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Journal of Chromatography B, 825 (2005) 124–133
JOURNAL OF CHROMATOGRAPHY B
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Review

Protein glycosylation analysis by liquid chromatography–mass spectrometry

Manfred Wuhrer*, André M. Deelder, Cornelis H. Hokke

Biomolecular Mass Spectrometry Unit, Department of Parasitology, Leiden University Medical Center, P.O. Box 9600, 2300 RC Leiden, The Netherlands

Received 7 December 2004; accepted 28 January 2005
Available online 11 February 2005

Abstract

Liquid chromatography (LC)–mass spectrometry (MS) has developed into an invaluable technology for the analysis of protein glycosylation. This review focuses on the recent developments in LC and combinations thereof with MS for this field of research. Recently introduced methods for the structural analysis of released glycans (native or derivatised) as well as glycopeptides, on normal phase, reverse phase and graphitized carbon LC columns with online MS(/MS) will be reviewed. Performed on nano-scale or capillary-scale, these LC–MS methods operate at femtomole sensitivity and support the further integration of glycosylation analysis in proteomics methodology.

Keywords: Glycopeptide; Glycoprotein; Graphitized carbon; Normal phase; Reverse phase; Sensitivity

Contents

* Corresponding author. Tel.: +31 71 526 5077; fax: +31 71 526 6907.
E-mail address: m.wuhrer@lumc.nl (M. Wuhrer).

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doi:10.1016/j.jchromb.2005.01.030

1. Introduction

Protein glycosylation analysis has since long been a challenging task for the analytical biochemist. Compared to other classes of biomolecules, in particular peptides and lipids, the primary structure of glycans, either alone or in association with a protein, is not easily solved with a single technique. Variable composition, linkage, branching and anomericity of the constituent monosaccharides in combination with the general heterogeneity due to the indirect, non-template control of their biosynthesis are the basis of the structural complexity of glycoprotein glycans. As a consequence, a multitude of techniques is often necessary to fully determine the structure of a glycan. These techniques may include ^{1}H- and ^{13}C-nuclear magnetic resonance spectroscopy (NMR) [1,2], gas chromatography (GC) [3] and liquid chromatography (LC) [4,5], electrophoresis [5] and mass spectrometry (MS) [6–8], all possibly in combination with chemical or enzymatic degradation and derivatisation methods.

Inherent to the complexity of protein glycosylation, the sensitivity with which it can be analysed, as well as the relatively extensive and time-consuming analytical procedures needed, have long been a limiting factor for applications in many biomedical and glycobiological studies. The maturating proteomics field requires better methods for the analysis of protein glycosylation. Partly responsible for the continuing progress in proteomics research are the technological developments in the MS field [9,10] and LC–MS based methods for glycosylation analysis have also benefited significantly from these developments in recent years.

Glycosylation of proteins can be analysed at different levels of detail, depending on the specific research question asked. In some cases it may be sufficient just to determine whether a protein is glycosylated or not. In order to get a more detailed picture of a glycoprotein however, glycosylation analysis may include the complete primary structure determination of all glycans on a given glycoprotein or glycoprotein mixture, including site-specific data on occupation and microheterogeneity of each N- and O-glycosylation site.

Glycosylation analysis is recognised as one of the main current challenges in proteomics [10], and in particular the integration of compatible approaches for proteomics and glycoproteomics in terms of scale, detail and sensitivity is a rapidly developing field. To achieve this compatibility, LC–MS based methods are invaluable. The combination of LC for the separation, and MS(/MS) for the detection and further structural analysis of glycans and glycopeptides provides detailed information at high sensitivity. Various mass analyzers (for example, ion trap (IT), quadrupole-time-of-flight, triple quadrupole) may be chosen for these analyses, each exhibiting specific advantages/disadvantages [11]. Although the mass spectrometer is an equally important factor in the combined LC–MS technology, this review will primarily focus on recent developments in miniaturized LC, which together with online nano-electrospray allow the sensitive LC–MS analysis of labelled or non-derivatised released glycans (see Section 2), and of glycopeptides (see Section 3), as schematically represented in Fig. 1.

