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REVIEW

Top-down proteomics: applications, recent developments and perspectives

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Now-a-days, top-down proteomics (TDP) is a booming approach for the analysis of intact proteins and it is attaining significant interest in the field of protein biology. The term has emerged as an alternative to the well-established, bottom-up strategies for analysis of peptide fragments derived from either enzymatically or chemically digestion of intact proteins. TDP is applied to mass spectrometric analysis of intact large biomolecules that are constituents of protein complexes and assemblies. This article delivers an overview of the methodologies in top-down mass spectrometry, mass spectrometry instrumentation and an extensive review of applications covering the venomics, biomedical research, protein biology including the analysis of protein post-translational modifications (PTMs), protein biophysics, and protein complexes. In addition, limitations of top-down proteomics, challenges and future directions of TDP are also discussed.

Keywords: Top-down, protein biology, mass spectrometry, fragmentation techniques, data analysis.

Introduction

In recent years the mass spectrometry (MS) ionization techniques such as ESI [1] and MALDI [2] have been applied for the detection of a wide variety of large biopolymers, such as proteins [3,4], lipids, and nucleic acids. Proteomics has significant number of applications in many fields, for instance, food science [5], clinical science, forensic science, biology and medical industry [6].

There are two well-known proteomic approaches for various proteins analysis, i.e. bottom-up proteomics (BUP) and top-down proteomics (TDP). From earlier days, mass spectrometry-based proteomics has been carried out in a bottom-up fashion. In these BUP experiments, proteins are separated on the basis of their isoelectric point (pI) and molecular weight, respectively [7]. Top-down mass spectrometry allows identification and characterization of proteins and protein networks by direct fragmentation. The 'top-down' [8] approach provides far higher specificity at the expense of far higher experimental requirements by directly introducing the proteins into the mass spectrometer. The top-down method is being developed progressively in specific applications, with 18% of proteomics papers/posters at the 2007 meetings of American Society for Mass Spectrometry [9].

Top-down proteomics is different from the conventional bottom-up strategy of protein analysis which starts with intact mass measurement **(Figure 1)**. In simple words, if anybody is interested just protein detection or its identification from a database, then bottom-up approach tends to be easier with a broad range of well-honed tools from the lion's share of efforts from academic and as well as commercial vendors. However, if anyone is interested in the full characterization of protein including its amino acid sequence and modifications, then top-down strategy is the more efficient one. Bottom-up mass spectrometry has following disadvantages: a peptide or even several peptides may not be specific to an individual protein or protein form. Large regions of the protein may not be which can leave behind important information regarding PTMs, modifications and other sequence variations may

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occur on disperate peptides, causing their relation to one another to be lost following digestion. Top-down proteomics can eliminate these problems by the introduction of an intact protein into the mass spectrometer where both its intact and fragment ions masses are measured **(Figure 1)**. This strategy usually covers 100% sequence coverage and full characterization of proteoforms including PTMs and sequence variations. Nowadays, a combination of both software and hardware is available to acquire the top-down data. Top-down proteomics in which a large number of intact proteins are fragmented directly in a mass spectrometer (without using a proteolytic enzyme)-is becoming more feasible every year. There are many more top-down studies have been reported in these years in comparison with the top-down studies prior to 2007. Some of them observed a few hundreds to even thousands of proteins in their top-down studies [10-14]. **Figure 1** shows the workflow of both top-down and bottom-up proteomic approaches for the protein analysis using mass spectrometry. **Figure 1** describes that traditional Bottom Up approach involves the digestion of proteins into peptides prior to introduction into the mass spectrometer where they are then detected and fragmented. In top-down mass spectrometry, the protein is ionized directly, allowing for improved sequence coverage and detection of post-translational modifications (PTM). Currently many labs like Amgen and Amylin are using top-down strategy for the characterization of recombinant antibodies and endogenous secretory peptides. Many other labs are using top-down MS for the analysis of endogenous proteins like histones and biomarkers under 30 KDa and protein based therapeutics. In BUP

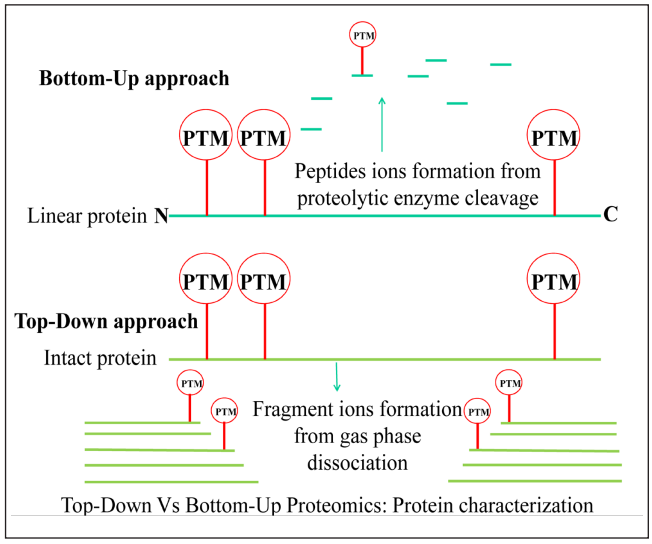

Figure 1. Schematic presentation of top-down vs bottom-up approaches in proteomics.

experiments, proteins of interest are separated on the basis of their isoelectric point and molecular weight, respectively and followed by enzymatic digestion before MS analysis [7]. This digestion can be performed either directly in solution or in-gel digestion after separation by 1D- or 2D gel electrophoresis. Top-down approach will provide protein sequence information directly without preliminary digestion. In contrast to bottom-up method, top-down method is attaining a pivotal position nowadays due to its versatile simplicity in the analysis of larger biomolecules. It has many advantages in comparison with bottom-up method, i.e., (i) no need of enzyme digestion (ii) high protein sequence coverage, etc. However, the current discovery mode TDMS has size limitation and it can't go beyond a certain molecular weight (MW) and a typical top-down proteomics experiment in discovery mode, the MW cut-off is around ~30 kDa [15]. However, this cut-off may go higher for targeted-mode TDP. In this top-down mass spectrometry method, intact proteins are fragmented directly (without using a proteolytic enzyme cleavage) in the mass spectrometer to attain both protein identification and characterization, even capturing information on combinatorial post-translational modifications. An emerging top-down MS-based proteomics approach provides an overview of all intact proteoforms, which has exclusive advantages for the identification and localization of PTMs and sequence variations [16-19]. The constitution of the new electron-based MS/MS techniques, like electron capture dissociation (ECD) [20] and electron transfer dissociation (ETD) [21], exemplify a substantial advancement for top-down by providing dependable methods for the localization and characterization of labile PTMs [20-27]. In the past 6-7 years, advanced instrumentation and software's for the top-down analysis were developed, and top-down MS has also experienced a major improvement from refined separations of whole proteins in complex mixtures that have both high recovery and reproducibility. It might be possible to see a high throughput work flow which covers intact proteins and polypeptides up to 70 kDa in near future with the combination of advanced commercial MS instrumentation and data processing [26].

Methodology

Sample preparation

To perform top-down experiments using fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) instruments, it is necessary to have pure protein samples or simple mixtures. Even though one can use complex mixtures and separate the proteins by using a quadrupole mass analyzer that is hyphenated with the FTICR rather than HPLC. However, the dispersion of signals during ionization and the suppression of individual proteins reduce the signal-to-noise ratio. Thus, sample purification is usually an important step before analysis. Usually, during the electron-capture dissociation, formation of many fragments observed due to the cleavage at the backbone N–Cα bond in a highly but not completely nonspecific manner. Therefore, spectral averaging over a number of scans is usually needed to improve the signal-to-noise ratio.

