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MINIREVIEW ARTICLE

Recent developments and applications of electron transfer dissociation mass spectrometry in proteomics

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Abstract Electron transfer dissociation (ETD) has been developed recently as an efficient ion fragmentation technique in mass spectrometry (MS), being presently considered a step forward in proteomics with real perspectives for improvement, upgrade and application. Available also on affordable ion trap mass spectrometers, ETD induces specific N–Cα bond cleavages of the peptide backbone with the preservation of the post-translational modifications and generation of product ions that are diagnostic for the modification site(s). In addition, in the last few years ETD contributed significantly to the development of top-down approaches which enable tandem MS of intact protein ions. The present review, covering the last 5 years highlights concisely the major achievements and the current applications of ETD fragmentation technique in proteomics. An ample part of the review is dedicated to ETD contribution in the elucidation of the most common posttranslational modifications, such as phosphorylation and glycosylation. Further, a brief section is devoted to top-down by ETD method applied to intact proteins. As the last few years have witnessed a major expansion of the microfluidics systems, a few considerations on ETD in combination with chip-based nanoelectrospray (nanoESI) as a platform for high throughput top-down proteomics are also presented.

Keywords Electron transfer dissociation · Mass spectrometry · Phosphorylation · Glycosylation · Top-down proteomics

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Introduction

Protein identification requires total or partial determination of its sequence, which previously was achieved by Edman degradation or, in some particular cases, based on protein recognition by specific antibodies. The discrepancy between the increasing analysis complexity and the minute analyte quantity available from biological sources, gave rise lately to massive efforts for the development of novel methods for protein de novo identification. Now, in the post-genome era, electrospray (ESI) mass spectrometry (MS) develops as one of the most powerful analytical techniques in proteomics. Its potential increased even more after the advancement of efficient dissociation techniques (Jones and Cooper 2011; Dodds 2012; Palumbo et al. 2011) based on tandem MS (MS/MS) and multiple stage MS (MS^n) which provide the possibility to sequence complex ionic species and carry out a straightforward structural analysis at subpicomolar sensitivity levels.

The conventional MS strategy for protein analysis is the so-called bottom-up approach, based on enzymatic

digestion, which renders a collection of peptides to be identified through the peptide mass fingerprint provided by ESI MS. A more comprehensive variant is the one including also the information acquired by collision-activation techniques, among which the popular collision-induced dissociation (CID), for peptide sequencing in MS^n. In this method, gas-phase peptide cations are internally heated by multiple collisions with rare gas atoms. CID leads to peptide backbone fragmentation of the C–N bond resulting in a series of b-fragment and y-fragment ions. Due to the slow heating, an energetic feature associated with this method, internal fragmentation and neutral losses of H_2O, NH_3, and labile post-translational modifications (PTM) are common (Zauner et al. 2012). Consequently, for protein analysis CID exhibits two major drawbacks: (a) incomplete sequence information for large peptides (>15 amino acids) and intact proteins; (b) limited possibility to characterize biologically essential PTM and their site(s) since, by CID, these attachments readily cleave off.

To eliminate these shortcomings, in the last decade the research was focused on the development of MS/MS methods such as electron capture dissociation (ECD) and electron transfer dissociation (ETD) able to provide the entire peptide/protein sequence, and a complete characterization of the protein and any of its PTM in either a top-down or a bottom-up approach.

ECD and ETD

Pioneered by the group of McLafferty (Zubarev et al. 2000; Zubarev 2006) in 1998 and continuously developed, upgraded and optimized ever since (Breuker et al. 2004; Pan and Borchers 2013; Manri et al. 2013; Mao et al. 2013), ECD is based on the interaction of a free electron with a multiply protonated molecule to induce specific N–Cα bond cleavages of the peptide backbone. As compared to CID, ECD provides an extensive fragmentation resulting in improved sequence coverage. Moreover, the nonergodic feature of ECD preserves the labile PTM and consequently enables the generation of ions diagnostic for the modification site(s). The shortcoming of ECD resides in its availability merely on costly and sophisticated high-resolution Fourier-transform ion cyclotron resonance (FTICR) MS.

Because it is extremely challenging to conceive, design and operate the ECD method on an another instrument than FTICR MS (Deguchi et al. 2007), an ECD-like activation technique for use with versatile, widespread and less expensive ion trap mass spectrometers was developed in 2004 by the group of Hunt at the University of Virginia (Syka et al. 2004). Unlike ECD, this method, called ETD, uses gas-phase ion/ion chemistry to transfer electrons from radical anions with low electron affinity to multiply protonated peptide or protein molecules as illustrated in relation 1:

$$[M + nH]^{n+} + A^- \rightarrow \left[[M + nH]^{(n-1)+}\right]^{\cdot} + A \rightarrow fragments \quad (1)$$

where M is the analyte (peptide or protein) molecule and A the radical anion.

