 species on the peptide K9me3K14acK18acK23acK27me2K36me2 (Figure 5b).

As researchers have turned their attention to improving instrumentation and MS/MS fragmentation methods for Top Down MS analysis, another area of the MS experiment that needs work is the ionization of protein molecules. Unlike peptides, when proteins are electrosprayed into the gas phase, it is not uncommon to observe high charge states (>+10). This multiple charge state observation is somewhat of a problem because the protein concentration is diluted into many different fractions, and different charge states can produce slightly different MS/MS spectra, which might produce problems for downstream bioinformatics efforts. Therefore, being able to shift the charge state distribution of proteins to higher charge states would be beneficial. Collapsing the charge states of a protein would act to gas-phase concentrate the proteins and would also be helpful in MS/MS experiments, especially with ECD or ETD fragmentation, as these processes are enhanced by a higher charge state precursor. Methods (both physical and chemical) to manipulate the charge state of proteins have been the focus of many investigations [61–64]. The present work has shown that the addition of m-nitrobenzyl alcohol to MS buffer solvents increases the charge state of peptides, facilitating ETD fragmentation as well as increasing charge state, while not disturbing noncovalent interactions [65, 66]. Future work in the area of charge fixing or "supercharging" proteins and polypeptides will definitely be useful for Top Down MS.
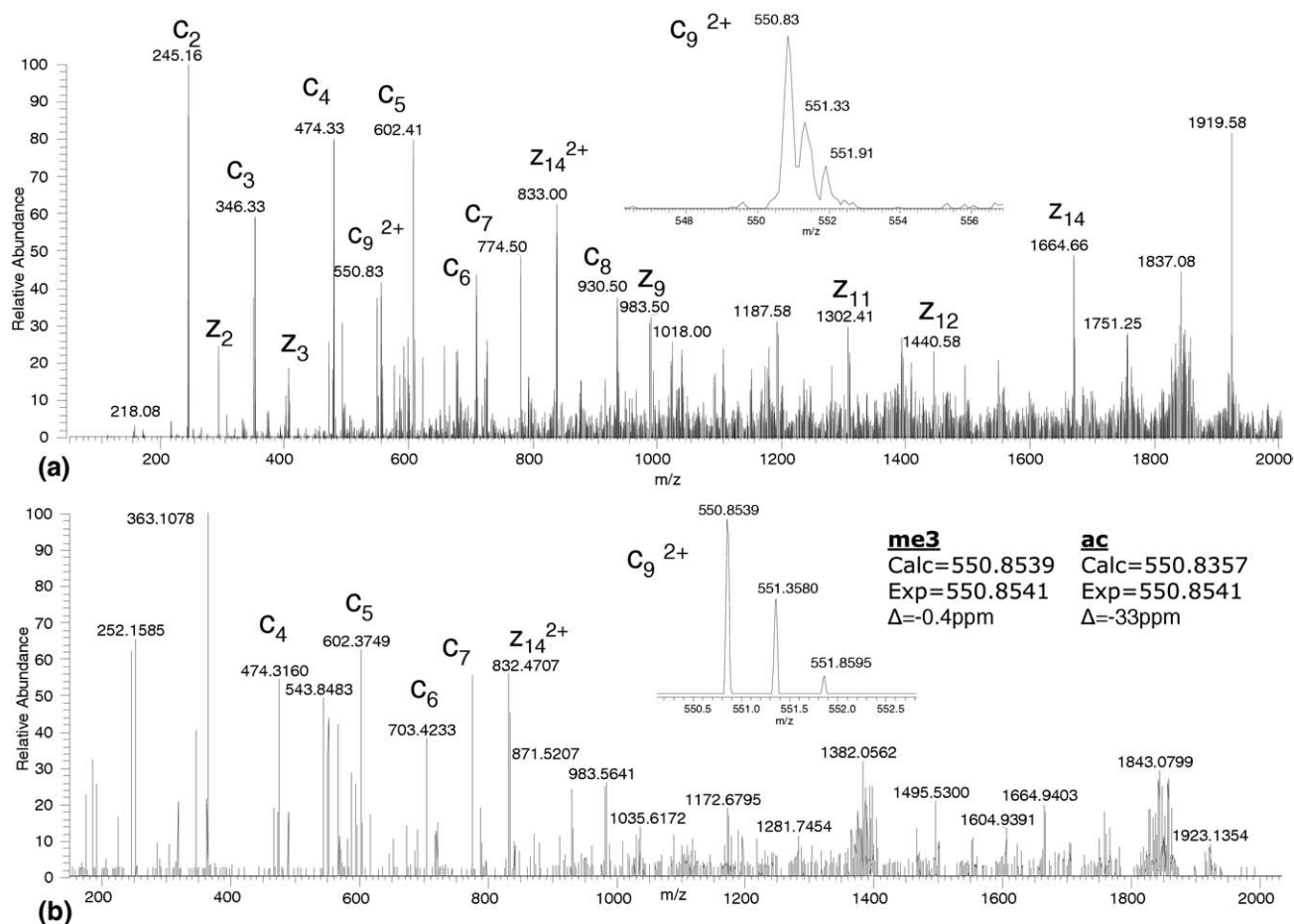
Figure 5. Data-dependent ETD-MS/MS spectrum of the 1-50 AA polypeptide of histone H3.1 recorded on an (**a**) LTQ ion trap and (**b**) LTQ-Orbitrap mass spectrometer. The high-resolution of the Orbitrap allows for distinguishing between an acetylation and trimethylation, such as at K9 (shown in inset) on the sequence K9me3K14acK18acK23acK27me2K36me2.