Unlike the peptide analysis in a bottom-up approach, each protein is a unique case in top-down analysis. The fragmentation conditions are established in advance in bottom-up peptide identification and not changed during the LC-MS/MS analysis. But, in case of proteins, optimum results, however, are achieved by tuning the optimal conditions to fragment each parent protein ion. Thus, top-down is difficult to conduct with on-line HPLC separation. Due to this reason, TD has some difficulties for high throughput analysis wherein coupling of sample separation and FTICR with ECD is necessary but sometimes incompatible with the shorter time scales of LC separation and the longer times of FTICR-ECD spectral averaging. Hence, fraction collection (such as protein fractionation methods) is very often introduced between protein separation and FTICR measurement. Despite the difficulties, there is strong motivation to develop further top-down analysis by increasing the speed and mass resolving power of current instrument platforms. These improvements are required to enhance through-

put to supplement bottom-up approaches that are inadequate for analyzing proteins with multiple modifications.

Separation science for intact proteins

Due to the high complexity of proteomic mixtures ranging from sub-organellar complexes to whole-cell proteomes, mass spectrometry is not solely sufficient to characterize a proteome. Therefore, efficient separation methods are necessary to reduce the sample complexity and increase the dynamic range of detection. Many separation methods can be applied offline or independent of the mass spectrometer [28]. From the collection of eluted fractions followed by their injection into the mass spectrometer, more instrument time can be spent on collecting the data of a single protein or simple protein mixture. On the other hand, offline separations are more adaptable as they do not need to be mass spectrometry compatible. In comparison, online separations which are hyphenated with mass spectrometry, would allow increased throughput and reduced sample handling but with limitations to data acquisition and separation conditions. Taking into the consideration of the complexity of most proteome samples, multiple separations are required to achieve sufficient separation, often using an off-line approach coupled with an online separation.

While there are many well-known separation methods available, based on the type of MS analysis, top-down or bottom-up, will determine what type of separation method is suitable for the better separation and as well as MS analysis. Usually separations are based on the protein-intrinsic parameters, which include charge, size and hydrophobicity, etc.

Liquid chromatography

Liquid chromatography (LC) is one of the most common methods for the separation of intact proteins, peptides, and small molecules. The separation principle relies on differential partitioning of analytes between liquid mobile phase and a stationary phase. In many cases, LC can often hyphenated to electro spray ionization (ESI) and proved to be an efficient on-line analysis method [27]. While there are variety of LC methods have been developed, reverse-phase LC (RPLC), hydrophobic interaction LC (HILIC) and ion exchange chromatography are three most common LC approaches applied for intact protein separations [28].

Reverse Phase Liquid Chromatography [RPLC]

RPLC uses a polar mobile phase and non-polar stationary phase which allows the most hydrophobic analytes elute first. Common stationary phases are alkyl chains (C_4, C_5, C_8 and C_{18}) linked to silica particles, where shorter alkyl chains are preferred for the intact protein separations as these shorter alkyl chains are less retentive and offer high recovery [28]. Similarly, polymeric reverse phase materials (PLRP) offers increased mechanical strength, uniform hydrophobicity, and high recovery and PLRP materials have also been utilized widely for the intact protein separations [27,29,30].

In contrast to RPLC, hydrophobic interaction liquid chromatography (HILIC) uses a polar stationary phase and gradients increasing water content which results in the elution of more hydrophobic species first [31,32]. Modified histone forms separation using HILIC prior to top-down MS has been reported [33,34].

Ion exchange chromatography used differences in the charge of the analyte, while RPLC and HILIC rely primarily on differences in hydrophobicity to achieve separation. Ion exchange chromatography has been considered as a widespread separation method because its widespread familiarity and relatively high loading capacity. Increasing the mobile phase ionic strength is the key principle to elute the analyte from the charged stationary phase.

Kellie et al. reported the use of a combination of ion exchange chromatography with reverse phase liquid chromatography and top-down mass spectrometry and they separated and identified 133 protein forms from human white blood cells [35]. Similarly, a novel 3-dimension liquid chromatography strategy by coupling of hydrophobic interaction chromatography (HIC) and reverse phase chromatography with top-down MS separated and identified a total of 640 proteins from HEK 293 cell lysate protein fractions [36]. On the other hand, chromatofocusing and solution isoelectric focusing (both utilizes pH gradient) are able to separate proteins with high isoelectric point correlation and are considered as alternative methods to salt gradient ion exchange chromatography for top-down proteomic separations [35,37].

Strong anion exchange coupled to RPLC-MS for the separation of intact proteins has also been reported for the study of E. Coli [38]. Similarly, anion exchange RPLC has been used for the top-down study of yeast [39], human leukocytes [40], and Shewanella oneidensis [41]. Chromatofocusing, a variant of ion-exchange chromatography which uses a pH change rather than ionic strength to perform elution, has been coupled with off-line RPLC for fractionation of proteins from Methanosarcina acetivorans [42]. In contrast to conventional ion-exchange chromatography, chromatofocusing elutes proteins as a function of their isoelectric point [pI] for the intact proteins separation.

Electrophoresis

In addition to chromatography, electrophoresis is considered as an extremely popular approach for intact proteins separation which relies on the differential migration of proteins in an applied electric field [27,43]. The most common electrophoretic method is SDS PAGE, in which SDS coated proteins migrate through a polyacrylamide gel matrix in an electric field, achieving molecular weight based separation [44]. This is commonly used in bottom-up proteomics via the digestion of proteins out of the gel and followed by on-line LC-MS run [45, 46].This approach can be extended to two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) in which isoelectrofocusing is used as a 1st dimension separation and SDS-PAGE is as 2nd dimension separation of intact protein separation [47].

2D-PAGE is the most known platform for intact protein separation due to its unrivalled peak capacity; however, intact proteins extraction from gels results in low recovery. Therefore, separation of intact proteins prior to top-down MS analysis is necessary to fractionate the various proteins in solution phase.

While conventional gel-based separation methods are not applicable to top-down proteomics, similar separation strategies such as tube gel electrophoresis have been developed. Continuous elution gel electrophoresis utilizes a tube gel column which can separate intact proteins which are then collected as they elute from the end of the gel column [48]. Meng et al. demonstrated this approach for the fractionation of Saccharomyces cerevisiae proteome using acid-labile surfactant rather than using SDS, as it could be degraded upon acidification, limiting the downstream interferences. These fractions were further separated using offline RPLC on a C_4 column before MS analysis. This tube gel electrophoresis further extended with the invention of gel-eluted liquid fraction entrapment electrophoresis (GELFREE). The GELFREE fractionation system is a novel protein fractionation system designed to maximize protein recovery during molecular weight based fractionation. The system consists of sample capacity cartridges with sample loading and sample collection chambers and a bench top GELFREE Fractionation Instrument. A constant voltage is applied between the anode and cathode reservoirs during the protein separation, and each protein mixture is electrophoretically driven from a loading chamber into a specially designed gel column gel. Proteins are concentrated into a tight band in a stacking gel, and separated based on their respective electrophoretic mobilities in a resolving gel. As proteins elute from the column, they are trapped and concentrated in liquid phase in the collection chamber [i.e., free of the gel]. This novel technology facilitates the quick and simple separation of several complex protein mixtures simultaneously. This technology offers broad mass range (5–100 kDa) of intact proteins separation on the basis of molecular weight, retaining important physiochemical properties of the analyte. This liquid phase entrapment of proteins provides for high recovery while eliminating the need for band or spot cutting, making the fractionation process highly reproducible. The GELFREE device first applied to a top-down study by Lee et al. 2009, in which SDS was used and then removed using methanol/chloroform/water precipitation prior to online nano-LCMS run [49,50].

Similar like GELFREE system, an approach MSWIFT [51] (membrane-separated wells for isoelectric focusing and trapping (**Figure 2**.) can also be employed for sample clean-up in bottom-up proteomics [52] and for removal of neutral buffering materials in protein sample [53]. The major advantage of MSWIFT is that peptides/proteins with different isoelectricaI values are bracketed by membranes of fixed pH values, and the sample can be directly used for MS analysis unless the sample mixture is too complicated and needs further separation. This approach is well suitable for top-down analysis.

