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Review

Glycoproteomics based on tandem mass spectrometry of glycopeptides☆

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Abstract

Next to the identification of proteins and the determination of their expression levels, the analysis of post-translational modifications (PTM) is becoming an increasingly important aspect in proteomics. Here, we review mass spectrometric (MS) techniques for the study of protein glycosylation at the glycopeptide level. Enrichment and separation techniques for glycoproteins and glycopeptides from complex (glyco-)protein mixtures and digests are summarized. Various tandem MS (MS/MS) techniques for the analysis of glycopeptides are described and compared with respect to the information they provide on peptide sequence, glycan attachment site and glycan structure. Approaches using electrospray ionization and matrix-assisted laser desorption/ionization (MALDI) of glycopeptides are presented and the following fragmentation techniques in glycopeptide analysis are compared: collision-induced fragmentation on different types of instruments, metastable fragmentation after MALDI ionization, infrared multi-photon dissociation, electron-capture dissociation and electron-transfer dissociation. This review discusses the potential and limitations of tandem mass spectrometry of glycopeptides as a tool in structural glycoproteomics.

Keywords: Collision-induced dissociation; Electron-capture dissociation; Electron-transfer-dissociation; Glycopeptide; Glycosylation

Contents

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1. Introduction

In proteomics, the incorporation of methods to target and analyze protein glycosylation has initially been a largely neglected area. This has radically changed in the last few years, with the development of more sophisticated MS techniques, as well as through the realization that many research questions addressed by proteomics approaches cannot be answered without taking protein glycosylation into account.

A number of reviews have been published in the last few years on analytical methods including chromatography, electrophoresis and MS for the characterization of glycans and glycoproteins [1–4] and on general MS-based proteome and peptide analysis methods [5–7]. The current review specifically summarizes recent developments and insights into the direct targeting of protein glycosylation at the level of glycopeptides, focusing on methods for sample preparation of glycopeptides and their analysis with different MS ionization and fragmentation techniques.

2. Sample preparation and chromatography

2.1. Glycopeptide enrichment

Conventional MS-based proteomics strategies often include the analysis of tryptic peptides and glycopeptides created from a diversity of samples ranging from individual purified (glyco)proteins or sets of co-migrating proteins in an SDS-PAGE gel, to the complete proteome of an organism. In these mixtures, glycopeptides are usually hard to analyze since most glycosylation sites carry a multitude of glycans (microheterogeneity) giving rise to different glycoforms of glycosylated peptides, each present at a relatively low concentration in the total peptide pool. Therefore, several approaches have been developed to target general or specific glycoproteomes and to enrich glycoproteins and glycopeptides from complex samples such as plasma, urine or cells.

The methods that may be chosen to study glycoproteins or glycopeptides in MS-based analyzes vary according to the specific research question. In many cases, these methods have been developed with the aim of selectively obtaining the *N*-glycoproteome of a particular sample by MS analysis of the corresponding (deglycosylated) tryptic peptides to identify the underlying proteins [8–24]. In these types of studies, *N*-glycosylation sites in (enriched) tryptic glycopeptides usually are identified by conventional LC–MS/MS or matrix-assisted laser desorption/ionization (MALDI)–MS/MS analysis based on the conversion of Asn to Asp upon treatment with *N*-glycanase (PNGase) [9,10,15,20,21,23–25], or on the localisation of the remaining GlcNAc-Asn tag upon treatment with endo-*N*-acetylglucosaminidases [19]. Large or medium scale enrichment techniques applied in combination with advanced MS/MS methods for the direct analysis of intact glycopeptides to obtain sequence information of both the glycan and the peptide moiety have so far been less commonly used [26–31]. Detailed knowledge of protein glycosylation at the proteome-scale is, however, becoming an important aspect of post-genomic research. The development and application of intact glycopeptide analysis techniques to increase this knowledge will strongly benefit from advanced MS/MS technologies, which are summarized and discussed in this review (see Sections 3 and 4).

For the isolation of glycoproteins or glycopeptides by affinity chromatography various lectins can be used [8,32]. Lectin chromatography using concanavalin A (Con A) has been reported for the enrichment of *N*-glycoproteins from diverse sources such as human urine or serum, *Caenorhabditis elegans* plasma membrane proteins and glycoproteins in the murine dermis [9–11,26]. Con A binds with preference to oligomannosidic, hybrid and di-antennary *N*-glycans, either unconjugated or attached to proteins or peptides. Con A has a relatively broad specificity, but triantennary and tetraantennary complex-type glycans are not retained on Con A, and binding is sometimes also hindered by fucosylation of GlcNAc in the antennae [32]. Moreover, *O*-glycopeptides, or glycoproteins that contain exclusively *O*-glycosylation sites will not be bound at all by this lectin. To afford a more complete coverage of the glycoproteome of human plasma or serum, lectin columns that combine Con A with wheat germ agglutinin (WGA), which binds to many complex-type sialylated and non-sialylated glycans, have been used [10,13,27]. In the study of Yang and Hancock [12], jacalin was used in combination with Con A and WGA in a multi-lectin column in an attempt to include *O*-glycoproteins in the enriched fractions. It is not clear from this study [12] whether jacalin, which binds to mucin-type *O*-glycopeptides that contain GalNAc-Ser/Thr (not substituted at C6 [33]), contributes significantly to the enrichment of plasma or serum glycoproteins.

Multi-dimensional lectin chromatography is an effective tool for comparative analysis of sub-glycoproteomes [14]. Sialylation and branching of tryptic *N*-glycopeptides from human serum has recently been reported in an ICAT-type study using serial lectin affinity chromatography with *Sambucus nigra* agglutinin (SNA) which binds to α2-6-sialylated glycopeptides, and Con A [15].

Regarding a more focused affinity approach, elegant studies specifically targeting the lysosomal glycoproteome were recently published [17,34]. Using immobilised mannose-6-phosphate (Man6-P) receptor, Man6-P containing glycoproteins, normally targeted to the lysosome based on this modification, were purified from human brain [19] and plasma [17]. In a similarly focused glycoproteomic study, mouse liver glycoproteins that bind to galectin-1 have been identified [35]. In this case, a conventional peptide sequencer instead of MS was used to identify the captured glycopeptides.

In addition to the specific lectin-affinity glycopeptide enrichment methods, approaches based on general physical and chemical properties of glycopeptides and glycoproteins are most valuable. Among them, the simple fact that most tryptic glycopeptides in a complex peptide/glycopeptide mixture have a relatively high mass is very useful. Glycopeptides can be significantly enriched by size exclusion chromatography [18]. Using the hydrophilicity of the glycan moiety, another enrichment method is based on hydrophilic interaction with carbohydrate gel matrices such as cellulose or Sepharose [29]. Extraction by hydrophilic interaction has been performed on glycopeptides from individual glycoproteins of human serum or plasma

[28,30] and to total serum glycoproteins [29]. Alternatively, hydrophilic interaction-liquid chromatography (HILIC; or normal phase-chromatography) of (glyco-)peptides may be performed. As glycopeptide retention is mainly determined by the hydrophilic character and size of the glycan moiety, glycopeptides are singled out in the late-eluting fractions. HILIC can be on-line coupled to nano-electrospray (ESI-)MS, allowing direct analysis of the enriched glycopeptides [19,28,36,37]. Recently, beads functionalized with di-boronic acid have been introduced for the enrichment of glycoproteins and glycopeptides from serum and other body fluids [38]. Since boronic acid can form diesters with all glycans and glycoconjugates that contain *cis*-diol groups (as in Man, Glc, Gal) [39], this matrix may be particularly useful for the unbiased enrichment of both *N*- and *O*-glycopeptides. A similarly generally applicable matrix for the enrichment of glycopeptides which is largely independent of the structure of the glycan moiety is graphitized carbon [40]. Graphite microcolumns have successfully been applied to selectively purify glycopeptides from gel-separated glycoproteins [31]. Finally, a good matrix for trapping glycopeptides and glycoproteins by covalent bonding after oxidation with periodate appears to be hydrazide functionalized beads [20]. Peptide moieties of the covalently captured glycopeptides are released by treatment with PNGase F to allow peptide and glycosylation site identification. Information on the glycan part can, however, not be obtained. The method has been applied to analyze the glycoproteome of plasma [21] and platelets [22].