For anionic peptide/protein species a few years ago, the group of Coon (2005) has developed negative electron-transfer dissociation (NETD) in which an electron from an anionic analyte is transferred to the cationic reagent. Subsequently to the loss of the electron, the anion undergoes internal rearrangement and fragmentation, from which the structural elements of acidic peptides and proteins may be determined (McAlister et al. 2012).

In contrast to CID, previously the only available fragmentation technique on ion traps, however, similar to ECD, ETD induces N–Cα bond cleavages of peptide backbone with the preservation of PTM and generation of ions that are diagnostic for the modification site(s) (Zhurov et al. 2013; Kim and Pandey 2012; Zhou et al. 2011).

Applied together, ETD and CID as well as alternate ETD/CID (Guthals and Bandeira 2012) may significantly increase the sequence coverage and add confidence to peptide/protein identification and characterization of their PTMs even on low-resolution instruments such as the majority of affordable ion traps that are available on the market. As in ETD the ion–ion reaction is highly efficient and fast, taking place in milliseconds, this method can easily be performed with femtomole quantities on a chromatographic time-scale (Halim et al. 2013).

Although ETD was first reported less than a decade ago and became commercially available with ion trap and Orbitrap instruments only in the last few years, so far this method was optimized and successfully applied on a variety of proteomics studies: from post-translationally modified peptides and polypeptides to top-down protein analysis. In this context, the next sections illustrate briefly the current state-of-the-art in ETD MS applicability and highlight significant achievements, which marked the method breakthrough in proteomics.

Determination of PTM by ETD

PTM play important roles in the structure and function of proteins, such as protein folding, protein localization, regulation of protein activity, mediation of protein–protein interaction and also influence the enzyme activity. Until now, more than 200 different types of PTM have been identified; however, only a few of them are reversible and important for the regulation of biological processes. The

most common protein PTM include phosphorylation and glycosylation.

Alteration of PTM patterns such as *N*-glycosylation and *O*-glycosylation or phosphorylation may have tremendous pathological effects such as cancer, cardiovascular and neurodegenerative diseases. Since PTMs play such an important role in the regulation of cellular environment, there is a constant preoccupation for the development of novel, highly sensitive, and sophisticated PTM identification techniques. Among all the later developments, MS with ETD emerged recently as one of the most powerful.

Phosphorylation

Protein phosphorylation is a reversible post-translational modification that regulates a broad range of cellular activities including signal transduction, cell differentiation and development, cellular metabolism, protein–protein interaction, enzyme reactions, and protein degradation, which results in intracellular signaling cascades (Ghosh and Adams 2011). A primary role of phosphorylation is to act as a switch to turn "on" or "off" a protein activity or a cellular pathway in an acute and reversible manner. Today, it is well known that almost all processes regulated by protein phosphorylation are reversible and controlled by the combined action of protein kinases and phosphatases. In addition, abnormal phosphorylation events are implicated in many pathologies. In the last years, a special attention was paid to the role of protein phosphorylation/dephosphorylation in cancers and their huge impact in disease pathophysiology (Hernandez-Aya and Gonzalez-Angulo 2011; MacLaine and Hupp 2011; Julien et al. 2011). For all these reasons, nowadays major efforts and resources are invested for phosphosite identification and localization, in studies on temporal dynamics, kinetics and stoichiometry.

Currently, the number of analytical methods, of which some well established for the analysis of proteins, are applicable also to the phosphorylated species. Edman degradation, the techniques based on phospho-specific antibodies, phosphoprotein staining, enzyme-linked immunosorbent assay (ELISA), radioactive labeling of proteins with ^{32}P isotope and MS (Delom and Chevet 2006) are nowadays intensively used for the characterization of phosphorylated proteins.

Phosphoprotein analysis using one of the first five methods is rather challenging because of the following drawbacks: (a) these analytical techniques present a limited dynamic range, which means that although major phosphorylation sites might be located, no straightforward data on the minor sites can be acquired; (b) as biochemical tools, most of these methods may lead to dephosphorylation during sample preparation; (c) the sensitivity, reproducibility and data accuracy provided by these classical methods are below the limit required for trace level analysis of complex samples originating from biological matrices.

With the development and introduction of new analytical systems in proteomics, the assessment of protein phosphorylation and phosphorylation site discovery has greatly evolved. A particular breakthrough in the field was achieved by the implementation of MS-based assays (Mijakovic and Macek 2012; Eyrich et al. 2011). Besides the accuracy of the data generated by exact molecular weight determination in high-resolution mode and the sensitivity in the atto-to-femtomole range, modern MS methods use combined dissociation techniques able to determine the peptide sequence and the phosphorylation site(s).