Usually, precursor ion having higher charge states is beneficial for better fragmentation. Evan Williams first reported that m-nitrobenzyl alcohol (mNBA) and glycerol can be included in the spray media to achieve "supercharging" of electro-sprayed protein ions [54]. The proposed mechanism is that addition of the reagent into the sample solution enhances the surface tension of the droplets during evaporation of the solvent. Increased surface tension allows the droplets to accommodate more charges before reaching the Rayleigh limit [55]. Loo's group screened sulfolane(tetramethylene sulfone) and other reagents for supercharging of native proteins and noncovalent protein complexes [56].

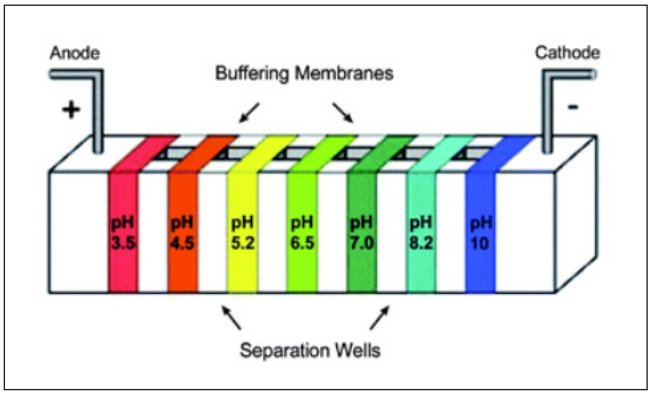

Figure 2. MSWIFT device for isoelectric point-based separation. Reprinted from with permission from [52].

Intact proteins mass spectrometry

A proteome wide-level intact protein detection and identification require a high performance mass spectrometer. Especially, high mass resolution and mass accuracy is essential to separate and accurately assign spectral peaks generated from complex protein precursor spectra containing multiple intact proteoforms. So extremely high resolution may be required to differentiate these undistinguishable peaks. Similarly, sensitivity plays also a key role as high molecular weight proteins will possess broad isotopic distributions, distributing the signal from a single protein across many peaks. In addition, a distribution of different charge states of analyte will be displayed in electro-spray ionization (ESI). From the combination of these two effects, the signal arising from an individual proteoform may be split into hundreds of channels, which reduces the signal at any mass-to-charge ratio (m/z). Perhaps, this becomes more prominent at high mass values as the number of isotopic peaks and charge states increase.

Usually, a protein must be first ionized well into the gas phase before its detection and fragmentation. So far, MALDI and ESI both are the two most common protein ionization techniques. MALDI generates single charged ion species and requires a mass analyzer capable of detecting high m/z species using time-of-flight (TOF) instrumentation. However, MALDI-TOF high throughput proteomics has limitations such as poor fragmentation, low resolution and the requirement of relatively purified samples and difficulty coupling to separations [57,58]. ESI usually generates multiple charged ion species and is considered as the preferred method for both peptides and intact proteins analysis. Taking advantage of mass accuracy and high mass resolution, top-down proteomic studies have majorly been implemented ESI coupled either with fourier transform ion cyclotron resonance (FTICR) or orbitrap mass analyzers and these two techniques will be the focus of further discussion.

Fourier transform ion cyclotron resonance mass spectrometry

Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) relies on the ion excitation at its cyclotron frequency within a strong magnetic field [59]. A spatially coherent packet of ions were generated by this excitation, which orbit at an increased radius, allowing for the detection by monitoring the image current on a detection plate. The detected signal (also termed a transient) is converted from the time domain to the frequency domain through a Fourier transform and then to m/z through mass calibration.

For the first time in 1989, ESI-FTICR-MS was used for the intact protein analysis with a detection of multiple charge states on a single protein [60]. Further top-down studies demonstrated the isotopic resolution on proteins using a 2.8 Tesla instrument which showed accurate mass determination [61]. The same instrument was also utilized to perform collision-induced dissociation (CID) and nozzle-skimmer dissociation (NSD)of ubiquitin, using the high resolving power of the instrument to determine charge state and identity of the fragment ions [62]. An advanced hyphenation to FT-ICR technology, often focused on improving the analysis of intact proteins, included increases in magnetic field [63,64], an accumulation octupole for ion storage before transmission to the ICR cell [65,66], addition of a resolving quadrupole for mass selection [67]. These modifications, allowing for increased sensitivity, dynamic range, and resolution were used on a 9.4 T instrument for the detection and identification of proteins from M. jannaschii and S. cerevisiae [68]. This instrumentation design has been used for a variety of top-down proteomic studies [42, 69, 70].

A linear quadrupole ion trap/FTICR-mass spectrometer construction was first reported in 2004 [70] and this instrument allowed for the storage and manipulation of ions from a continuous ion source in the linear trap before injecting them into the ICR cell. Mass accuracy was improved through the accurately controlling the number of ions (use of automatic gain control (AGC)) that are allowed to enter the ICR cell even from variable ion flux into the instrument which is typical of LC–MS. Fragmentation is also performed within the ion trap and fragment ions able to be detected using the high resolution and mass accuracy of the ICR analyzer or the ion trap speed. This instrument was commercialized using a 7 T (Tesla) magnet, achieving 100000 resolving power (m/z 400) and as well as <2 ppm mass accuracy without internal calibration. The use of 7 T LTQ-FTICR for top-down proteomics for the analysis of the S. cerevisiae proteome [39] and membrane proteins [72] has been reported. Similarly, a 12 T version of the instrument has been used for increased throughput studies using the automated on-line/off-line RPLC-MS platform for the study of human leukocytes [40], and M. acetivorans [73] as well as the GELFrEE platform for the analysis of S. cerevisiae [74]. A 14.5 T version of the LTQ-FTICR instrument has also been reported, featuring approximately 4-fold higher mass accuracy and twice the resolving power of the 7 T instruments [75].

Orbitrap mass spectrometry

The Orbitrap mass analyzer, a new type of Fourier trans-

form mass spectrometer was described in 2000 [76]. This trap features a pair of axially symmetric electrodes: a central "spindle-like" electrode and an outer "barrel-like electrode". In this electric field, ions rotate around the central electrode while oscillating down the length of the electrode. The frequency of these oscillations is proportional to $(m/z)^{-1/2}$. Image current on the outer electrodes is monitored and the resulting time domain signal is converted to frequency and then to m/z as in FTICR. Also similar to FTICR, the orbitrap mass analyzer has been coupled to a LTQ allowing for use of a continuous ion source (e.g. ESI), increasing mass accuracy with automatic gain control (AGC), and enabling efficient fragmentation [77]. Coupling of the two analyzers was achieved by the use of a transfer octupole following the ion trap into a curved rf-only quadrupole (C-trap) used to eject ions axially towards the orbitrap analyzer. This new instrument was capable of obtaining 60000 resolving power (m/z 400) using a one second transient, achieving isotopic resolution of myoglobin and carbonic anhydrase.

In 2006, the use of the LTQ-orbitrap for more extensive analysis of intact proteins was first reported [78]. The authors reported reproducible <10 ppm mass accuracy with this instrument on intact proteins and confidently identified the proteins using CID fragmentation (MS2 and MS3). The study of low molecular weight proteins from human blood including the quantitation of apolipoprotein proteoforms [79,80] using LTQ-orbitrap instrumentation. The LTQ-orbitrap was also used to distinguish several glycoforms from intact recombinant antibodies (150 kDa) and fragment the reduced light and heavy chains using CID [81]. Similarly, the structural characterization of intact antibodies using higher-energy collisional dissociation (HCD) based high-resolution LTQ-orbitrap mass spectrometry has been reported [82]. A new branded instrument LTQ Velos with significant modifications to the linear ion trap is also used for the top-down analysis [83]. Improved ion injection optics allowed for 5-fold reduction in ion injection times and the use of two linear ion traps allowed for more efficient trapping and CID fragmentation in a higher pressure trap and higher resolution scanning in a lower pressure trap.

The LTQ-orbitrap Velos has been used for the analysis of disease causing hemoglobin variants from dried blood droplets for potential clinical use [84]. Antibodies have also been analyzed using this instrument, allowing for improved sequence coverage through the use of electron transfer dissociation (ETD) of the disulfide intact species [85]. ETD is an electron-based fragmentation technique similar to ECD, but utilizes gaseous anions to transfer low-energy electrons to protonated analytes [86].