Non-biased matrices that do not rely on the presence or absence of particular structural elements in glycoproteins or glycopeptides, such as HILIC material, Sepharose, carbon or boronic acid are the matrices of choice for the purpose of overall enrichment. On the other hand, the use of lectin-chromatography methods may be advantageous for targeting particular sub-glycoproteomes. The use of specific carbohydrate binding molecules such as the mannose-6-phosphate receptor, galectins, anti-carbohydate monoclonal antibodies and other carbohydrate binding proteins will help to identify sub-glycoproteomes based on particular glycosylation-defined properties.

2.2. Glycopeptide separation

All glycopeptide enrichment techniques described in Section 2.1 are compatible with MS in an off-line mode, for example after clean-up using C_{18} tips or cartridges. For on-line MS applications, some techniques may not be suitable because of the use of MS-incompatible elution media such as concentrated saccharide solutions in the case of lectin-chromatography. Liquid chromatography systems compatible with on-line MS of glycopeptides have recently been reviewed [36].

In addition, capillary electrophoresis with on-line MS has become an established separation and detection technique for the analysis of peptides and other biomolecules (reviewed in [41]) and some reports on the use of capillary electrophoresis (CE)–ESI–MS/MS for the analysis of glycopeptides from a complex matrix such as urine [42,43] or antithrombin as a simple test substance [44] have appeared. The separation power of CE suggests that this technique will be very useful for the analysis of glycopeptides in complex mixtures.

2.3. Diagnostic fragment ions of glycopeptides

MS analysis of glycopeptides may be performed after extensive purification (see also above). In practice, however, glycopeptides are often analyzed from complex peptide/glycopeptide mixtures. When such samples are subjected to LC–ESI–MS/MS analysis with collision-induced fragmentation (see Section 3.1), data evaluation methods which highlight the relevant glycopeptide MS data within the complex overall data set are required. Strategies applied to achieve this comprise the generation of diagnostic fragment ions in the MS-mode (without precursor selection) and/or MS/MS-mode (with precursor selection). Glycopeptide-marker ions in collision-induced dissociation are normally low-molecular-weight oxonium ions (B-type fragmentation according to Domon and Costello [45]) of *m/z* 204 (*N*-acetylhexosamine; abbreviated as HexNAc), *m/z* 366 ($hexose_1HexNAc_1$), *m/z* 292 (*N*-acetylneuraminic acid; abbreviated as NANA), and *m/z* 657 ($hexose_1HexNAc_1NANA_1$), among others. In particular, the marker ion at *m/z* 204 [46–49] as well as ions at *m/z* 186 and *m/z* 168 arising from the elimination of 1 or 2 water molecules from the HexNAc oxonium ion [50] have been shown to be indicative for both *N*-glycans and *O*-glycans. Scanning for these diagnostic fragment ions have been classically performed on triple-quadrupole mass spectrometers in the precursor-ion scanning mode [46–48]. Glycopeptide identification can also be achieved by selected ion monitoring (SIM) with ion trap (IT–)MS [51,52], by magnetic sector MS instrumentation [49] and quadrupole-TOF mass analyzers [53,54]. In addition, mass spectra datasets can be screened for constant neutral losses of terminal monosaccharides, which likewise highlights glycopeptides [37,55].

3. Electrospray ionization–tandem mass spectrometry of glycopeptides

3.1. Collision-induced dissociation

Early experiments by Carr and co-workers [46,47] with ESI and collision-induced dissociation (CID) on a triple-quadrupole mass spectrometer have already established several of the key features of CID of glycopeptides. Similarly, Burlingame and co-workers [56–59] have shown similarities in fragmentation behaviour and information content with liquid secondary ion MS using high-energy CID. On the basis of this pioneering work, ESI with CID of glycopeptides has become a key tool in glycoproteomics.

3.1.1. CID of N-glycopeptides

The fragmentation of electrospray-ionized *N*-glycopeptides may be performed by CID using a variety of instruments and experimental setups. A common approach is IT–MS/MS, which allows repetitive ion isolation/fragmentation cycles. In most cases, IT–MS/MS of *N*-glycopeptides is dominated by the B-type and Y-type fragmentation of glycosidic linkages,

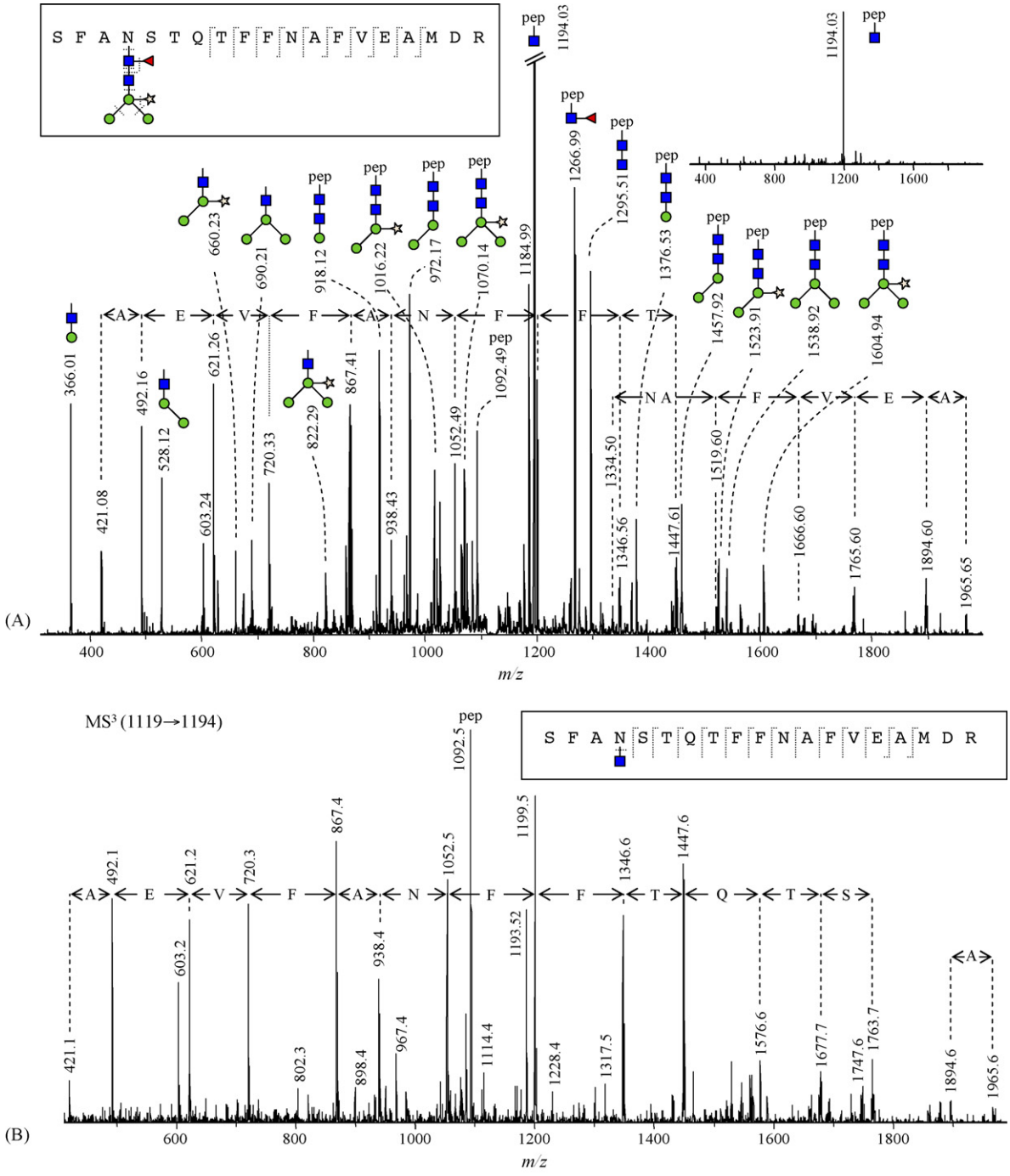

Fig. 1. ESI–IT–MS/MS with CID of the tryptic glycopeptide Ser_{295}-Arg_{313} from HRP. The RP-HPLC-purified tryptic glycopeptide Ser_{295}-Arg_{313} containing a core-fucosylated and core-xylosylated trimannosyl *N*-glycan attached to N_{298} was analyzed by direct infusion with nano-ESI and IT selection of the $[M+3H]^{3+}$ species at *m/z* 1119 and (A) collision-induced dissociation with helium gas using a Bruker high capacity trap (HCT) ultra. (B) The fragment ion at *m/z* 1194 was subjected to a second ion isolation/fragmentation cycle (MS^3 experiment). The b-ions at *m/z* 1894.6 and 1965.6 were both retaining the *N*-linked *N*-acetylglucosamine. Monoisotopic masses are given. Square, *N*-acetylglucosamine; circle, mannose; triangle, fucose; star, xylose; pep, peptide moiety.