Unlike general proteomics, where protein identification by MS combined with bioinformatics is used routinely, phosphoproteomics requires other specific approaches for the identification of phosphorylation sites.

Phosphorylation analysis by MS is generally accomplished by a two-step bottom up approach: (a) digestion of the target protein with enzymes, such as trypsin, which cleaves C-terminal of the basic amino acid arginine and lysine to obtain oligopeptides; (b) LC–MS and CID MS/MS analysis of oligopeptide mixture for the determination of the sites and types of modification and, at the same time, identification of the protein.

Although this popular method is relatively straightforward, not all phosphopeptides can be identified because: (a) even for non-phosphorylated proteins, the peptide maps are frequently incomplete; (b) under all CID conditions, the labile phosphate moiety competes with the peptide backbone as the preferred cleavage site; (c) phosphoric acid is cleaved off, which prevents further fragmentation of the peptide backbone; (d) as the phosphate group is lost, the exact location of this peptide modification cannot be determined; (e) it was observed that besides the potential for phosphate group losses, phosphopeptide ions subjected to CID exhibit phosphate group scrambling before dissociation, leading to erroneous assignment of the site of phosphorylation within the peptide (Palumbo and Reid 2008).

These particular fragmentation features of CID hinder the protein identification because of the reduced set of ions corresponding to peptide fragment series and the absence of the phosphorylated peptide ions. For these reasons, most of the CID tandem mass spectra of phosphorylated peptides provide insufficient information for confident sequence assignment (Swaney et al. 2009).

In contrast to CID, ETD preserves the localization of the labile phosphate group (Han et al. 2008; Hansen et al.

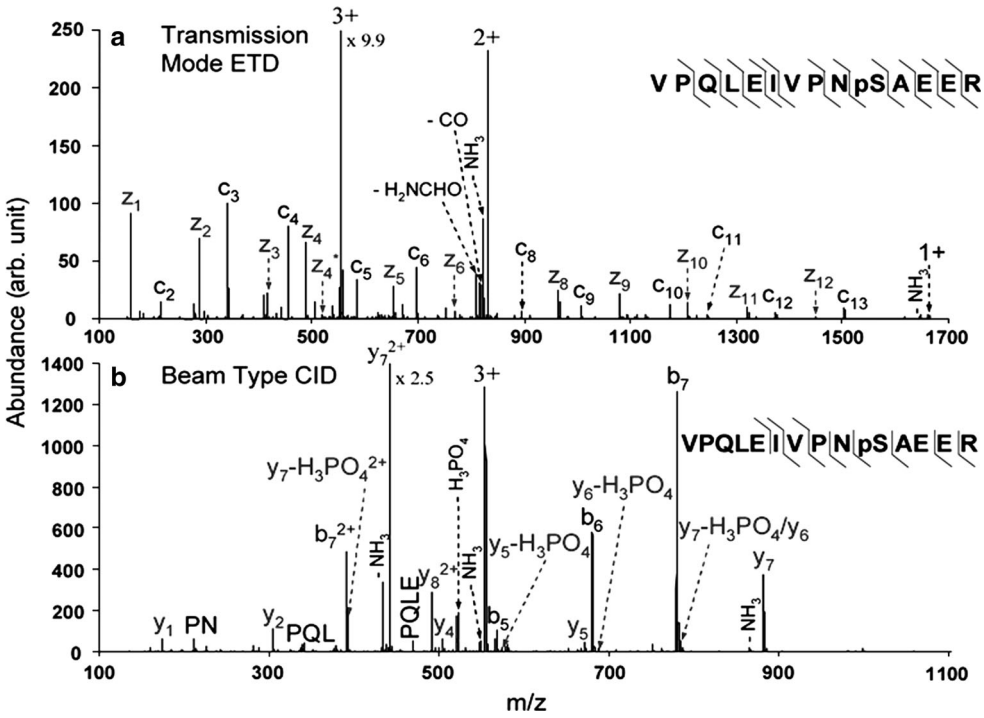

Fig. 1 MS/MS of the tryptic phosphopeptide VPQLEIVPNpSAEER from alpha-casein: **a** transmission mode ETD of the triply charged peptide with azobenzene radical anions, **b** beam type CID of the triply charged peptide (CE = 54 eV). z^* denotes the O_2 adduct of z ions. Reprinted with permission from (Han et al. 2008)