Data Analysis Software for Top Down Mass Spectrometry

Tandem mass spectra generated from Top Down mass spectrometry experiments are very complex to interpret, due to certain characteristics of the data that are not intrinsic properties of Bottom Up-based tandem mass spectra. For example, since 100% sequence coverage is obtained with Top Down MS, a larger number of PTMs (such as N-terminal modifications) and even single nucleotide polymorphisms need to be seriously considered in database searches. Up until the last year or so, ProSight PTM and earlier versions were the only software programs available for identifying proteins and their modified forms from the MS/MS spectra of intact proteins [67, 68]. In ProSight, the user inputs the high-resolution neutral parent ion mass and all MS/MS fragment masses, and this data is then searched against a "shotgun annotated" proteome database or known protein sequences. A Poisson distribution model is then employed to find statistically significant matches between observed and theoretical masses. A shotgun annotated proteome database [69] is one where PTMs

and other potential sequence altering modifications are assigned before searching to generate all possible modified forms, and are included in the database and retrieved during the actual database search. Now that Top Down mass spectrometry is becoming a more widely accepted technique, other computational approaches for interpreting intact protein tandem mass spectra are beginning to emerge. An approach based on using spectral alignment to assign protein forms including positional isomers that may be present in the same spectra has been reported [70]. A new version of the peptide identification program Mascot, named BIG Mascot, has been utilized for Top Down MS database searching of many types of intact protein MS/MS data (CID or ECD), correctly identifying a large 669 kDa protein, thyroglobulin [71]. BIG Mascot has user-enabled features similar to the standard peptide Mascot program, but has been engineered for entering large precursor ion masses above 16,000 Da, and other key parameters needed in standard Mascot searches such as enzyme specificity are efficiently bypassed for accepting Top Down MS data. Other computational tools for Top

Down MS discovery of biomarkers or for interpreting ETD fragmented polypeptides and proteins are also increasing number and overall usage [72–75].

Can High-Throughput Top Down Proteomics be Reached?

Top Down MS-based strategies have been elegantly applied for the characterization of single purified proteins and mostly moderate to low complexity protein samples, often from organisms with relatively small proteomes. Although large-scale analysis of proteomes by Top Down methods is increasing at a good rate, the question still remains if Top Down MS can overcome its current limited throughput status and become a truly high-throughput compliment to Bottom Up MS? There are hints in the last few years that indicate that high-throughput Top or Middle Down MS proteomics may be an achievable goal in the not too distant future. For example, Roth et al. used a multidimensional protein characterization by automated Top Down platform which detected over 600 unique protein masses [76]. Subsequent on-line MS/MS experiments revealed the identities of 133 proteins from 67 distinct genes, several proteins of which contained some type of modification such as a PTM, single nucleotide polymorphism, or were detected as in vivo proteolysis products. This same research group also performed the first Top Down proteomics experiment using on-line liquid chromatography on a linear ion trap Fourier transform system and identified 22 yeast proteins with "on-the-fly" CID-based tandem mass spectrometry [77]. Muddiman and coworkers also achieved on-line LC-MS/MS for Top Down analysis of differential expression of proteins from the fungus *Aspergillus falvus* using SILAC labeling and nanoflowLC also coupled to an LTQ-FT-MS [78]. Over 1300 protein masses (659 SILAC pairs) were detected, and 22 proteins (up to 36 kDa) were subsequently identified following CID experiments. Stephenson and coworkers have published an automated high-throughput Top Down proteomic analysis of *E. coli* proteins [79]. In those experiments, proteins were first fractionated by anion-exchange chromatography and then analyzed on an LTQ ion trap with ETD fragmentation following nanoflowLC separation on a C18 column, leading to the identification of 174 proteins from 322 detected different protein forms. Hunt's research group has also demonstrated a high-throughput Top Down analysis of intact small ribosomal proteins from *E. coli* on an LTQ equipped with ETD fragmentation and PTR charge reduction, resulting in the characterization of 46 of the known 55 70S ribosomal protein complex members, several of which were covalently modified [80]. Although great advancements have been made, there are technological hurdles that must be passed before Top Down MS is turned into a robust proteomic platform. As mentioned above, advances in instrument have to be made in order for the analysis of >30 kDa proteins to be made routine and automated. Although mere speculation at this point, with the large advances in proteomic instrument witnessed in the last 5 years, it is not unreasonable to believe that the ~30 kDa limit will be surpassed in the future. Additional gains in dynamic range, duty cycle, and sensitivity will also aid to detect poorly ionizing intact proteins with multiple forms, and ion–ion chemistry to reduce the several charge states observed with proteins is a necessity.