thereby revealing predominantly information on the composition and sequence of the glycan moiety, as demonstrated for a tryptic glycopeptide from horseradish peroxidase (Fig. 1A). IT–MS/MS analyzes have been performed on *N*-glycopeptides exhibiting a variety of different glycan structures, including complex-type sialylated structures [44,60–62], asialo complex-type structures from invertebrates exhibiting antenna fucosylation [63,64], paucimannosidic structures [37,52,64,65], oligomannosidic species [66], as well as peptides carrying a single *N*-linked *N*-acetylglucosamine which may carry a fucose [52,67]. As a general feature, B-type and Y-type fragmentations of the glycan moiety at the glycosidic bond of *N*-acetylhexosamine residues tend to dominate the MS/MS spectra. Therefore, in the case of *N*-glycopeptides with short or no antennae, a dominant fragment is the Y-ion arising from chitobiose cleavage with additional loss of an eventually present core-fucose, resulting in a

(often multiply charged) peptide ion retaining a single, *N*-linked *N*-acetylglucosamine (see e.g. inset in Fig. 1A). Furthermore, fucoses (especially in 3-linked form) are easily eliminated as neutral losses.

Besides the cleavage of glycosidic linkages, the fragmentation of peptide backbone bonds may be observed, leading to a series of y-ions and/or b-ions (nomenclature as in [68]), as shown in Fig. 1. In most biological and medical applications, however, the low relative abundance of these peptide fragment ions hinders their use for peptide sequence determination in *N*-glycopeptide analysis by ESI–IT–MS. An alternative approach for a more detailed characterization of *N*-glycopeptides by IT–MS combines MS/MS and MS^3 experiments, as shown in Fig. 1. In this approach, the glycopeptide ion is selected and fragmented resulting in a variety of fragment ions predominantly due to the cleavage of glycosidic linkages (Fig. 1A). The peptide ion carrying a single *N*-acetylglucosamine (*m/z* 1194 in Fig. 1A), which is often the most abundant fragment ion, is subjected to a second ion isolation/fragmentation cycle (often performed in the automatic mode), resulting in fragmentation of the peptide moiety (Fig. 1B). Notably, y-type and b-type fragment ions comprising the *N*-glycosylation site tend to retain at least in part the *N*-acetylglucosamine residue, thereby often allowing the deduction of the glycosylation site [52,60,69].

Quadrupole–TOF MS/MS has been more widely used for the CID fragment-ion analysis of *N*-glycopeptides than IT–MS/MS. For example, it has been applied to the analysis of sialylated complex-type *N*-glycopeptides [70–74], asialo complex-type structures exhibiting antenna fucosylation [70,75,76], glycopeptides with oligomannosidic *N*-glycans [77,78] and paucimannosidic *N*-glycans [79], and glycopeptides obtained after enzymatic truncation of the glycan chain to a single *N*-acetylglucosamine by endoglycosidase treatment [19]. While most of the MS/MS analyzes of *N*-glycopeptides with quadrupole–TOF analyzers almost exclusively exhibited cleavages of glycosidic linkages thus revealing mainly information on the glycan moiety, the analyzes by Harazono et al. [74] and Nemeth et al. [77] also exhibited a significant level of y-type and b-type peptide backbone cleavages, thereby providing peptide sequence information as well as information on the glycan attachment site. These differences in fragmentation characteristics on quadrupole–TOF instruments seem to be largely influenced by the applied collision energy. As an example, the CID mass spectra of the horseradish peroxidase (HRP) model *N*-glycopeptide at low (Fig. 2) and elevated (Fig. 3) collision energies are presented. Low energy CID results predominantly in cleavage of glycosidic bonds, whilst the peptide backbone remains largely intact (Fig. 2). Besides conventional

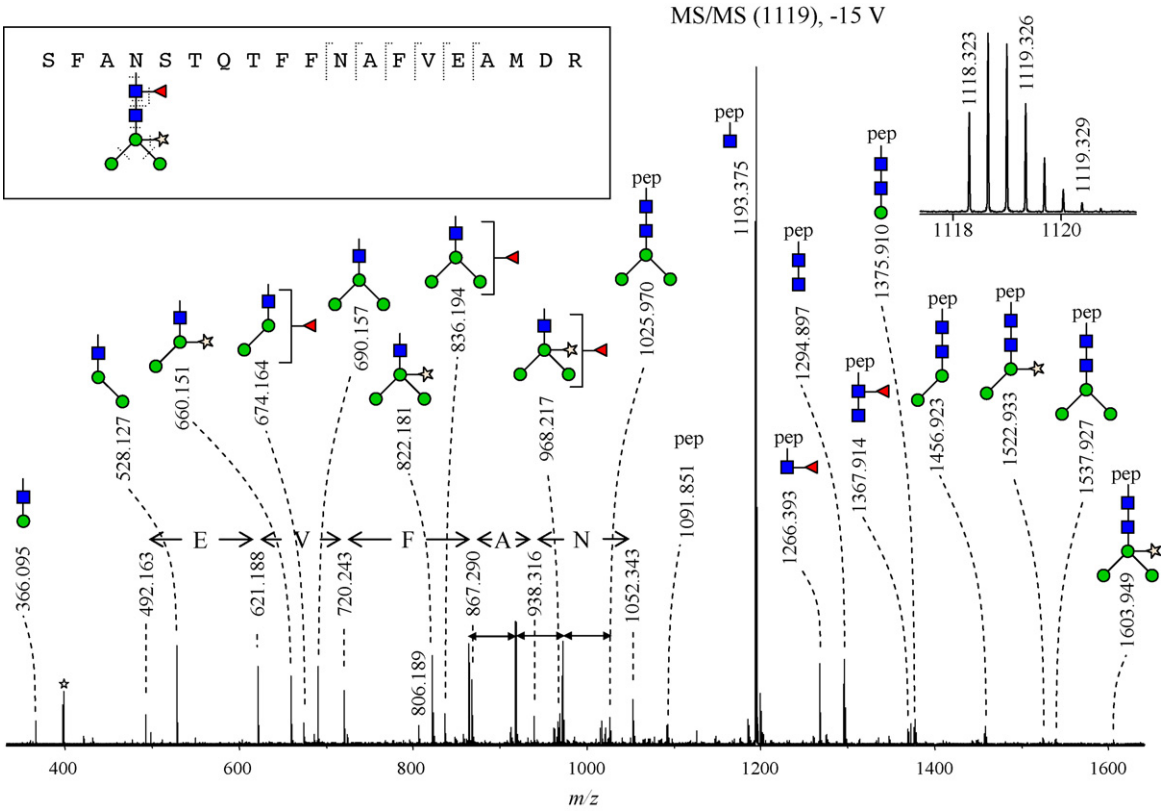

Fig. 2. ESI–Q–FT–ICR–MS/MS with collisional hexapole low-energy CID of the tryptic glycopeptide Ser_{295}-Arg_{313} from HRP. The RP-HPLC-purified tryptic glycopeptide Ser_{295}-Arg_{313} containing a core-fucosylated and core-xylosylated trimannosyl *N*-glycan attached to N_{298} was analyzed by direct-infusion ESI with quadrupole selection of the $[M+3H]^{3+}$ species at *m/z* 1119 and was fragmented by CID with argon gas in the collisional hexapole (15 V offset). Fragments were analyzed by FT–ICR–MS (Bruker Apex-Qe with 9.4 T magnet and Combisource). Monoisotopic masses are given rounded to three digitals. Square, *N*-acetylglucosamine; circle, mannose; triangle, fucose; star, xylose; pep, peptide moiety.