2012) and also provides peptide-sequence information (Fig. 1a, b). However, one downside of ETD is that it fails to fragment peptide bonds adjacent to proline, which are readily cleaved by CID. Therefore, ideally, in the case of phosphorylated peptides, CID and ETD are to be used complementarily. For instance, in a study by the group of McLuckey (2008) on a triply charged tryptic phosphopeptide from α-casein, ETD and CID spectra were acquired in a single rapidly alternating scan function. As shown in Fig. 1b, the loss of H_3PO_4 from the peptide precursor ion and y-type fragment ions indicates that the peptide is phosphorylated. However, more information was needed in order to characterize the sequence of the phosphopeptide and the phosphorylation sites. Employment of ETD (Fig. 1a) for sequence analysis of the triply charged phosphopeptide VPQLEIVPNSAEER clearly disclosed the phosphor location at serine. Besides the identification of the phosphorylation site, 22 out of 26 possible c-type and z-type fragment ions are observed in the ETD spectrum (85 % sequence coverage), compared to only 10 out of 26 possible b-type and y-type fragment ions observed in the CID spectrum (62 % sequence coverage). These results show that ETD alone could localize the phosphorylation site. ETD spectrum of this triply charged phosphopeptide generated more sequence information than the corresponding CID spectrum. However, by combining the information derived from both ETD and CID data acquired in a single scan function, superior sequence coverage (92 %) was gained (Han et al. 2008).

Glycosylation

Glycosylation is one of the most common posttranslational modifications of proteins which play an important role in cell–cell interactions (Fan et al. 2012), white blood cell recognition (Andresen et al. 2012), and protein folding (Mendoza et al. 2012). *N*-glycosylated and *O*-glycosylated proteins have an aberrant expression in malignancies, such as stomach carcinoma (Chirwa et al. 2012), lung cancer (Zeng et al. 2010), ovarian cancer (Alley et al. 2012) and pancreatic cancer (Remmers et al. 2013). Glycosylation may also be involved in cell adhesion, embryonic development, immune function, and cell division. Vinaik et al. (2013) have demonstrated that the complex functions of glycoproteins are strongly correlated to their structure.

Combining ETD and CID is particularly useful for glycopeptide/glycoprotein structural analysis since ETD and CID provide complementary information: ETD generates data related to the peptide sequence and glycosylation sites, while CID provides information on the glycan structure.

O-glycopeptides

O-linked glycans are attached to the hydroxy oxygen of serine, threonine, tyrosine, hydroxylysine, or hydroxyproline side-chains. Analysis of *O*-glycosylated peptides/proteins by MS is a complex task because of the high diversity of the protein sequence and glycan composition and branching. Fragmentation of *O*-glycopeptides using CID