Michalski et al. reported a compact high-field orbitrap, coupled with an improved Velos PRO dual ion trap mass spectrometer and advanced signal processing, capable of a nearly 4-fold increase in resolution [87]. Top-down proteomic analysis of H1299 human cancer cell line proteome using this instrument has also been reported and identified 690 unique proteins from the H1299 human cancer cell line [88]. Similarly, top-down analysis and the identification of 1976 unique proteins from an H1299 cell line has also been reported [89].

Other types of mass spectrometry instruments (e.g., time-of-flight mass spectrometers and ion-traps) can also be applied for intact proteins analysis, but till date they are less effective than FTICRs, and hybrid linear ion trap (LTQ)–orbitraps [78] or LTQ-FTICRs [90]. In fact, the new-design orbitrap from Thermo Fisher utilizes a higher field orbitrap analyzer and improved ion-trap, providing another opportunity for top-down proteomics [89]. The combination gives improved resolving power over older designs and higher speed, making it competitive with FTICR-based instruments [91]. Fragmentation so far is by either ECD or ETD, but the relative effectiveness of ETD remains unknown for large proteins. Nevertheless, a significant advantage of orbitraps compared to FTICRs is that mass resolving power decreases as the square root of m/z not as its first power, making resolving power on orbitraps less sensitive to m/z [48]. MALDI-TOF is less competitive than FTICR and orbitraps and several mass spectrometry groups utilized this approach for top-down analysis by employing the strategy of in-source decay (ISD) [88,92-94].

Fragmentation methods

In proteomics study, gas phase chemistry has played a crucial role in mass spectrometry (MS). The ion dissociation or transformation to the characteristic tandem mass spectrometry spectra (MS2) fragmentation patterns is the key step generating the structure information of a protein or a peptide. Even though, collision-induced dissociation (CID) is the most widely applied fragmentation method for proteome identification and quantification analysis, it is not suitable for fragmentation of intact proteins, and peptides with labile post-translational modifications, such as phosphorylation and S-nitrosylation [95]. Usually tandem mass spectrometry (MS/MS) involves the characterization of a precursor ion based on its fragments. MS/MS involves isolation of the parent ion followed by exposure to an external stimulus to induce controlled fragmentation. It is necessary to fragment the parent ion in a sequence-specific manner to gain sequence information about a peptide, i.e., to cleave be-

tween each amino acid residue with a single cleavage per intact peptide or protein ion.

There are many different MS/MS fragmentation techniques. Most commonly used fragmentation techniques are (i) collision-induced dissociation (CID) [96,97] (ii) electron capture dissociation (ECD), and (iii) electron transfer dissociation (ETD). CID, ECD and ETD methods are used in top-down proteomic analysis.

Collision-induced fragmentation

Collision-induced dissociation (CID) is also referred as collision activated dissociation (CAD). It was first described by Jennings [98] and McLafferty and Bryce [97]. In CID, parent ions are allowed to collide with neutral collision gas atoms or molecules (typically helium, nitrogen or argon which result the formation of 'b' and 'y' -type ions [98]. **Figure 3** shows the basic fragmentation behavior of CID (b- and y-ions), ECD and ETD (c and z ions).

Usually, CID is more effective for small and low-charged

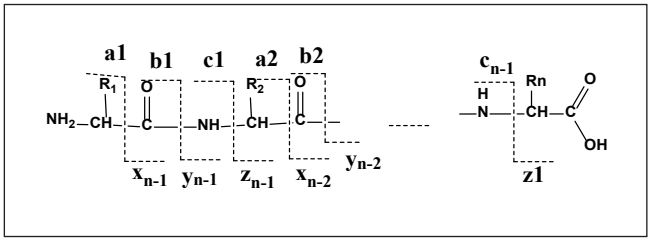

Figure 3. Peptide/protein fragmentations nomenclature. Formation of b, y (CID), 'c' and 'z' ions (ECD and ETD).

peptides. The basic residues which are present in a peptide sequence may also prevent dissociation and generating few sequence ions. In addition, CID is not suitable for fragmentation of intact proteins, and peptides with labile post-translational modifications, such as phosphorylation and S-nitrosylation. In general, CID does not generate complete fragmentation in order to fully characterize proteins, but rather produces sufficient fragments or sequence tags to identify the protein. Furthermore, if the protein contains post-translational modifications (PTMs), low-energy CID most likely will not be appropriate to localize the modified site or the PTM. As the CID fragmentation method is found to be less effective fragmentation method for top-down analysis, there are two major fragmentation techniques that are used in top-down MS/MS analysis, i.e. electron capture dissociation (ECD) and electron transfer dissociation (ETD) fragmentation. However, top-down MS is still in its early developmental stage and in the process of overcoming several technical challenges. The inclusion of dissociation methods such as electron capture dissociation (ECD) greatly improves the top-down capabilities. ECD is especially utile for mapping labile post-translational modifications which are well-preserved during the ECD fragmentation process. Top-down MS with ECD has been successfully applied to cardiovascular research with unique benefits in separating the molecular complexity, quantifying modified protein forms, complete mapping of modifications with full sequence coverage, discovering unexpected modifications, and identifying and quantifying positional isomers and determining the order of multiple modifications [99-104]. The non-ergodic electron-based MS/MS techniques, ECD and electron transfer dissociation (ETD), are particularly suitable for the localization of labile PTMs like phosphorylation [100, 104].

Even though, there are several electron-based fragmentation methods available, only few fragmentation methods i.e. ECD and ETD fragmentation methods are discussed in this paper.

Electron capture dissociation

Electron-capture dissociation (ECD) is one of the fragmentation methods in which gas phase ions will be fragmented to elucidate the structure of the precursor compound. It was first time reported by Roman Zubarev and Neil Kelleher [105]. The ECD technique involves the capture of electrons by positive precursor ions to form radical cations with subsequent specific cleavages for peptide N-Cα bonds (fragments). **Figure 4** shows the ECD fragmentation scheme of protonated protein.

$$[M + nH]^{n+} + e^{-} \rightarrow [\,[M + nH]^{(n-1)+}]^{*} \rightarrow \quad \text{fragments}$$

ECD is proved as a significant MS/MS technique for biomolecules analysis [106] predominantly for peptides and proteins. Major advantage of ECD for peptide/protein analysis is (i) ECD fragmentation results random cleavage. Therefore, sequence coverage tends to be higher for ECD than other fragmentation techniques [107,108], and ECD fragmentation efficiencies allow sequencing of longer peptides (20-30 residues), whereas CAD fragmentation is limited to 20 or fewer residues (ii) labile PTMs are retained on peptide/protein backbone fragments [109]. In ECD, interested multiply charged ions are irradiated with low energy electrons (<0.2 eV) producing charge-reduced species, which dissociate along radical-driven pathways. The major product of electron capture by peptide $[M+nH]^{n+}$ cations is typically the charge-reduced species $[M+nH]^{(n-1)+}$, that is, the precursor peptide ion that has captured an electron but has not dissociated. Often, that is accompanied by hydrogen loss,

i.e. formation of $[M+(n-1)H]^{(n-1)+}$. The dominant peptide fragmentation pathways proceed via cleavage of the backbone N–Cα bond to give c-and fragment ions [110] (which may be accompanied by hydrogen atom transfer to give and, more commonly, z-fragment ions [110] and disulfide bonds [112]. The fragments observed during ECD are shown in **Figure 4**. The mechanism by which c and z fragments are generated following electron capture has been, and remains, the subject of intense debate. The original ECD experiments utilized a heated metal filament, e.g., tungsten or barium. In order to produce sufficient fragments for detection, it was necessary to irradiate precursor ions for 30 s. As such, ECD was incompatible with LC and therefore unsuitable for bottom-up proteomics. The problem was addressed by the introduction of the heated dispenser cathode by Tsybin in 2001 [113]. The timescale for ECD was reduced significantly (ms) and it became possible to couple ECD with online liquid chromatography [114-116].