2. Analysis of released glycans

The most convenient approach to determine the structures of the glycoprotein glycans present in a given protein preparation regardless of their position on the protein backbone(s) requires their release by enzymes (peptide N-glycosidases F and A, endo-glycosidases) or by chemical procedures (β-elimination or hydrazinolysis) [8]. The released glycans can then be analysed by LC–MS in their reducing form, as alditols, after permethylation, or after addition of a label to the reducing end. Typically, electrospray MS as well as MS/MS, on-line coupled to LC are widely applied to the analysis of oligosaccharide derivatives [7,8,12–14],

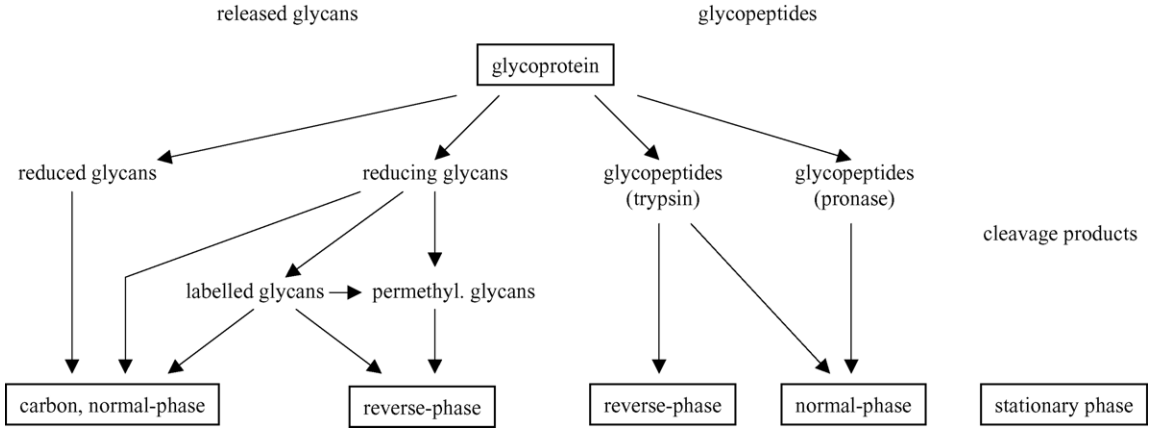

Fig. 1. LC–MS approaches for the analysis of protein glycosylation. Glycan release and protein degradation lead to various cleavage products, which can be analysed by LC–MS using different stationary phases. Enrichment techniques and approaches using deglycosylated peptides are not included in the scheme, permethyl, permethylated.

with MS(/MS) being performed both in the positive- and negative-ion mode. In the positive-ion mode, the fragmentation of alkali or proton adducts of the glycans is commonly used to obtain sequence information. Although we will focus this section on the chromatography rather than the MS, it should be noted that the fragmentation of proton adducts of fucosylated glycans often leads to "internal residue loss", and the resulting MS/MS spectra should be interpreted with caution [7,15]. Commonly used stationary phases for oligosaccharide LC–MS are reverse-phase (RP) (Section 2.1), normal-phase (NP) (Section 2.2), and graphitized carbon (Section 2.3), each suitable for coupling with MS (see Fig. 1). Capillary electrophoresis (CE) and high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), both separation techniques for oligosaccharides, which also can be combined with on-line MS, will not be discussed in this review.

2.1. Use of reverse-phase columns

As oligosaccharides generally exhibit poor retention on C_{18}-RP-LC columns, derivatisation with a hydrophobic agent is required to allow their efficient separation. For this purpose, various tags can be introduced (for example, by reductive amination), as outlined by Anumula [4]. The choice for a particular derivatisation agent may be determined by different factors. The tag will influence not only the retention behaviour of the derivatised glycan on a particular stationary phase (RP or otherwise), but it may also facilitate sensitive and selective detection by UV absorption or fluorescence, and it will influence the ionisation and fragmentation behaviour in MS. Japanese scientists introduced 2-aminopyridine (2-AP) as a fluorescent label for glycan analysis and detection in two- or three-dimensional HPLC separation systems, including RP-chromatography [4,16–21]. Mapping of glycans with these systems, combined with exoglycosidase treatment, allows their identification and structural characterization. A similar system comprising RP and NP chromatography was established for N- and O-glycans labelled with 2-aminobenzamide (2-AB) [22–24].

During the past 5 years, various methods for derivatisation, RP separation and online MS detection of protein-derived glycans have been described. 1-Phenyl-3-methyl-5-pyrazolone (PMP)-labelling of N-glycans followed by RP-LC (4.6 mm i.d.; running solvents containing 0.05% trifluoroacetic acid, elution with organic solvents) and ESI-MS with in-source decay fragment-ion analysis was performed by the group of Perreault [25,26]. Recently, an alternative derivatisation of glycans with phenylhydrazine in order to allow RP-LC–MS was introduced by the same group [27–29]. Li and Kinzer established RP-LC–MS of oligosaccharides upon reductive amination with 2-amino-5-bromopyridine, which due to the characteristic isotope profile of brome allows the straightforward differentiation between B-type and Y-type fragments [30,31]. Gennaro et al. [32] described a RP-LC–MS method (column of 1 mm i.d.) for the analysis of oligosaccharides derivatised with 8-aminonaphatalene-1,3,6-trisulphonic acid (ANTS), which is based on ion-pairing chromatography (10 mM triethylammonium acetate in the running solvents) and negative-ion mode MS detection. This system is able to resolve structural isomers. ANTS is a versatile tag which can be used for the analysis of N-glycans not only by RP-LC–MS but also by normal-phase LC–MS [26] and CE–MS [33,34].