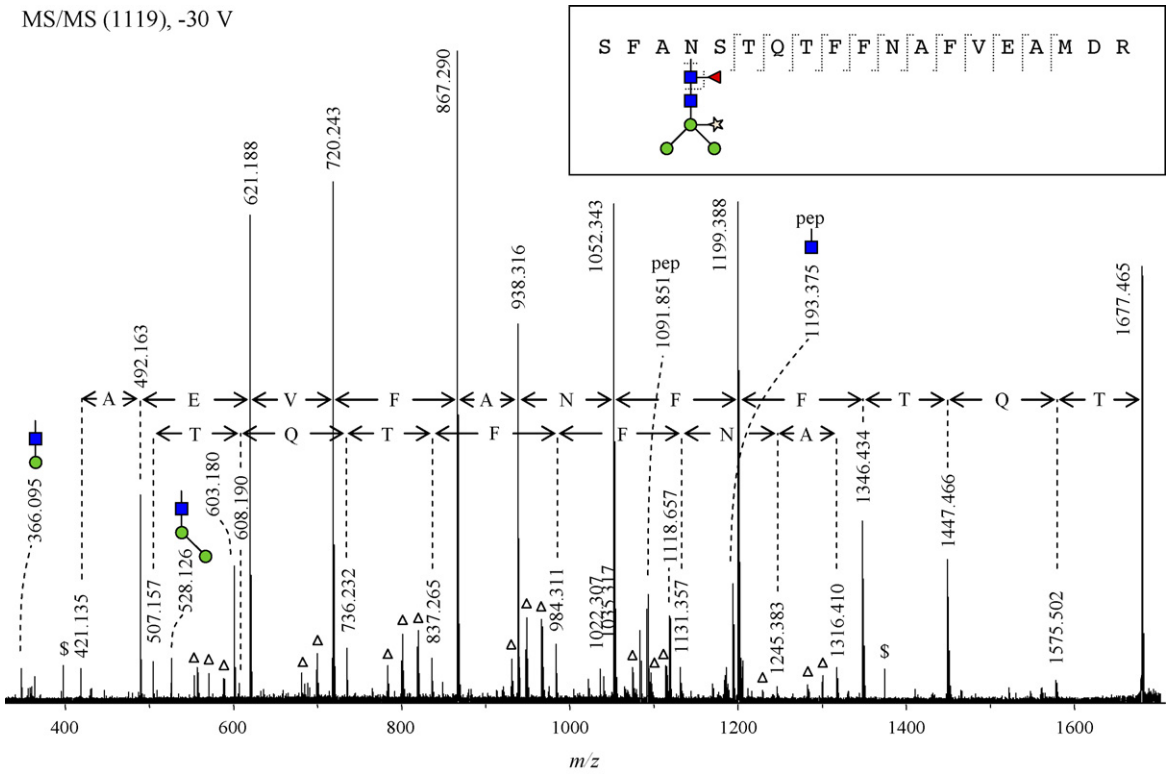

Fig. 3. ESI–Q–FT–ICR–MS/MS with collisional hexapole enhanced-energy CID of the tryptic glycopeptide Ser_{295}-Arg_{313} from HRP. The RP-HPLC-purified tryptic glycopeptide Ser_{295}-Arg_{313} containing a core-fucosylated and core-xylosylated trimannosyl *N*-glycan attached to N_{298} was analyzed by direct-infusion ESI with quadrupole selection of the $[M+3H]^{3+}$ species at *m/z* 1119 and was fragmented by CID with argon gas in the collisional hexapole (30 V offset) and analysed by FT–ICR–MS (Bruker Apex-Qe wiith 9.4 T magnet and Combisource). Monoisotopic masses are given rounded to three digitals. Square, *N*-acetylglucosamine; circle, mannose; triangle, fucose; star, xylose; pep, peptide moiety; △, ion exhibiting additional loss of water and/or ammonia; $, signal not related to the glycopeptide precursor.

Y-type and B-type glycan ions, fucose rearrangement products are observed (*m/z* 674, 836, and 968), which complicate the interpretation of the fragmentation spectrum and may be misleadingly interpreted as conventional B-type ions of a structural isomer (see Section 5.2). At higher collision energy, oligosaccharide ions were hardly observed any more. Instead, a series of y- and b-peptide ions were observed, revealing an 11 amino acid sequence tag (Fig. 3). Notably, the peptide fragments of the observed b-ion series (ions at *m/z* 507, 608, 736, 837, 948, 1131, 1245 and 1316) were found to be completely deglycosylated under the applied conditions. Interestingly, fragmentation of another model glycopeptide, which carries the same glycan structure but exhibits a different peptide moiety, resulted with identical experimental parameters in partial retention of the *N*-linked *N*-acetylglucosamine residue on y-ions comprising the *N*-glycosylation site (Fig. 4). The latter observation is in accordance with the analyzes of Harazono et al. [74], which showed peptide fragment ions comprising the *N*-glycosylation site and retaining the *N*-linked *N*-acetylglucosamine.

In addition, glycopeptides may be analyzed by CID in deprotonated form (negative-ion mode) resulting in complementary structural information [80]. In particular, the CID of multiply deprotonated glycopeptide species resulted in valuable information on the glycan moiety obtained in MS^3 experiments.

3.1.2. CID of O-glycopeptides

The potential of nano-ESI with a quadrupole–TOF mass analyzer for the characterization of *O*-glycopeptides has been demonstrated by Peter-Katalinic and co-workers [81–84]. Nano-ESI–quadrupole–TOF MS/MS has been shown to be a sensitive tool that provides information on glycan structure, glycan attachment site, and peptide sequence for mucin-type glycopeptides with serine- or threonine-linked *O*-glycans. This method has been successfully applied to the characterization of *O*-glycosylated peptides carrying the Tn-antigen (GalNAcα1-), the T-antigen disaccharide, or other, slightly more elongated *O*-glycans based on α-linked GalNAc, attached to serine or threonine residues (e.g. [85–87]). Moreover, *O*-fucosylation [88] as well as *O*-linked *N*-acetylglucosamine [89–91] can likewise be studied using this approach. Glycopeptides with *O*-linked *N*-acetylglucosamine have also been analyzed with ion trap [55] and triple-quadrupole instrumentation [92].

Fragmentation mass spectra of mucin-type *O*-glycopeptides generated with ESI–quadrupole–TOF generally allow the deduction of the *O*-glycosylation site(s) based on y-type and/or b-type peptide ions comprising the glycan attachment site. These y-type and b-type ions are usually reported in heterogeneous form: (1) with the intact glycan moiety, (2) with a truncated glycan chain, as well as (3) in deglycosylated form after loss of the complete *O*-glycan chain [81–87]. This heterogeneity, together