Palmblad et al. [115] coupled LC with ECD for the analysis of a tryptic digest of bovine serum albumin and Davidson and Frego [114] demonstrated LC-ECD analysis of a pepsin digest of cytochrome c. Zubarev and co-workers demonstrated an approach on a modified LTQ FT hybrid linear ion trap FTICR in which a low resolution survey scan in the ion trap was followed by analysis of CID and ECD in the ICR cell [117]. Creese and Cooper [118] undertook a direct comparison of LC-ECD-MS/MS with LC-CID-MS/MS revealed that, CID resulted in greater overall protein coverage, whereas

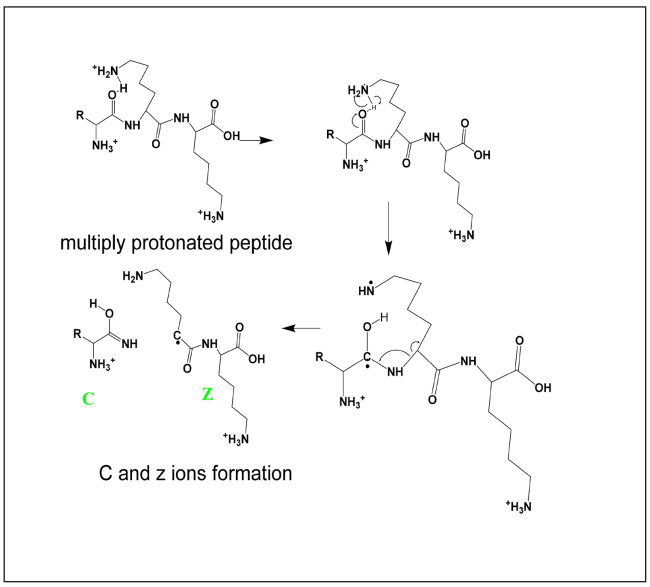

Figure 4. ECD fragmentation scheme for production of c- and z-type ions after reaction of a low-energy electron with a multiply protonated peptide.

ECD gave higher confidence in peptide sequence, thus showing the complementary nature of CID and ECD for bottom-up proteomics. ECD has been used frequently used for top-down analysis of proteins. The original paper by Zubarev et al. [105] showed that ECD of ubiquitin (8.6 kDa) resulted in cleavage of 50 of the 75 backbone N–Cα bonds; however, it was noted that ECD efficiency decreased with increasing protein size [108].

From these above-mentioned achievements, ECD will be certainly considered as an extraordinary tool in the up-coming future. The electron-based dissociation techniques deliver valuable structural information on different types of precursor ions. Hence, based on the information, the structure of these ions can be deduced from the fragment ion distribution. Since the inception of ECD, many review articles have been published on ECD based ion electron reaction mechanism [108,118,119,106, 103].

Electron tansfer dissociation

In a similar MS/MS fragmentation technique called electron-transfer dissociation the electrons are transferred by collision between the analyte cations and reagent anions [120-122]. ETD is known as electron transfer dissociation is one of the fragmentation methods in which fragmentation of the precursor ion will take place via the transfer of the electrons from an electron donor, reagent ion. Usually, in ETD method, reagent compounds such as fluoranthene, anthracene and azobenzene etc. were employed.

ETD is a method of fragmenting ions in a mass spectrometer [100,101]. Similar to electron-capture dissociation; ETD induces fragmentation of cations (e.g. peptides or proteins) by transferring electrons to them. It was invented by Donald F. Hunt, Joshua Coon, John E. P. Syka and Jarrod Marto at the University of Virginia [123]. ETD does not use free electrons but employs radical anions (e.g. anthracene or azobenzene) for this purpose:

$$[M+nH]^{n+} + A^{-} \rightarrow [[M+nH]^{(n-1)+}]^{*} + A \rightarrow \text{fragments}$$

where A is the anion. ETD cleaves randomly along the peptide backbone (c-and z-ions) while side chains and modifications such as phosphorylation are left intact. The technique only works well for higher charge state ions (z>2), however relative to collision-induced dissociation (CID), ETD is advantageous for the fragmentation of longer peptides or even entire proteins. This makes the technique important for top-down proteomics [123]. Much like ECD, ETD is believed to be particularly effective for peptides with modifications such as phosphory-

lation [98]. **Figure 5** shows the schematic view of ETD enabled Thermo LTQ Velos Pro mass spectrometer, ETD ion source, ETD reagent vials with heated inlets are located at the back side of the instrument.

Differences between ECD and ETD

Even though ECD has been proven to be a most useful and powerful fragmentation technique, ECD is not suitable to the all types of instruments. There is a restriction for ECD fragmentation i.e. low mass cut-off restrictions of the RF field in ECD, electrons cannot be captured in quadrupole ion traps. To overcome this restriction, specific anions (anthracene anions) were used as electron donors by Syka, Coon and Hunt to transfer electrons to multiple charged peptide cations, creating the electron

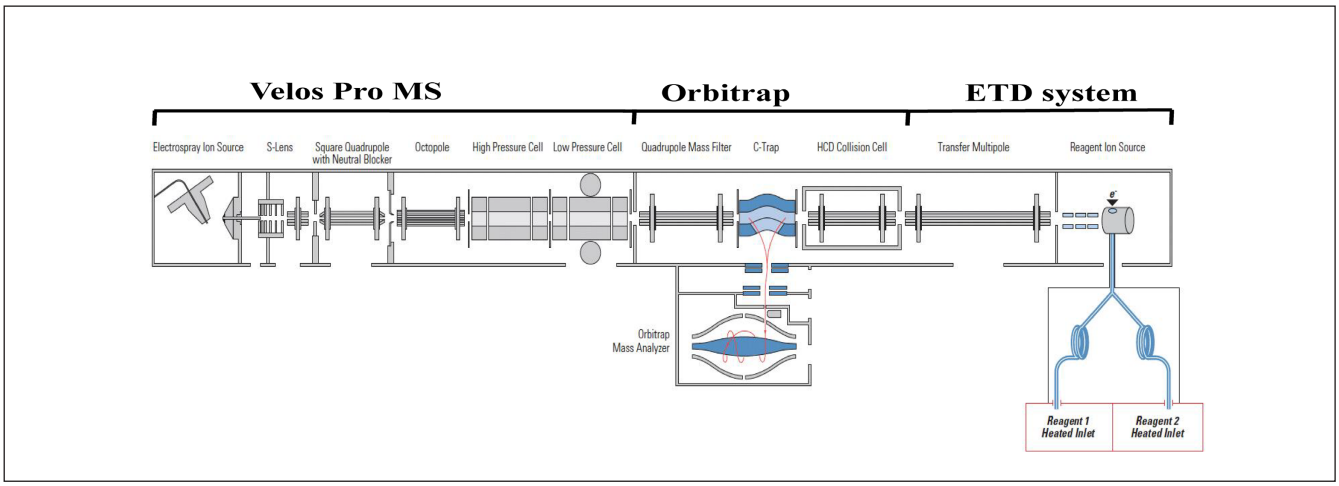

Figure 5. Schematic presentation of Orbitrap Velos Pro ETD mass spectrometer; Reagent ion source and reagent heated inlets are located at the back side of the instrument. Reprinted from Thermo Inc. operating manual.

transfer dissociation (ETD) technique [125]. Preliminary data from a modified 3D ion trap showed that N–Cα or disulfide bonds were cleaved via ETD by interactions of multiply charged ions with anions (**Figure 6**).

However, the ETD fragmentation technique was found to be lower efficient than that of ECD, because the recombination energy for ETD requires the electron to overcome the binding energy barrier from the anion before transferring to the cations. However, implementation of ETD in popular low-cost ion trap instrument was a significant discovery for the mass spectrometry society.