The systems discussed so far all use columns of 1–4.6 mm i.d. To improve sensitivity in RP-LC–MS of glycans to a level, which is more in the range of that in peptide LC–MS, we recently performed RP-LC–MS of 2-AB labelled N-glycans using a nano-RP column (75 μm i.d.) (Fig. 2). Coupled to an ion trap-MS, this system exhibited sensitivities in the low femtomole range, both in the MS and MS/MS modes (unpublished results). Thus, the sensitivity of the system is similar to that of NP-nano-LC–MS systems (see Section 2.2) [35,36]. Nano RP-LC–MS methods with impressive resolution for malto-oligosaccharides and milk oligosaccharides derivatised with 4-aminobenzoic acid in its methyl ester, ethyl ester and butyl ester form have been established by Schmid et al. [37], but have not yet been tested for the analysis of protein-derived glycans.

A different approach for RP-LC–MS of oligosaccharides was followed by Delaney and Vouros [38], who set up a system for the separation of unlabelled or 2-AB labelled N-glycans in permethylated form (Fig. 1). Permethylated glycans are prone to sensitive detection by ESI-MS and the MS/MS fragment ion patterns are relatively informative [39,40].

In conclusion, reverse-phase nano-LC–MS of glycans after reducing-end labelling or permethylation is versatile, information-rich and sensitive. Importantly, standard RP-nano-LC–MS systems used in the various fields of life sciences can be used for the study of protein-derived glycans (Fig. 2). In particular, laboratories already working with a proteomics setup including RP-LC–MS should be able to include protein glycosylation analyses with minimal effort and investments.

2.2. Use of normal-phase columns

Hydrophilic interaction HPLC or normal-phase HPLC are part of the two-dimensional mapping systems for oligosaccharides derivatised with 2-AP or 2-AB (described in Section 2.1). In these systems, fluorochrome-labelled glycans are taken up in solvents with a high content of organics and subsequently retained by hydrophilic interaction with silica-based stationary phases terminating in amino or amide groups [4,16–24]. Elution is achieved by increasing aqueous buffer concentrations. Retention is usually measured relative to glucose units (dextran hydrolysate), enabling oligosaccharide identification. As a convenient rule of thumb, addition of monosaccharides to a glycan increases its retention. Substitution of an oligosaccharide with an increasing amount of fucose residues, however, can result in a decrease of retention