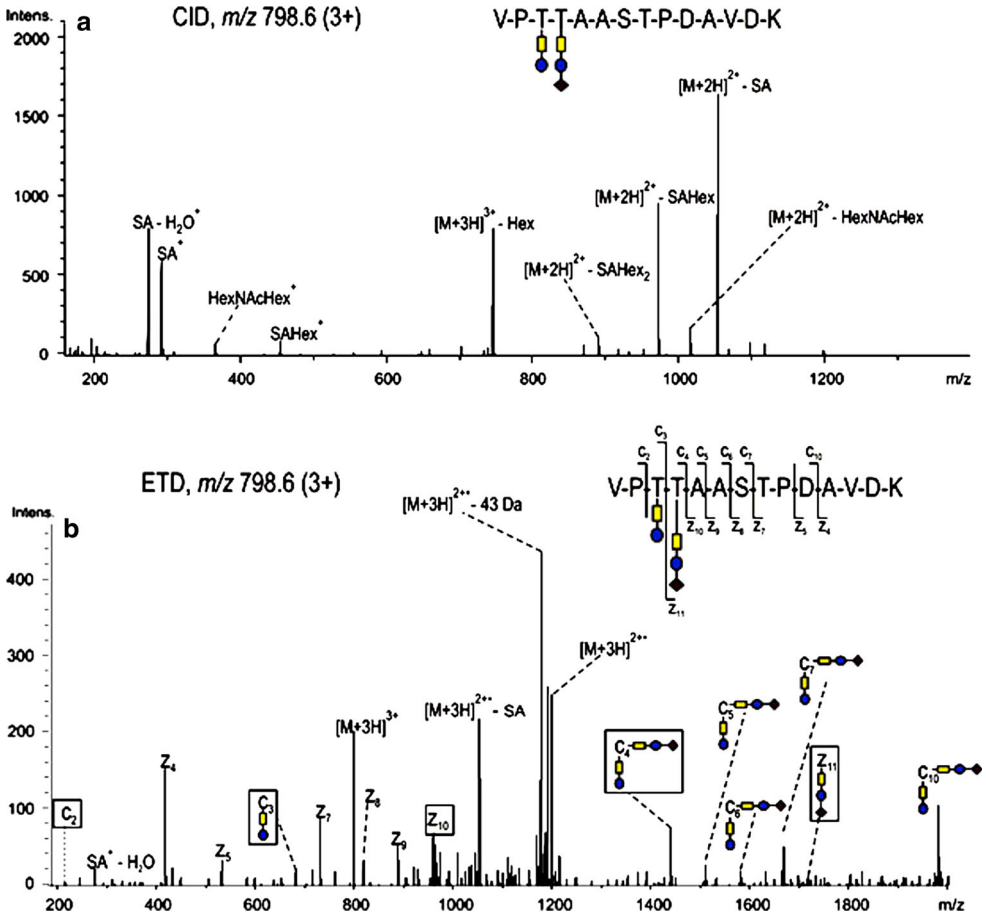

Fig. 2 **a** CID and **b** ETD spectra of the precursor ion *m/z* 798.6 (3+) corresponding to glycopeptides 289–302 of the full length APP695, showing the amino acids Thr 291 and Thr 292 occupied with two distinct Core 1 type glycans. Data dependent acquisition; activation energy 0.07 V; The fragment ions relevant for determination of the glycosylation site(s) are indicated with *black boxes*. Capillary voltage 2,000 V, capillary exit 180 V, anionic reagent: fluoranthene. Reprinted with permission from (Perdivara et al. 2009)

MS^n is characterized by an extensive cleavage of glycosidic bonds. Hence, CID provides abundant information on the attached glycan structure, however, less data regarding the glycosylation site(s) and peptide sequence. ETD technique, inducing fragmentation along the protein core with the preservation of the glycan modification, was introduced in glycoproteomics and extensively used for unambiguous identification of glycosylation sites and complete structural characterization of the peptide backbone (Alley et al. 2009; Hanisch 2012; Singh et al. 2012). However, the most efficient procedure for structural elucidation of complex *O*-glycosylated proteins is the one based on alternate ETD and CID MS^n (Hanisch 2012; Perdivara et al. 2009). In this protocol, glycosylated peptides are subjected to ETD MS/MS. After ETD sequencing, the peptide fragment ions bearing the glycan can be isolated and submitted to an additional fragmentation step by CID (Alley et al. 2009) or high-energy collision-dissociation (HCD) (Darula and Medzihradszky 2009; Darula et al. 2011). ETD product ion spectrum provides the information on the peptide backbone and glycosylation sites while CID on the structure of the *O*-linked carbohydrates (Hanisch 2012). In a variant of the alternate ETD/CID, after ETD activation, the unfragmented precursor ions exhibiting reduction in their charge, as a consequence of electron transfer from ETD reagent, are isolated and subjected to CID. This dissociation by collisions, applied to the charge-reduced species, is called CRCID. The advantage of CRCID is the great increase of c-ion and z-ion series and a better sequence coverage (Wu et al. 2007).

Due to the high speed in data-dependent analysis exhibited by commercial ion trap/Orbitrap instruments equipped with CID and ETD kits, complex *O*-glycopeptide mixtures showing small structural differences may be successfully separated and investigated using liquid chromatography (LC) online coupled to ETD and ETD/CID MS^n (Perdivara et al. 2009; Darula et al. 2011). For instance, *O*-glycosylated structures of β-amyloid precursor protein were elucidated by LC-CID/ETD MS/MS (Perdivara et al. 2009). Such a combination of LC ETD and CID allowed for the identification of three glycosylated sites: Thr291, Thr292 and Thr576 with heterogeneity of glycosylation (Fig. 2).

One of the ETD disadvantages is that the effective fragmentation relies on a high-analyte charge state as well as on the quality and stability of the electrospray signal. Therefore, extension of ETD applicability entailed its combination with efficient systems for ESI such as

microfluidics, which proved superior performance in terms of ionization efficiency, spray steadiness, analysis reproducibility, sensitivity and pace. In this context, the group of Yoshimura (2012) coupled the TriVersa NanoMate robot based on chip-nanoESI with a LTQ Orbitrap and investigated by ETD MS/MS the mucin-type *O*-glycosylation. The facile generation by the chip-nanoESI of multiply protonated molecules enhanced ETD analysis with the identification of the sequence and GalNAc attachments.

Although the use of ETD for determination of the heterogeneous *O*-glycosylation at specific sites in regions of multiple occupancy is still at the beginning, many studies (Thaysen-Andersen et al. 2011; Darula et al. 2011) demonstrated the capabilities of ETD MS/MS to characterize glycosylated peptides with high abundance of *O*-linked glycan structures, showing a high peptide sequence coverage and confident determination of the glycosylation site(s).