Techniques and search engines used in top-down analysis

Most popularly used search engines for identification of proteins via bottom-up approaches are MascotTM, SequestTM, X!tandemTM, PhenyxTM, and ProteinProspectorTM. In peptide mass fingerprinting experiments the protein identification is done by comparing experimental enzymatic digests m/z values with theoretical m/z values of silico protein digestion. Alternatively database searching with experimental MS/MS data sets of selected ions in the low mass range can also be used for identification. To decrease the number of complications these search engines are constantly improved [126]. Although various proposals are under stage of experimentation, for instance to combine searches from various engines is still at early stage of experimentation. The software solutions for the analysis of intact protein fragmentation and top-down analysis are not fully developed. There is a huge demand for software that used for web-based identification and characterization of proteins by direct comparison of parent and fragment ions masses against elucidated proteomic databases. The first search engine that used for top-down identification of proteins was ProSight [127,128]. This software is a combination of search engines and a browser environment for the analysis of fragments above 10 kDa. ProSight focused on organism specific databases of protein and site-specific information. Fragment ion masses of high mass parent ions are used to perform searches against the constantly updated databases, which include post-translational modifications and single nucleotide polymorphisms (SNPs). Apart from ProSight, new web application tools have been developed. The most widely used web-based database search engine for bottom-up approach is Mascot due its probability-based scoring method and its

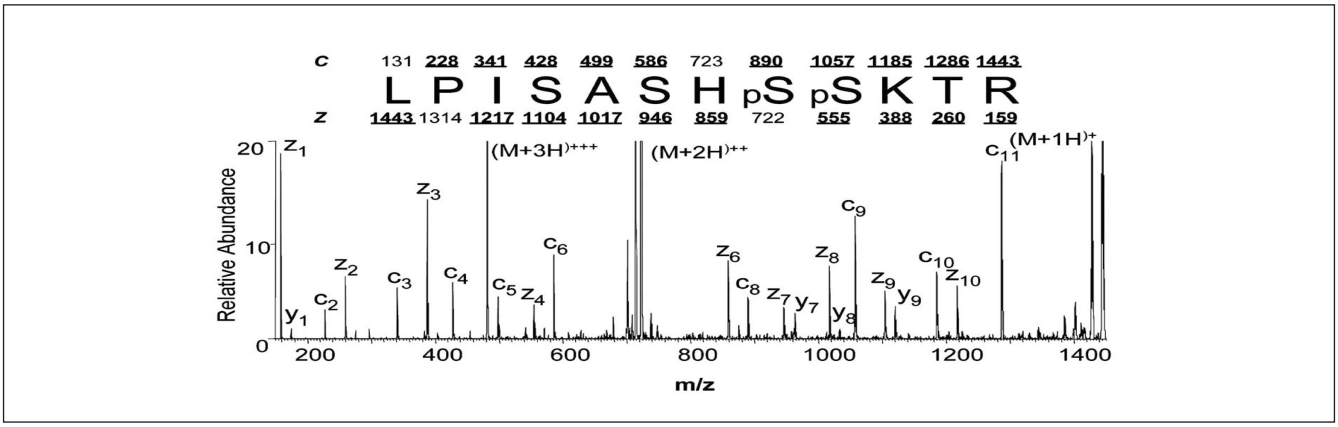

Figure 6. Single-scan ETD MS/MS spectrum of triply charged phosphopeptide, LPISASHpSpSKTR (m/z 482) via the reaction with anthracene anions (activation time 50 ms). All other possible c and z type ions appear in the spectrum. Peptide sequence based on the fragmentation is also shown in the figure.Total experiment time was ≈300 msec. Reprinted with permission from [125].

fast operation algorithm, is limited to 16 kDa for precursor ion selection. BIG Mascot has been exclusively designed for top-down analysis and is therefore used for identification of large proteins fragmented via CID or ECD [129]. Align spectra is another approach used to assign the different isomers of protein [130]. This is an algorithm based approach that can find optimal alignments between an experimental mass issued from spectrum and a theoretical mass of protein sequence by using a constant number of mass values that correspond to post-translational modifications and mutations.

Top-down proteomics applications

From the review of published literature, it is well-known that top-down (TD) proteomics has various applications in different fields such as snake Venomic proteomics, intact protein analysis, cross-link protein analysis, cardiovascular, biomedical research, etc. Even though, TD has many applications in various biology fields, only few selected applications are given in this review.

TDP in snake venomic proteomics

The study on snake venoms has inspired many scientists to discover or develop number of pharmaceutical drugs, diagnostic kits, and research molecular tools. A nice review has been presented with details of different methodological approaches used so far to quantify all proteoforms present in any given snake venom [131,132]. The recent advancements in mass spectrometric instrumentation, dissociation strategies, and bioinformatic tools including top-down mass spectrometry instrumentation is increasingly attaining momentum in proteomic analysis [133-142]. More recently, Daniel P. et al was the first person who applied top-down mass spectrometry for the snake (Indonesian King Cobra, Ophiophagus Hannah) Venomic proteomics applications [143].

TDP in intact proteins analysis

A rapid purification, reliable quantification, and comprehensive characterization of α-actin isoforms due to genetic variations together with PTMs was recently developed [144]. It was reported that using top-down MALDI-FTICR-MS platform, the mass analysis of intact human serum peptides and small proteins with isotopic resolution up to ≈15 kDa and identified new proteoforms from an accurate measurement of mass distances [145]. It was proved that mass spectrometry (MS)-based top-down proteomics, multi-dimensional liquid chromatography (MDLC) strategies can effectively separate intact proteins with high resolution and automation are highly desirable [36]. Usually in proteomics, the major problem is the structural characterization of proteins expressed from the genome. To solve this problem, first we have to separate the interested protein from a complex mixture, identification of its DNA-predicted sequence, and the characterization of sequencing errors and post-translational modifications. And finally, top-down mass spectrometry (MS) approach with electron capture dissociation (ECD) will be applied to characterize proteins. A similar approach for the characterization of proteins involved in the biosynthesis of thiamin, Coenzyme A, and the hydroxylation of proline residues in proteins has been reported [146].

TDP in crosslink protein analysis

Top-Down proteomics is getting more attention in the field of crosslink proteins analysis. Chemical cross linking hyphenated with mass spectrometry can be a pow-

erful methodology for the identification of protein-protein interactions and for providing constraints on protein structures. However, enrichment of cross linked peptides is crucial to reduce sample complexity before mass spectrometric analysis.

The heterogeneity of a complex protein mixture from biological samples becomes even more difficult to deal with when a person's attention is shifted towards different protein complex topologies, transient interactions, or localization of protein-protein interactions (PPIs). A well-known method, i.e. chemical cross-linking of proteins, which gives not only identities of interactors, but could also provide information on the sites of interactions and interaction interfaces. Kruppa et al. reported the top-down mass spectrometric analysis of proteolytic digested crosslink proteins that have been covalently modified by bifunctional cross-linking reagents which can be useful for protein structure determination [147]. Similarly, Novak et al. also has reported a top-down approach for the protein structure studies using FT mass spectrometry and chemical cross linking [148]. **Figure 7** shows the schematic illustration of cross-link proteins analysis using top-down approaches such as ECD and ETD, etc. for their structural identification using data analysis softwares such as proteome discoverer, Stavrox and Prosight softwares, etc. A nice review was published in 2006 which reported the use of top-down approaches for the three dimensional protein structure identification and as well as protein-protein interaction studies [149].

TDP in Biomedical Research

Top-down MS has been successfully applied to cardiovascular research with the unique advantages such as quantifying modified protein forms, complete mapping of protein modifications with full sequence coverage, discovering unexpected modifications, and identifying and quantifying positional isomers and determining the order of multiple modifications [99]. Since top-down MS analyzes whole proteins instead of small peptides, it can easily reveal the full extent of molecular complexity of a protein [13]. Top-down mass spectrometry was applied by Zhang et al. to study phosphorylation of cardiac troponin in a set of post-mortem and transplant human heart tissue samples with chronic heart failure [150]. This group also characterized different phosphorylation sites and splice variants present in human and mouse cardiac tissues [151].