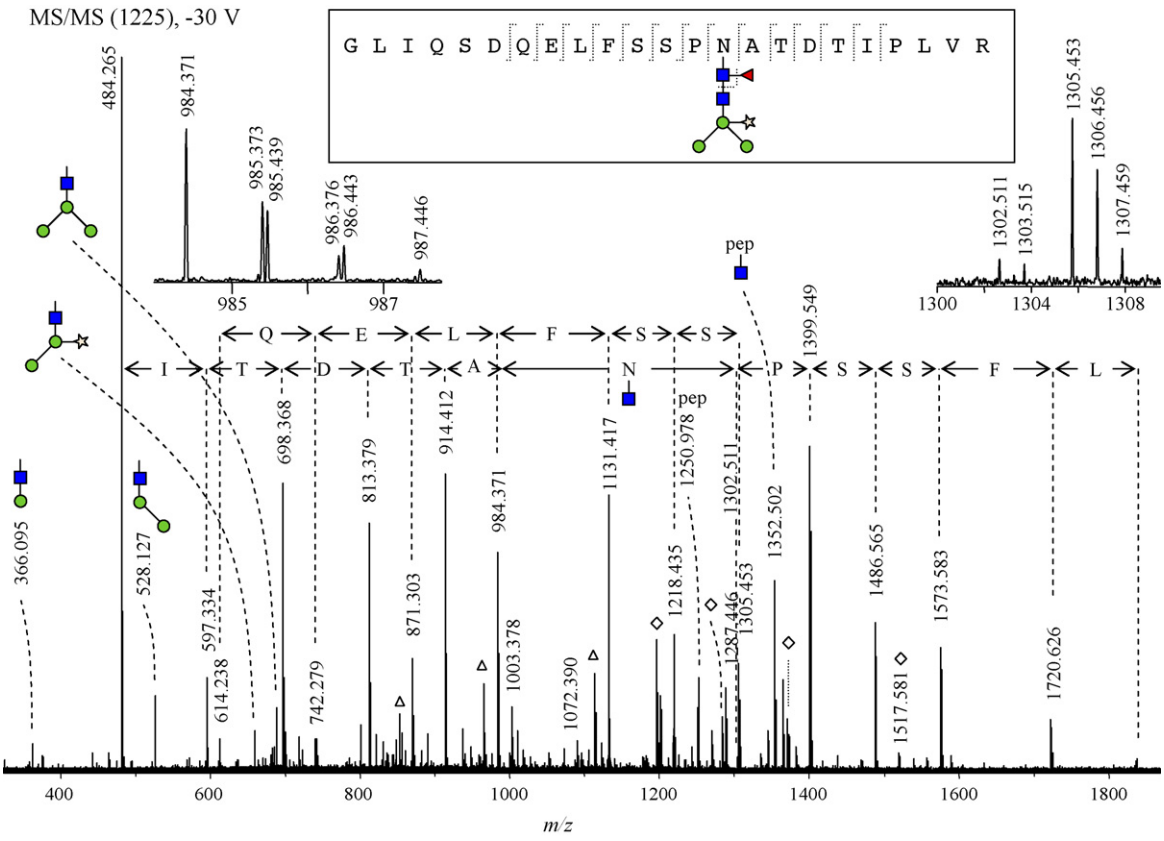

Fig. 4. ESI–Q–FT–ICR–MS/MS with collisional hexapole enhanced-energy CID of the tryptic glycopeptide Gly_{272}-Arg_{294} from HRP. The RP-HPLC-purified tryptic glycopeptide Gly_{272}-Arg_{294} containing a core-fucosylated and core-xylosylated trimannosyl *N*-glycan attached to N_{285} was analyzed by direct-infusion ESI with quadrupole selection of the $[M + 3H]^{3+}$ species at *m/z* 1225 and was fragmented by CID with argon gas in the collisional hexapole (30 V offset) and analysed by FT–ICR–MS (Bruker Apex-Qe with 9.4 T magnet and Combisource). Monoisotopic masses are given rounded to three digitals. Square, *N*-acetylglucosamine; circle, mannose; triangle, fucose; star, xylose; pep, peptide moiety, △, ion exhibiting additional loss of water and/or ammonia; diamond, fragment ion comprising the *N*-glycosylation site which lacks the complete glycan chain.

with the superimposition of various charge stages, makes the fragment ion spectra rather complex.

Another type of glycosylation, which has been analyzed by nano-ESI–quadrupole–TOF is C-mannosylation. Mannose is found linked to the C_2-atom of the tryptophan indole ring in various proteins, e.g. the human complement system [93,94]. The C-linked mannose appeared to be very stable in CID, in contrast to *O*-glycans and *N*-glycans.

3.2. Sustained off-resonance irradiation collision-induced dissociation (SORI-CID) and infrared multi photon dissociation (IRMPD)

Two so-called "slow-heating" techniques available in Fourier transform ion cyclotron resonance (FT-ICR) MS, which are useful for the analysis of glycopeptides, are sustained off-resonance irradiation collision-induced dissociation [95] and infrared multi photon dissociation [96,97]. In SORI-CID, precursor ions collide with an inert gas leaked into the ICR cell while they are excited slightly off-resonance. In IRMPD, precursor ions are heated by infrared irradiation until they begin to dissociate. Both fragmentation mechanisms result in similar fragmentation spectra of peptides or proteins, i.e., b-type and y-type ions are observed as well as water and ammonia neutral losses. The peptide fragmentation spectra are thus similar to the CID spectra in other types of instruments (see above).

Glycan sequencing of glycopeptides by SORI-CID MS/MS has been shown for *O*-glycosylated sialylated peptides from urine of patients suffering from Schindler's disease (hereditary *N*-acetylhexosaminidase deficiency). It has to be mentioned that in these experiments the peptide moiety was restricted to either a single Ser/Thr amino acid or the di-amino acid peptide Thr-Pro [98,99], and therefore, glycan attachment was readily obtained. For the structural characterization of *N*-glycopeptides, IRMPD has been shown to preferably cleave glycosidic linkages rather than peptide linkages, thus offering structural information on the glycan moiety. This preference has been shown for paucimannosidic [100,101] as well as for complex type glycopeptides [101]. More recently, Adamson and Hakansson [102] have shown that for high-mannose type glycopeptides, peptide backbone fragmentation can effectively compete with glycosidic backbone cleavages.

We have performed SORI-CID MS/MS of an electrospray-ionized glycopeptide after selection in the quadrupole and with helium as collision gas in the cell of the FT-ICR MS (Fig. 5). The obtained MS/MS spectrum showed predominantly cleavages of the glycosidic linkages with mainly neutral losses of one or several terminal monosaccharide units. In addition, a

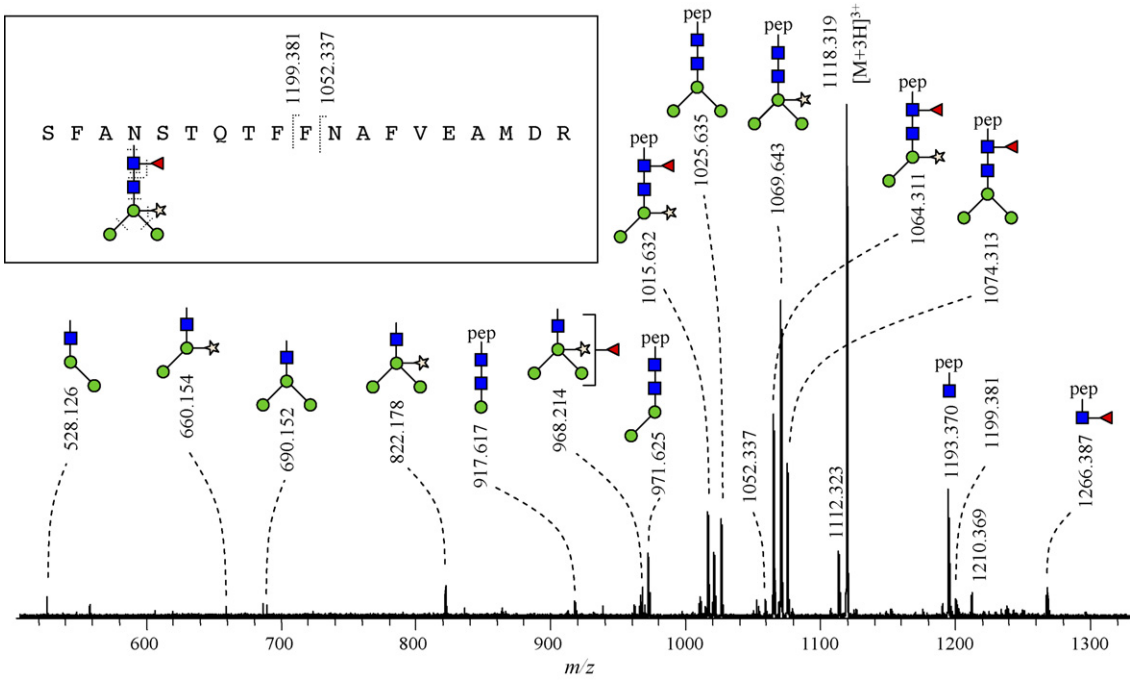

Fig. 5. ESI–Q–FT–ICR–MS/MS with SORI-CID of the tryptic glycopeptide Ser_{295}-Arg_{313} from HRP. The RP-HPLC-purified tryptic glycopeptide Ser_{295}-Arg_{313} containing a core-fucosylated and core-xylosylated trimannosyl *N*-glycan attached to N_{298} was analyzed by direct-infusion ESI with quadrupole selection of the $[M + 3H]^{3+}$ species at *m/z* 1119. Fragmentation was performed by sustained off-resonance irradiation CID in the cell of the FT–ICR–MS (Bruker Apex-Qe with 9.4 T magnet and Combisource). Monoisotopic masses are given rounded to three digitals. Square, *N*-acetylglucosamine; circle, mannose; triangle, fucose; star, xylose; pep, peptide moiety.

fucose rearrangement product was observed (*m/z* 968 in Fig. 5; see Section 5.2 for discussion). Only two ions of low intensity arising from fragmentation of peptide backbone linkages were observed.