N-glycopeptides

N-linked glycans are attached to the protein backbone through the nitrogen from the asparagine structure being present in a tripeptide sequence Asn-X-Ser or Asn-X-Thr where X could be any amino acid except proline. Such a consensus sequence facilitates ETD analysis of *N*-glycosylated peptides/proteins and site-specific glycosylation profiling. Usually, a universal core glycan structure, $GlcNAc_2Man_3$ is conserved across species (Darula and Medzihradszky 2009; Yu et al. 2011). This modification is typically found in membrane and secreted proteins and can be required for proper protein folding.

ETD was for the first time introduced for structural analysis of *N*-glycopeptides in 2005 using a 3D-quadrupole ion trap (Hogan et al. 2005). Elucidation of glycan structure and peptide sequence of *N*-glycosylated peptide employed sulfur dioxide and nitrobenzene anions for electron transfer reactions. It was shown that by ETD, almost every amino acid residue may be cleaved, with the preservation of the glycosidic linkages. When sulfur dioxide anions are used for electron transfer reaction, the spectrum is dominated by z-type ions, while c-type ions are absent. In the case of nitrobenzene anions, a few c-type ions are visible together with almost all z-type ions.

Similar to *O*-glycosylation, the combination of ETD and CID, for *N*-glycopeptides, provides complete information about the amino acid sequence and glycan structure (Han et al. 2008; Catalina et al. 2007). Moreover, in many instances the sites of glycosylation and the *N*-glycosylation microheterogeneity can be assessed as well (Alley et al. 2009).

Combination of ETD with CRCID based on the enhanced fragmentation of molecules with ETD-reduced charging is highly efficient also for *N*-glycopeptides (Wu et al. 2007). Hence, in ETD and CRCID steps, a large number of peptide bond cleavages with a continuous ion series at the *N*-terminal segment of the peptide are generated. CRCID was shown to provide additional product c-ion and z-ion series to augment the information from ETD fragmentation process. Furthermore, combination of ETD with CRCID followed by regular CID of the peptide fragment ions linked to the glycan chain, generates complementary information necessary for *N*-glycosylation site and structure identification, as well as peptide sequence determination (Wu et al. 2007).

For *N*-glycopeptide analysis, ETD was effectively applied also in conjunction with liquid phase separation methods such as LC and nanoLC (Alley et al. 2009; Catalina et al. 2007) (Fig. 3), chip-LC (Alley et al. 2009), enrichment (Snovida et al. 2010; Scott et al. 2011) and affinity chromatography techniques (Zhang et al. 2007).

However, probably the most attractive applications of ETD in *N*-glycosylation analysis are those related to biomedical and clinical research. Of particular importance is the determination of aberrant *N*-glycosylation, found in association with oncogenesis, which represents a challenge for all MS techniques. In this regard, a systematic approach for MS analysis of aberrant *N*-glycosylation in serum of human hepatocellular carcinoma patients was conducted using CID and ETD fragmentation (Chen et al. 2012). The outcome of this work was the discovery and characterization of 69 aberrant sites in the serum of the patients. ETD and alternate ETD/CID techniques in combination with LC were successfully employed in another clinical case: the non-small cell lung malignancy. Here, the information derived from ETD mass spectra allowed an ultrasensitive characterization of site-specific *N*-glycosylation of haptoglobin from plasma of the lung cancer patients included in the testing contingent (Wang et al. 2011).

Top-down proteomics

Presently, protein identification by MS/MS is carried out by two basic strategies: the "classical" bottom-up and the newly introduced for middle- and top-down methods. In bottom-up approach, proteins undergo proteolytic cleavage and the peptide products are analyzed by LC MS/MS with either CID, ECD or ETD. In top-down method, intact protein ions, typically formed by ESI, are trapped in either a Fourier transform ion cyclotron resonance (Penning trap) or quadrupole ion trap (Paul trap) mass spectrometer and fragmented directly in tandem MS. For many years, ECD (Zubarev 2004) conceived for FTICR MS, was the only available fragmentation tool for top-down proteomics. Later, it was demonstrated that under carefully optimized