An overview of the recent applications of top-down proteomics in biomedical research has been provided and outlined about the challenges and opportunities facing top-down proteomics strategies aimed at understanding and diagnosing human diseases [152]. Using a top-down MS strategy, Ying Ge and co-workers linked the altered Post-translational modification of cardiac troponin-I (cTnI) to Heart Failure (HF)-associated contractile dysfunction in both animal models of HF and human clinical samples [153].

Recent times, the type II diabetes mellitus (T2D) cases have increased dramatically. Therefore, there is a signif-

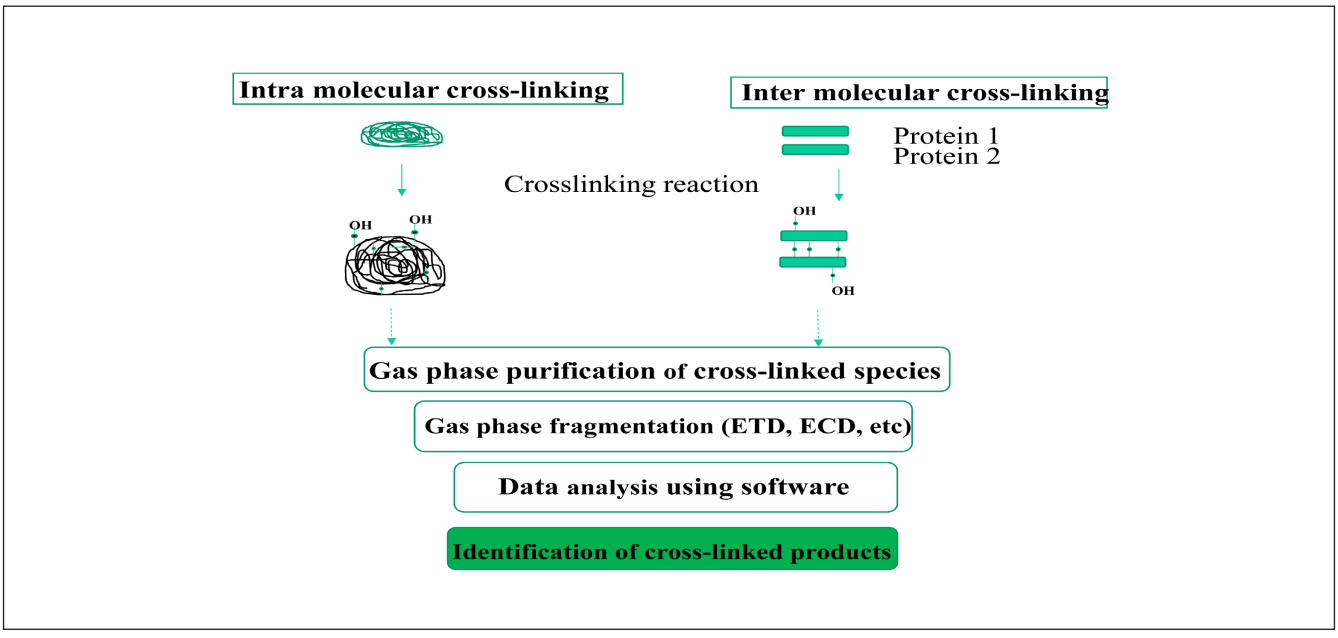

Figure 7. A schematic illustration of Intra and inter- molecular crosslink proteins analysis using Top-Down MS approaches (ECD and ETD, etc).

icant interest in understanding the mechanisms contributing to the development of this condition [154]. Borges et al. using a top-down proteomics approach identified a panel of PTM-based biomarkers to distinguish the spectrum of cardio vascular disease (CVD) and T2D co-morbidities [155].

TDP in micro-organisms identification

The recent top-down MS applications have focused on the role of bacterial protein PTMs in infection [156-158]. Top-down MS was employed to study the PTM of intact pilin from the pathogenic bacterium Neisseria meningitidis [158]. A study by Ansong et al. discovered a specific protein S-thiolation switch in Salmonella typhimurium in response to infection-like conditions [156]. More recently, a top-down MS method for the rapid and high-confidence identification of intact Bacillus spore species was developed. In this method, fragment ion spectra of undigested [whole] protein biomarkers are obtained without the need for biomarker pre-fractionation, digestion, separation, and cleanup [159]. Several proteins from E. coli, including an over expressed one directly from a cell lysate, using their charge stripping approach on an ESI/ion trap instrument were identified by McLuckey and co-workers [160]. The ability of the top-down technique rapidly progressed to characterize eukaryotic proteins with more complicated combinations of PTMs, especially as improved hardware and software become widely available [161].

TDP in neurodegenerative diseases and cancer research

Neurodegenerative diseases treatment strategies focus only on symptom management [162]. The top-down MS approach can be a preferred strategy for the elucidation of PTM-associated to disease mechanisms underlying neurodegenerative disorders [163-165].

A number of modified PTMs including phosphorylation and acetylation have been associated to the constitutive activation of cellular signaling pathways involved in the growth, proliferation, and survival of tumor cells. Perhaps, due to this link between the modified PTMs with tumor growth, several groups have already utilized top-down mass spectrometry for the identification of disease biomarkers and to study the effects of chemotherapeutics. [166-168].

TDP in human body fluids analysis

Human body fluids are considered as great sources of biological markers, especially, as sources of potential protein biomarkers of the diseases. Analytical tools that permit rapid screening, low sample consumption, and accurate protein identification are of great significance in studies of complex biological samples and clinical diagnosis. Nowadays, mass spectrometry is considered as one of the most important analytical tools with applications in a wide variety of fields. Mass spectrometry, especially, top-down MS has been used to find post-translational modifications and to identify key functions of proteins in the human body.

For example, a top-down LC/MS-based methodology for the separation and analysis of alterations in histone PTMs in primary leukemia cells from patients with refractory or chroniclymphocytic leukemia in response to treatment with depsipeptide, a histone deacetylase inhibitor was reported [167]. Similarly, top-down proteomic analysis of Cerebrospinal fluid evidenced the potential biomarker role of LVV- and VV-hemorphin-7 in posterior cranial fossa pediatric brain tumors. Top-down MS identified two peptides, originally believed to be CSF contaminants from the blood, as LVV- and VV-hemorphin-7, two opioid peptides produced from the enzymatic cleavage of hemoglobin. [168].

Transthyretin (TTR) amyloidosis and hemoglobinopathies are the archetypes of molecular diseases where point mutation characterization is diagnostically critical. Théberge et al. have developed a top-down analytical methodology for variant and/or modified protein sequencing and examined the possibility of using this platform for the analysis of hemoglobin/TTR patient samples and evaluating the potential clinical applications. For this analysis, they used a commercial high resolution hybrid Orbitrap mass spectrometer (LTQ-Orbitrap™) with automated sample introduction. The presence of a variant is revealed by a mass shift consistent with the amino acid substitution in the protein sequence.

Usually, Sickle cell disease and amyloidsis caused by single amino acid changes in hemoglobin and transthyretin (TTR). Therefore, it is essential the early detection of hemoglobin and TTR variants for the effective management of these diseases. A top-down MS platform utilizing affinity purification and direct injection of diluted whole blood for the detection of TTR and hemoglobin variants was developed by Costello and coworkers [169]. Also, Coelho Graca et al. developed a method for the rapid analysis of hemoglobin variants from patient blood samples [170].

Möhring T et al. described the top-down identification of endogenous peptides up to 9 kDa in cerebrospinal fluid and brain tissue by nanoelectrospray quadrupole time-of-flight tandem mass spectrometry. In this article, they described an approach using quadrupole time-of-

flight mass spectrometry (TOF-MS) as a highly efficient mass spectrometric purification and identification tool after off-line decomplexation of biological samples by liquid chromatography [170]. Post-translational modifications of proteins were identified by automated database searches and they identified thymosin beta-4 (5.0 kDa) and NPY (4.3 kDa) from rat hypothalamic tissue and ubiquitin (8.6 kDa) from human cerebrospinal fluid [171].