Taking the current state of Top Down mass spectrometry as still being in developmental mode for high-throughput proteomics, the question now turns to whether the hybrid Middle Down mass spectrometry approach could provide a compromise for those wanting to cross the Bottom Up or Top Down worlds. The concept of Middle Down mass spectrometry has been in place for a while now [14], but it has not been until recent work by the Karger research group that this methodology was recognized as having potential as a new type of high-throughput proteomics platform. Wu et al. showed that this Middle Down MS strategy termed Extended Range Proteomic Analysis could be used not only to obtain better sequence coverage than Bottom Up MS [81], but also could be performed under the same chromatographic conditions, and at similar sensitivities as Bottom Up MS. This group has gone on to improve large peptide separations and Middle Down MS analysis for the characterization of low levels of peptides carrying diverse kinds of PTMs [82]. There are several advantages of Middle Down MS compared with either Top Down or Bottom Up MS. First, Middle Down MS as mentioned earlier has Bottom Up-like sensitivities, chromatography, and MS acquisition characteristics, putting it at an advantage over conventional Top Down MS. However, the ability to sequence larger peptides puts it at an advantage over Bottom Up MS, with, in theory, improved abilities to sequence and identify long range combinatorial PTM patterns or detect small primary sequence changes that may be missed in a standard Bottom Up MS experiment. To this end, several applications of Middle Down MS proteomics have surfaced, including large-scale PTM identifications and use as a general new proteomics tool for protein identification [8, 83, 84]. Hidden among all these great applications is the ability of high-resolution Middle Down MS to improve confidence in polypeptide and, hence, protein identification. Boyne et al. have recently shown that a Middle Down MS platform using high-resolution MS and MS/MS measurements on a 12T LTQ-FT Ultra instrument can be efficiently used in high-throughput fashion to easily detect thousands of peptides ranging in size from 2 to 20 kDa [85]. Moreover, the ability to incorporate MS/MS fragmentation data with less than 2 ppm mass accuracy vastly improved database searching by sharply increasing peptide identification confidence, as well as allowing for facilitated analysis of multiplexed tandem mass spectra.

Conclusions

Top Down mass spectrometry has experienced a tremendous growth over the last several years into a well accepted and practiced proteomic technique due to the dedication and pioneering work of many research groups world-wide. Top Down MS has, accordingly, also made many significant contributions to various biological fields, mostly in the area of protein PTMs and especially in the histone biology realm. As both technology and methodology in various practical aspects (chromatography of intact proteins, fragmentation of large proteins, data analysis, etc.) of Top Down MS are improving, developing Top Down MS into a useful proteomics high-throughput platform may be achievable. In the meantime, momentum is gaining for the hybrid approach Middle Down MS to take center stage as a viable method capable of producing Top Down-like information on large regions of proteins (3–20 kDa) with Bottom Up-like like throughput, sensitivity, and ease. As all of these Top- or Middle Down methods continue through their growing pains similar to what small peptide MS went through several years ago, large molecule proteomics will evolve into techniques that will become more accessible to all types of scientists and will play pivotal roles in determining the biological structures of many proteins, protein complexes, including quantitatively characterizing PTMs and their influence on protein activity.

Acknowledgments

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