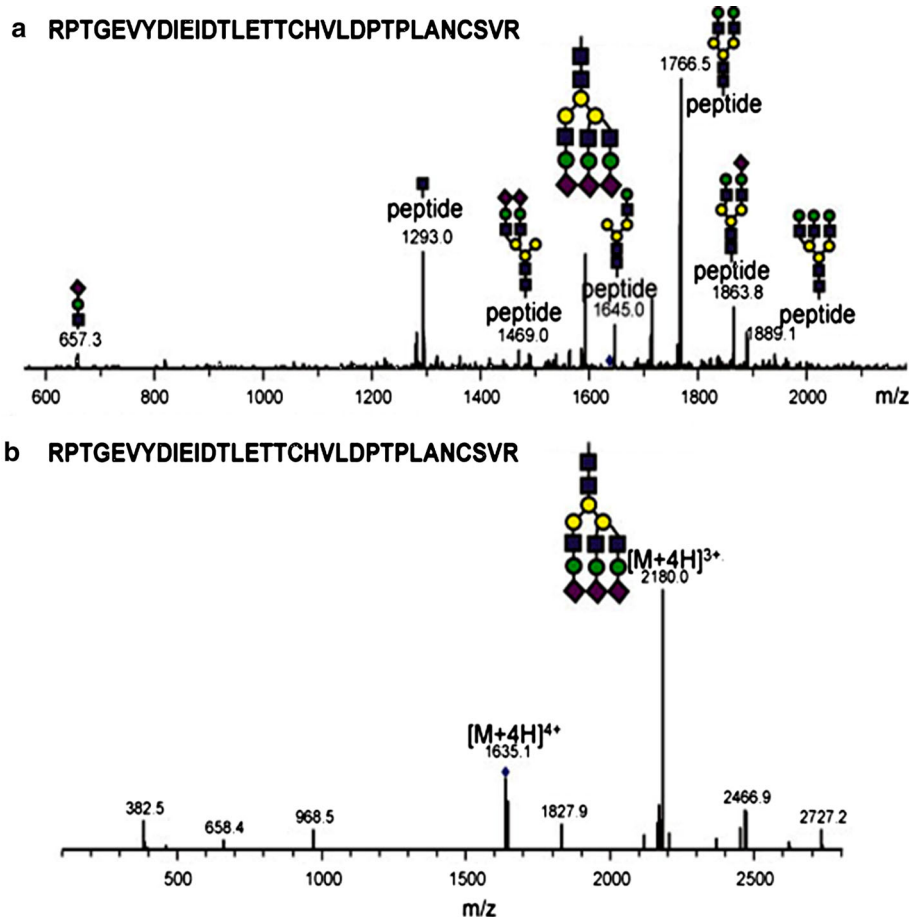

Fig. 3 LC CID (**a**) and ETD (**b**) MS/MS of the tryptic glycopeptide RPTGEVYDIEIDTLETTCHVLDPTPLANCSVR derived from bovine fetuin with a triantennary trisialyated complex glycan. ESI voltage: 1,825 V (activated graphitized carbon chip) and 1,975 V (C18 chip), ionization energy: 65 eV, emission current: 5.0 μA, anionic reagent: fluoranthene. Reprinted with permission from (Catalina et al. 2007)

conditions, a successful top-down analysis of intact proteins may be achieved also using CID and high-energy collisional dissociation (HCD) (Catherman et al. 2013). In the last couple of years, ETD on a high-capacity ion trap (HCT) and Orbitrap instruments (Fornelli et al. 2012; Drabik et al. 2012; Ahlf et al. 2012) emerged as a viable method in top-down proteomics. The speed and versatility, as well as the high confidence in protein identification, allowed the advancement of ETD-based top-down platform towards combination with ion-pair reversed-phase LC followed by protein quantification (Hung and Tholey 2012) and with chip-based nanoESI performed on a NanoMate robot (Flangea et al. 2013) in-laboratory coupled to a HCT instrument. In this study, only 5 μL protein solution in H_2O/HCOOH (1:1 v:v) at 0.5 pmol μL^{-1} concentration was loaded in the 96-well plate of the NanoMate robot and infused into HCT MS by applying 1.5 kV on the pipette tip. The spray was enhanced by the application of 0.3–0.40 psi nitrogen back pressure, and 50 psi pressure of nitrogen nebulizer on MS. The *in-source* fragmentation was prevented by keeping a potential difference between the end of the transfer capillary and the skimmer (capillary exit) at 50 V.

For top-down by ETD experiment, the protein precursor ion was isolated within a 2u window. ETD was carried out in manual fragmentation mode using fluoranthene as the reactant agent under 60 °C temperature, 20 ms accumulation time for top-down experiment on apomyoglobin and 75 eV ionization energy yielded a high coverage of sequence ions.

The top-down fragmentation (Fig. 4) was successfully accomplished within only 30 s and with a sample consumption of approximately 12 fmols.