Tian et al. has developed a top-down MS method to identify different histone variants from a core histone extract. They identified 41 histone variants and they were also able to identify 20 different modifications sites on the different histones [172].Contrepois et al. used ultrahigh performance LC coupled to Orbitrap to quickly identify histone variants and PTMs in unfractionated core histones via top-down analysis [173].

TDP in relative quantitation

Quantitative information in proteomics is often required from the comparative studies between test (disease) and control (healthy) states. Usually, In the bottom-up proteomic approach, isobaric tag for relative and absolute quantitation (iTRAQ), isotope-coded affinity tags (ICAT), stable isotope labeling by amino acids of cell culture (SILAC), and related isotope-based labeling methods will be used to label peptides for MS measurements [174]. Another strategy which is "label-free" strategy involves quantitation by counting the spectra taken for a given analyte (assuming that abundant analytes will be characterized by a larger number of spectra than low-level analytes) [175,176]. The information about protein isoforms is usually missing in bottom-up approach because it measures peptides. Top-down analysis provides quantitative data on different isoforms including individual and post-translation modifications. In addition, given that a protein modification gives several site-specific isoforms, a crude measure of the abundance of one isoform can be made by comparing the intensities of the peaks corresponding to unmodified and modified peptides [177]. Zabrouskov et al. [177] characterized sequentially the relative step-wise deamidation of bovine ribonuclease A at five sites: Asn67,Asn71, Asn94, Asn 34, and Gln4. In more detail, Ying Ge's group [102] considered the possible change of fragmentation efficiency owing to addition of a modification group to the protein and the variation of the modification sites. She introduced a quantitation scheme by using the fragments from the unmodified protein as "yardsticks" to normalize those unmodified fragments from the modified protein. Then the abundances of the modified fragments from the modified protein can be compared with those of unmodified fragments obtained in the same experiment.

TDP in native mass spectrometry

Native mass spectrometry (MS), or sometimes it is called as "native electro-spray ionization" allows proteins in their native or near-native states in solution to be introduced into the gas phase and investigated by mass spectrometry. This approach is found to be as a powerful tool to investigate protein complexes and allows the structural elucidation of protein complexes. It will not provide a structural model in atomic detail, but the sensitivity, speed, selectivity, dynamic mass range and mass accuracy of the analysis provides important advantages over other techniques. Its sensitivity permits the investigation of endogenous protein complexes, and another major benefit of the method is that it can simultaneously analyze several species in one spectrum. In simple words, if there is a heterogeneous population of protein complexes present in one sample, the interested one can be specifically isolated for further studies. Electro-spray ionization, a gentle ionization method allowing the preservation of quaternary protein structure, is the most popular technique to ionize the proteins/protein complexes of interest in this research field. Normally the protein complex is sprayed from a volatile buffer, compatible with the electro-spray process.A range of mass spectrometric approaches can be applied to investigate the biological systems. The exact mass of proteins can be determined, but also the stoichiometry of an assembly, its stability, dynamical behavior, conformation, subunit interaction sites and topological arrangement of the individual proteins within a complex. Zhang et al. reported a nice review [178] review which explained the background of native MS of protein complexes and described its strengths, taking photosynthetic pigment–protein complexes as examples.

Challenges, limitations and perspectives

Since 1998 to till date, many number of publications have published on "top-down approach" and this number is showing the capability of the approach. Nowadays, many more laboratories are adopting this approach. Its major strength is the utilization of electron-based fragmentation to determine various protein structures, structural modifications (for example, post-translational modifications and other changes (e.g., amino-acid substitutions) in proteins, protein-protein interactions, etc. However, this approach has also following limitations and challenges.

Protein chromatography issues

The major issue of protein chromatography is the solu-

bility of proteins. Usually, working with peptides is rather easy due to their high solubility and are easily separated on any reversed phase column. In case of proteins, they are not the same. Large number of proteins is not easily soluble. Furthermore, LC-MS based protein chromatography is not friendlier in comparison with LC-MS based separation of peptides, in terms of peak shape, retention times and resolution. Because, larger proteins, on C4 reversed phase columns, are tend to be bind irreversibly to the phase with eluents used for the MS analysis. Due to these reasons, the most of the top-down (TD) applications deal with complex protein solutions that are preliminary separated by classical chromatographic methods. Samples are then collected and analyzed by TD after appropriate buffer exchange. The sample preparation workflow is complex and thus preventing TD from high throughput data production that remains Bottom-Up (BU) approach alive.

Lower sensitivity of TD analysis

The second limitation of top-down approach is the lower sensitivity for detection of PTMs than that of bottom-up approach. Till date, TD proteomics suffers from a sensitivity issue. This is due to the low ion counts for a single TD fragmentation event. Hence, the sample amount required for TD may be 10-100 fold more in comparison with BU experiments for the complex proteome analysis. Advanced technological is highly desired to address these issues. From this review, it is clear that protein separation methods coupled with top-down mass spectrometry will be a breakthrough to characterize therapeutic proteins. We hope that improvements in the technology and as well as methodologies might be on the way to resolve these issues with the new advanced instrumentation such as Orbitrap Elite, etc. The required technological developments were headed by the Kelleher group to realize the hope that top-down MS can be applied to solve problems in systems biology.

Limited number of bioinformatics tools

Till date, lack of sufficient bioinformatics tools for the Top-Down data analysis, management and interpretation in comparison with BU approach. The most convincing web-based softwares for TD data generated by ESI are ProSight PTM, ProSight PC and ProSight Lite softwares from Prof. Kelleher group. These tools allow the identification of proteins, both single and in mixtures, searching TD data against the most common online databases, with the possibility to detect PTMs, alternative splicing products and single nucleotide polymorphisms. In the near future, however, bioinformatics for ESI TD should gain increasing space. As an FTMS instrument working on a 20–30 kDa protein has the feasibility to accomplish an almost residue-by-residue de novo sequencing of the entire protein, to date, only a few solutions are available for the efficient interpretation of this huge amount of data.

Due to the above-mentioned disadvantages, BU proteomics is still alive and will still produce important data flows for long time. It is clear from the recent discoveries that the unquestionable advantages of TD techniques are moving forward to reduce this gap and we hope that substantial improvements might be implemented in routine TD proteomics over the next few years.

Perspectives

The extension of ECD to other non-FTICR types of mass spectrometers should have advantages in expanding the mass spectrometry field. Recently, Voinov et al. [179] constructed a radiofrequency-free magnetic cell in which ECD products were generated and detected in a triple quadrupole instrument via the replacing the second quadrupole with this cell. This improvement brings ECD into the realm of beam-type instruments. By adjusting the voltages, ECD and CAD can be accomplished sequentially in the cell [180]. The hyphenation of Q-TOF instruments with their shorter duty-cycle may provide a more convenient means to achieve high-throughput ECD top-down analysis. In–source atmospheric pressure (AP)-ECD is another potentially interesting advanced instrumentation [181].

On other side, construction of high-field FTICR instruments for improved performance is already established with a 21 T magnet [182]. In the meantime, the top-down technology will coexist with bottom-up and middle-down approaches for application to identification and biophysical investigation of proteins and other biomolecular entities.

Conclusion

From the above-mentioned top-down MS applications, it is clearly noted that TD mass spectrometry is well-suited for target-compound analysis. The protein biophysics research is growing in combination with hydrogen/deuterium exchange and other chemical labeling strategies to probe protein conformation and dynamics. Similarly, determination of the stoichiometry and compositions of macromolecular assemblies has significant importance in structural biology, because many biological functions are accomplished by proteins in complexes rather than by isolated proteins. From our scientific review of existing literature, it is concluded that ECD analysis of intact complexes using FTICR delivers both identification and

structure information which holds significant importance in this direction. Also, a hyphenated ETD/ECD with the Q-TOF/ion mobility platform seems promising for the study of protein complexes; this integration gives cross sections, their changes for ETD/ECD fragments, and possibly CAD fragments to deduce structure.

In addition, the high costs of TD setups play a vital role in the TD proteomics implementation in worldwide MS core facilities. However, TD has the caliber to elucidate protein structure down to such an accurate level that advanced discoveries in biomedical and biological sciences are to be expected in the next future.

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