3.3. Electron-capture dissociation (ECD)

ECD [103,104] is a recently developed MS/MS technique that shows promising alternative fragmentation pathways for the analysis of peptides and proteins, and the localization of their possible post-translational modifications. ECD has several unique features compared to other fragmentation techniques. Unlike the traditional MS/MS techniques described above (CID and IRMPD), ECD appears to retain labile post-translational modifications (PTM) and preferentially cleave disulfide bonds in proteins ([105] and references therein). In ECD, multiply protonated peptide ions are irradiated with low-energy electrons (<0.2 eV). The need to confine ions while they are irradiated with electrons complicates the application of ECD on many mass analyzers and therefore it remains largely restricted to FT-ICR MS, though ECD has recently been achieved on an ion trap-MS [106]. The major product of electron capture in most cases is the charge-reduced species, i.e., the precursor ion captures an electron without dissociating in sequence-specific fragments. H-atom loss is also a common phenomenon during ECD experiments [107]. These facts place an intrinsic limitation in the efficiency of ECD in peptide backbone fragmentation. Yet, it has been demonstrated that ECD is applicable to the analysis of pure peptides at 50 fmol quantities [108]. ECD of peptides results in the cleavage of the amine backbone (N–C_α) to generate preferentially $\mathbf{c}'$ and $\mathbf{z}^\bullet$ fragments ions (nomenclature of Zubarev and co-workers [109]). A detailed treatment of the mechanistic aspects of ECD can be found in a recent review by Zubarev et al. [110].

The reduction of the time scale required for ECD from 3 to 30 s down to a few milliseconds, through the implementation of an indirectly heated dispenser cathode as source of e^- [111], has led to its compatibility with on-line chromatography and thus, the possibility of incorporating ECD into proteomics [112–115].

For the structural analysis of protein/peptide glycosylation, ECD has been applied to both *N*- and *O*-glycopeptides. Kjeldsen et al. [116] showed the use of ECD in the characterisation of a complex *N*-glycopeptide and the two *O*-linked glycans in the bovine milk protein PP3. More recently, the use of ECD for the characterisation of *N*-glycopeptides has been extended to the high-mannose type of the standard protein ribonuclease B [102]. In the latter report, an *N*-glycopeptide as large as 40 amino was characterised through ECD and IRMPD. Hakansson et al. [100,101] demonstrated the use of ECD and IRMPD for the structural analysis of a xylose type *N*-glycopeptide from an unfractionated tryptic digest of the lectin of the coral tree, *Erythrina corallodendron*. ECD showed all the peptide backbone cleavages (except the N-terminal side of proline) with no saccharide loss and thus revealed the peptide sequence and site of modification; whereas IRMPD showed extensive cleavage of the glycosidic bonds and thus provided structural information of the glycan composition and branching pattern.

For *O*-glycopeptides, Mirgorodskaya et al. [117] demonstrated the use of ECD for the unambiguous localization of the GalNAc *O*-substitution sites in several *in vitro* glycosylated peptides. They also characterised a dimannosylated peptide by ECD. Haselmann et al. [118] determined the positions of six GalNAc groups in a 60-residue model glycopeptide, and the five sialic acid and six *O*-linked GalNAc groups of a 25-residue model glycopeptide with the aid of ECD. More recently, Mormann et al. [119] studied the electron-capture dissociation of various mucin-derived peptides carrying glycans of different core-types. *O*-fucosylation, where fucose is directly attached to either serine or threonine through a *O*-glycosidic bond [119] has also been probed by ECD. Renfrow et al. [120] showed the use of ECD for identifying potentially aberrant *O*-glycosylation of IgA1. Again, ECD was shown to be an excellent tool for localizing the glycosidic modification.

3.4. Electron-transfer dissociation (ETD)

Similar to the peptide structural information obtained from ECD, electron-transfer dissociation has recently emerged as a MS/MS technique complementary to CID and IRMPD (see above). Peptide fragmentation is generated through gas-phase electron-transfer reactions from singly charged anions to multiply charged protonated peptides. These ion/ion reactions can readily be executed in radio frequency (rf) ion traps, which are less expensive than the FT–ICR–MS instrumentation used for ECD. Singly charged anions are used as vehicle for the electron delivery to the multiply protonated peptides. Analogous to ECD, dissociation from electron transfer results preferentially in peptide backbone fragmentation into $\mathbf{c}'$- and $\mathbf{z}^{\bullet}$-type ions. Peptide backbone fragmentation seems to be preferred over bonds related to PTM's as phosphorylation [121] and glycosylation [122]. This feature makes this technique, together with ECD, a very attractive tool for the localization of the PTM attachment.

Different instrument firms have chosen for the negative chemical ionization of fluoranthene as reactant for ETD in their rf quadropole IT instruments. ETD has been implemented in both linear- [121] and 3D- [123] electrodynamic IT. Moreover, data-dependent LC–MS/MS and the consecutive acquisition of CID and ETD tandem mass spectra during a chromatographic run are expected to be readily features in the upcoming commercial instruments.

This very recent fragmentation technique has been reported to date only in one account on the characterization of glycopeptides. Hogan et al. [122] demonstrate the powerful combination of CID and ETD in a 3D-quadrupole IT for the structural characterization of a paucimannosidic tryptic *N*-glycopeptide. ETD fragmentation yielded the cleavage of the peptide backbone with no loss of the glycan moiety whereas CID resulted in glycosidic bond cleavages and therefore in glycan structural information.

As an example, the ETD fragmentation spectrum of the tryptic glycopeptide Ser_{295}-Arg_{313} from HRP is shown in Fig. 6 (kindly provided by Dr. Carsten Bäßmann and Dr. Markus Lubeck, Bruker Daltonics, Bremen, Germany). Glycosidic bond cleavages were not observed. The *N*-glycan modification attached to Asn_{298} remained intact and the whole peptide backbone sequence was obtained in both the $\mathbf{c}'$- and $\mathbf{z}^{\bullet}$-ion series.