Hence, for protein identification in top-down analysis via ETD fragmentation, this system exhibited a number of advantages among which: (a) higher ionization efficiency as compared to classical ESI, with beneficial consequences upon the formation of multiply charged cations required by ETD; (b) reduction of the experiment time (down to 30 s) and of the sample consumption (down to 0.02 pmols); (c) high reproducibility, signal-to-noise ratio and quality of

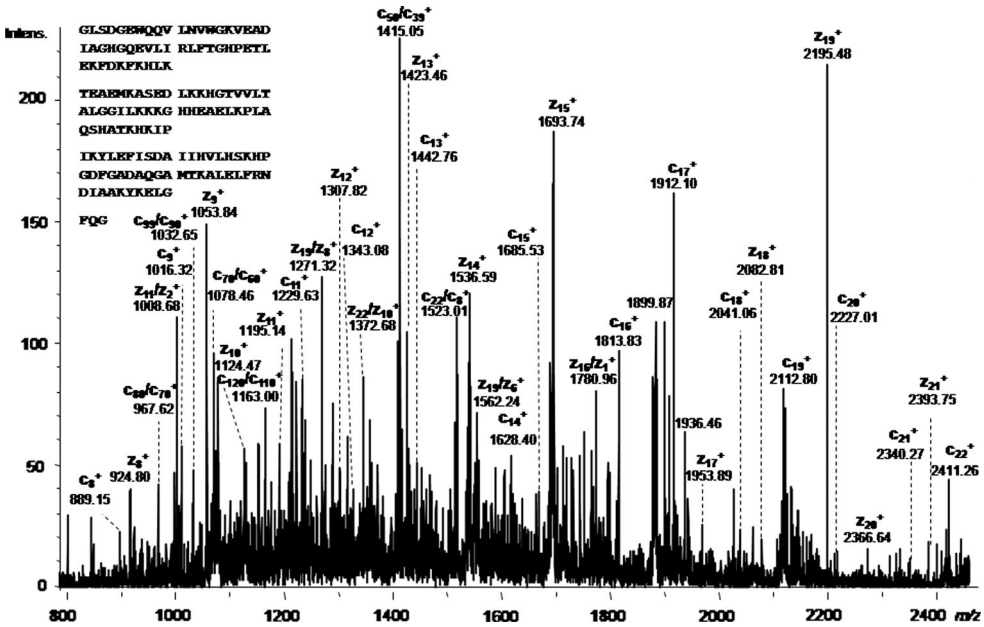

Fig. 4 Top-down by NanoMate-ETD MS^2 of the $[M + 16H^+]^{16+}$ ion at *m/z* 1060.66 corresponding to apomyoglobin. Inset: amino acid sequence of horse apomyoglobin. Acquisition time 30 s. Sequence coverage: 80 %. Reprinted with permission from (Flangea et al. 2013)

mass spectra; (d) high-throughput and potential for complete automation, from infusion to fragmentation and assignment of top-down protein mass spectra.

In CID and HCD on either ion trap, quadrupole time-of-flight mass spectrometer or FTICR MS via sustained off resonance (SORI) (Wells and McLuckey 2005) the apomyoglobin also undergoes relevant top-down fragmentation, with preponderant formation of y- and b-ions that characterize also the loose end of the protein (Wells and McLuckey 2005). In the case of the modern Orbitrap MS platforms, a high throughput top-down protein analysis is feasible with either ETD or HCD as recently reported by Ahlf et al. (2012).

Applied for top down, CID, HCD and ETD are able to offer complementary information in structural biology-based experiments. Conjugated ETD/CID in multistage fragmentation analysis emerged recently as a reliable tool for protein/peptide sequencing. For example, CID of the non-dissociative electron transfer products (ETnoD) was shown to increase significantly the yield of c-type and z-type of product ions (McAlister and Coon 2010).

As compared to top-down by CID and HCD, ETD may in particular address the structural issues of larger proteins. On the other hand, ETD exhibits a number of disadvantages, such as the requirement related to the minimum number of charges ($z > 3$) for efficient fragmentation and the complexity of the top-down spectra, occasionally difficult to interpret. Moreover, water, ammonium and carbon monoxide losses, which are sometimes important for *de novo* identification, are not visible in ETD mass spectra. Nevertheless, ETD remains a fragmentation technique that complements CID and provides a set of structural information inaccessible by exclusive use of CID.

Conclusions and perspectives

As an ion/ion analog of ECD, ETD exhibits over ECD the key advantage of the compatibility with the affordable, user-friendly and, consequently, very popular RF ion traps. Furthermore, ETD is well suited for the new generation of mass spectrometers based on Orbitrap analyzers, which merge the speed and versatility of ion traps with the high resolution and mass accuracy.

The applications emphasized in this mini-review demonstrate that presently many laboratories give full consideration to ETD for studies of high complexity, such as determination of phosphorylation and glycosylation, assessment of glycosylation microheterogeneity, identification of cancer biomarkers, fragmentation analysis of monoclonal antibodies and protein quantification. Moreover, several research groups initiated the combination of ETD with separation and enrichment techniques as well as with microfluidics systems in attempts to replace partially or totally the laborious bottom-up approaches by fast top-down analyses using ETD and ETD alternating with CID/CRCID.

In this context, although currently at the beginning, ETD appears to have a tremendous potential for further development and applications. It is even expected ETD to turn into a routine method for protein identification in the near future.

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