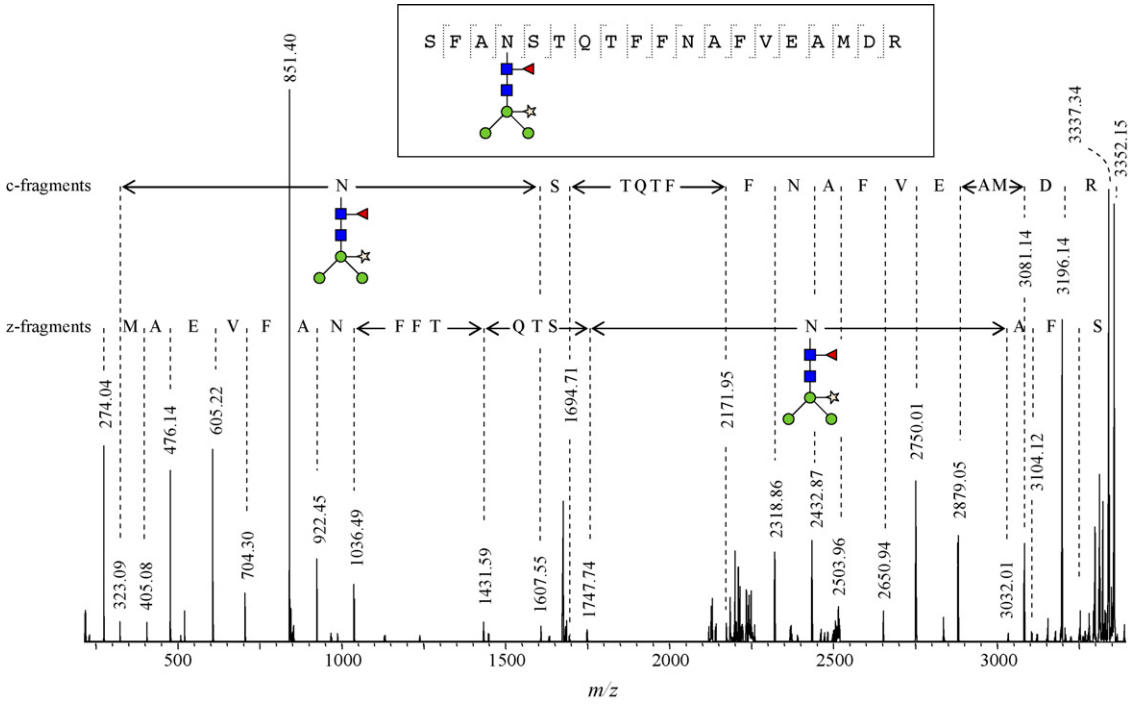

Fig. 6. ESI–IT–MS/MS with electron-transfer dissociation (ETD) of the tryptic glycopeptide Ser_{295}-Arg_{313} from HRP. The RP-HPLC-purified tryptic glycopeptide Ser_{295}-Arg_{313} containing a core-fucosylated and core-xylosylated trimannosyl *N*-glycan attached to N_{298} was analyzed by nano-LC–MS with ESI and IT selection of the $[M+3H]^{3+}$ species at *m/z* 1119 and electron-transfer dissociation using a Bruker HCT ultra equipped with an ETD facility. Data were kindly provided by Dr. Carsten Bäßmann and Dr. Markus Lubeck from Bruker Daltonics, Bremen, Germany.

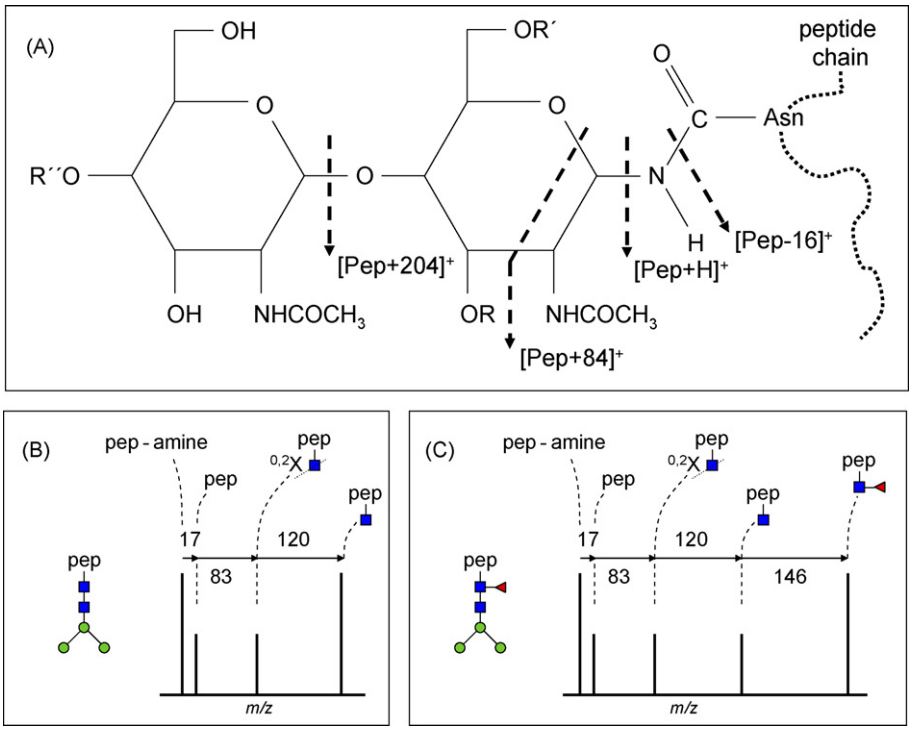

Fig. 7. Fragmentation observed in MALDI–TOF/TOF–MS of tryptic glycopeptides. (A) A schematic representation is given of the fragmentations occurring near the innermost *N*-acetylglucosamine in positive-mode MALDI–TOF/TOF–MS of *N*-glycopeptides, with retention of the intact peptide moiety. R or R′, H or fucose; R″, glycan chain; Asn, asparagine. Characteristic fragment patterns are observed for non-core-fucosylated (B) and core-fucosylated (C) *N*-glycopeptides. Circle, mannose; square, *N*-acetylglucosamine; triangle, fucose.

4. MALDI–MS/MS of glycopeptides

MALDI–MS/MS of glycopeptides has been performed with different instrumental configurations: MALDI–TOF with post-source decay (PSD), MALDI–TOF/TOF, MALDI–quadrupole–TOF, and MALDI–IT/TOF MS. MALDI–TOF/TOF MS of glycopeptides in protonated form has been established using 2,5-dihydroxybenzoic acid (DHB) as a matrix [26,124–127]. Observed fragments result from metastable decay of the precursor in the first field-free drift region of the MS. Fragmentation characteristics are very similar to those observed with MALDI–quadrupole/TOF–MS [128–130] and MALDI–IT/TOF–MS [60,131]. Three different groups of fragment ion signals are observed, which provide information on both peptide and glycan moiety of the glycopeptide:

MALDI–TOF/TOF–MS of *N*-glycopeptides results in a set of cleavages at or near the innermost *N*-acetylglucosamine residue, with all the fragment ions retaining the peptide moiety. A prominent signal usually arises from the cleavage of the side-chain amide bond of the glycosylated asparagine, resulting in a $[M_{pep} + H - 17]^+$ fragment (Figs. 7 and 8). Moreover, the $[M_{pep} + H]^+$ species is observed. A $^{0,2}X$-ring cleavage of the innermost *N*-acetylglucosamine gives rise to a $[M_{pep} + H + 83]^+$ signal. Another prominent signal arises from the Y-type cleavage (according to the nomenclature introduced by Domon and Costello [45]) of the chitobiose core, which results in the following signals: $[M_{pep} + H + 203]^+$ in case of a non-fucosylated core (Fig. 7B), $[M_{pep} + H + 349]^+$ in case of a monofucosylated core (Fig. 7C; see also Fig. 8), and $[M_{pep} + H + 495]^+$ for a difucosylated, innermost *N*-acetylglucosamine, as it is found for several egg glycoproteins of the human parasite *Schistosoma mansoni* [64,132]. A similar fragmentation pattern has been described for MALDI–quadrupole/TOF–MS of glycopeptides [128–130].

In MALDI–TOF/TOF–MS [26,124–127], MALDI–quadrupole/TOF–MS [128–130], and MALDI–IT/TOF–MS [131], peptide bond cleavages (predominantly y-type and b-type fragmentation of the backbone amide bonds; occasionally deamination or elimination of water) provide peptide sequence tags (Fig. 8). These types of ions have already been described in MALDI–TOF–MS with PSD of glycopeptides [82,84]. Peptide fragments comprising the *N*-glycosylation site generally retain the attached glycan moiety, thereby corroborating the attachment site of a specific glycan moiety [124].

Y-type and B-type cleavages of *O*-glycosidic linkages provide information about the glycan sequence, branching, and terminal motifs (*N*-glycan antennae structures) [26,124–131]. Y-type fragment ions of the glycan part generally comprise the complete peptide moiety. Fragmentation occurs preferably at the reducing-end side of *N*-acetylhexosamine residues (Fig. 8). Next to conventional Y-type and B-type fragments, rearrangement products may be observed, especially in the case of fucosylated glycopeptides, which should be taken into consideration with the interpretation of the MALDI–TOF/TOF–MS spectra (discussed in Section 5.2).

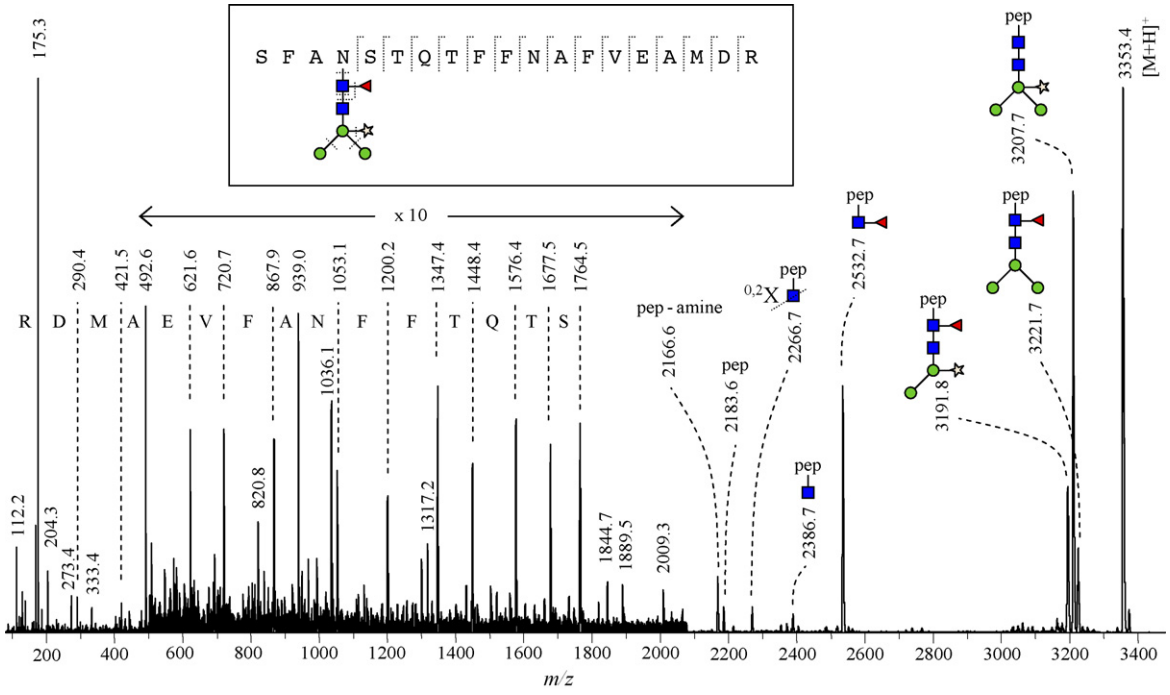

Fig. 8. MALDI–TOF/TOF–MS/MS of the tryptic glycopeptide Ser_{295}-Arg_{313} from HRP. The RP-HPLC-purified tryptic glycopeptide Ser_{295}-Arg_{313} containing a core-fucosylated and core-xylosylated trimannosyl *N*-glycan attached to N_{298}, was prepared with DHB and analyzed for fragment ions by MALDI–TOF/TOF–MS (Ultraflex II, Bruker Daltonics, Bremen, Germany) in positive-ion reflectron mode (precursor at *m/z* 3353.4). Monoisotopic masses are given rounded to one digital. The signal in the mass range *m/z* 480 and 2080 is enhanced by a factor of 10. Square, *N*-acetylglucosamine; circle, mannose; triangle, fucose; star, xylose; pep, peptide moiety; ^{0,2}X, ring cleavage of the innermost *N*-acetylglucosamine.

5. Discussion and perspectives

5.1. Which is the preferable mass spectrometric technique for glycopeptide analysis?

The tandem MS methods for glycopeptide analysis, which are covered by this review, vary considerably in the type and amount of structural information provided. Multiply protonated glycopeptide species obtained by ESI may be fragmented by CID. Low-energy CID experiments with IT instruments or in FT-ICR cells mainly result in the fragmentation of the relatively labile linkages between the sugars (glycosidic bonds), providing predominantly information on the structure of the glycan moiety. Similar fragmentation patterns are obtained by IRMPD dissociation in the ICR cell.

Particularly versatile instruments for glycopeptide analysis are quadrupole–TOF and quadrupole–FT–ICR mass spectrometers: with low-energy CID, the fragmentation of glycosidic bonds dominates, whilst elevated collision energies result in the removal of (most of) the glycan moiety and efficient y-type and b-type fragmentation of the peptide backbone.

Moreover, IT mass spectrometers have recently been shown to allow the very detailed characterization of glycopeptides using two different approaches: In a first approach, glycopeptides are analyzed by repetitive ion isolation/CID fragmentation cycles. The first cycle predominantly results in the cleavage of glycosidic bonds, and for *N*-glycopeptides often leaves the peptide moiety with a single monosaccharide as the dominant fragment ion. In a second cycle, this latter ion is fragmented providing information on the peptide sequence and glycan attachment site. In a second approach, ETD is used as a second fragmentation technique next to CID on IT–MS. Applying both fragmentation techniques sequentially to protonated glycopeptides provides information on the glycan structure (CID) as well as information on peptide sequence and glycan attachment site (ETD). The combination of these complementary data sets allows the detailed structural characterization of glycopeptide species. Similarly, complementary data sets can be obtained on FT-ICR mass spectrometers by fragmenting glycopeptide species with IRMPD (glycan structure) and ECD (peptide sequence and glycan attachment site).

Glycopeptide ionization by MALDI results in singly protonated species which are much more stable in CID than the multiply protonated glycopeptide species obtained by ESI. Fragmentation of these ions by metastable dissociation in a MALDI–TOF/TOF–MS or by CID in a MALDI–quadrupole–TOF instrument is therefore performed at higher energies. Resulting fragment spectra provide a wealth of structural information: y-type and/or b-type peptide backbone cleavages are observed next to the fragmentation of glycosidic bonds. Moreover, several characteristic fragmentations close to the glycan-peptide linkage result in a characteristic peak pattern, which allows the deduction of peptide and glycan mass.

Due to these pronounced differences in structural information on the glycan and/or peptide moieties provided by the various MS/MS techniques, the specific question which is to be addressed in an experiment will largely influence the choice of the MS technique: when glycan structural elements shall

be elucidated, low-energy CID, and IRMPD of multiply protonated glycopeptides will allow to specifically address this issue. When (additional) peptide sequence information is required, fragmentation should be performed at elevated energy within a collision cell or by MALDI–TOF/TOF–MS. Alternatively, radical-formation and consecutive fragmentation may be performed, i.e., ECD or ETD.

5.2. Structural characterization of glycans by MS/MS of glycopeptides

First of all, caution has to be applied when deducing glycan structures from CID spectra of glycopeptides. A major complication arises from fucose rearrangements, which are observed in MS/MS analysis of protonated glycans [133,134] and glycopeptides [64]. Rearrangement products may erroneously be interpreted as conventional B-type and Y-type fragment ions, and scientists may be tempted to postulate structures based on these misleading fragment ions.

While CID MS/MS techniques do routinely provide information on the glycan moieties of glycopeptides, they do only sometimes reveal information on peptide sequence and glycan attachment site(s). For a more detailed characterization of protein glycosylation, these techniques may be combined with other experiments. Glycopeptides may be treated with exoglycosidases revealing the nature and anomericity of terminal monosaccharide residues. Alternatively, by treatment of glycopeptides with peptide-*N*-glycosidase F or A, *N*-glycans can be released and deglycosylated peptide moieties are obtained. The peptides may then be subjected to mass spectrometric characterization. Notably, the conversion of the *N*-glycosylated asparagine into an asparatate residue on enzymatic deglycosylation introduces a tag which allows the MS analysis of the glycan attachment site at the level of the deglycosylated peptide. Enzymatically released glycans may be analyzed by various techniques including mass spectrometric analysis in sodiated or deprotonated form and linkage analysis by GC–MS providing detailed structural information, as reviewed by others [1–3,135,136]. These approaches may in particular be necessary for the detailed characterization of novel structural elements often observed in the analysis of glycoprotein samples from non-mammalian sources.

5.3. Towards a higher throughput in glycopeptide-based glycoproteomics

Proteomics software tools for the fully automatic assignment of the fragment spectra of unknown peptides are broadly used, and similar tools for the characterization of glycans are getting more and more popular [137–141]. Analysis of glycopeptides by the various MS methods, however, still requires man power for the assignment and interpretation of the spectra. Data evaluation is therefore the bottleneck in glycoproteomics via MS/MS of glycopeptides, and the development of suitable software tools is expected to make these techniques much more broadly applicable.

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