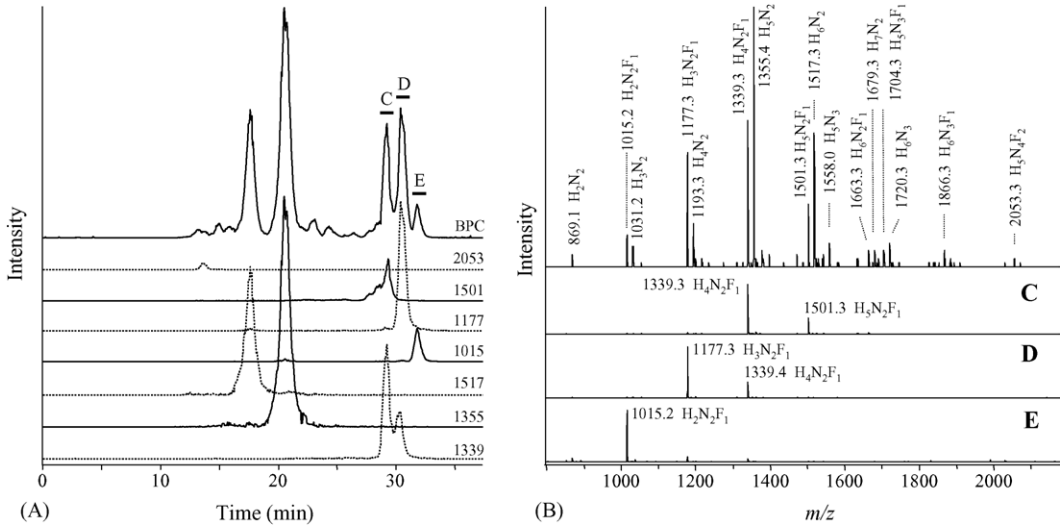

Fig. 2. RP-nano-LC–MS of a KLH N-glycans labelled with 2-AB. 2-AB-labelled glycans were separated on a PepMap column (75 μm × 100 mm; Dionex/LC Packings), which was equilibrated with eluent A (H_2O/acetonitrile 95:5, v/v, 0.1% formic acid) at a flow rate of 100 nl/min, using the instrumentation described elsewhere [35,36]. After injecting the sample, the column was run isocratically for 5 min, followed by a linear gradient to 30% eluent B (H_2O/acetonitrile 20:80, v/v, containing 0.1% formic acid) in 15 min and a final wash with 100% B for 5 min. The solvent was evaporated at 150 °C with a nitrogen stream of 6 l/min. (A) Base-peak chromatogram (BPC; mass range *m/z* 800–3000) as well as extracted ion chromatograms labelled with the selected mass for KLH 2-AB-N-glycans. (B) Total ion mass spectrum of KLH N-glycans eluted from 12 to 32 min. (C–E) Mass spectra of KLH 2-AB-N-glycans obtained for the time windows indicated by horizontal bars in (A). H: hexose; F: fucose; N: *N*-acetylhexosamine.

[41]. Running solvents usually contain 50–200 mM of a volatile buffer salt. In this way the chromatography is compatible with off-line MALDI-TOF-MS analysis of fractionated samples [24], without intermediate purification/desalting.

On-line LC–MS of 2-AB-labelled N- and O-glycans has been established at the Oxford Glycobiology Institute [42–44]. We have shown that by scaling down the LC-system to a 75 μm inner diameter TSK80-amide column with online nano-ESI-ion trap-MS, detection and fragmentation analysis of 2-AB-labelled glycans can be performed at low femtomol sensitivity [36]. Since nano-LC systems often suffer from run-to-run variations in retention times, this system may be calibrated by co-chromatography of 2-AB-labelled samples and an 2-AB-labelled dextran ladder. The glucose unit values determined for various glycans were comparable to those determined on a conventional 4.6 mm TSK80-amide column with fluorescent detection. This nano-LC–MS system [36] is 10 times more sensitive than conventional HPLC of 2-AB-labelled glycans with fluorescence detection [22], and approximately 100 times more sensitive than MALDI-TOF-MS of peak-fractions obtained by NP-LC (4.6 mm inner diameter) [24].

Although labelling of glycans with 2-AB or other fluorescent/hydrophobic tags has many advantages for their LC–MS analysis, in some cases reducing-end derivatisation is not feasible since multiple step work-up procedures are required. Moreover, in the case of O-glycan alditols obtained by reductive β-elimination, such derivatisation is not possible at all. Underivatised glycans can be chromatographed well on NP (amine/amide) columns, which have been applied in conventional HPLC settings since more than a decade [45,46]. Obviously, these methods that rely on UV-absorbance for detection were rather insensitive because of the lack of a good chromophore in native glycans.

With the same NP-nano-LC–MS setup as described above for application to 2-AB-labelled glycans, recently also the analysis of non-derivatised reducing oligosaccharides has been evaluated [35]. The sensitivity of the ion trap-MS detection for native glycans is approximately 1 fmol. Unlabelled glycans were found to elute slightly later from the NP nano-LC system than their 2-AB-labelled counterpart. Reducing oligosaccharides were found to give rise to peaks with a shoulder, probably due to partial separation of anomers. Despite this effect, NP-nano-LC–MS(/MS) of N-glycans released from the model glycoprotein keyhole limpet hemocyanin still displayed resolution of isobaric species [35].

NP-LC–MS of oligosaccharides has furthermore been applied to sulphated, mucin-type oligosaccharide alditols [47] as well as to small carbohydrate metabolites from plants [48].

Taken together, normal phase-LC–MS in its scaled-down form can be used for the sensitive analysis of labelled as well as unlabelled glycans. It has an outstanding resolution power, and elution positions can be determined in a standardized manner (expressed, for example, in glucose units) for chromatographic mapping of the glycans. Furthermore, NP-LC can be applied to the analysis of protein glycosylation at the glycopeptide level (see Section 3.4).

2.3. Use of graphitized carbon columns

The application of graphitized carbon HPLC columns for the separation of oligosaccharides, mostly in reduced

form, was established in the early nineties [49–52], using acetonitrile and trifluoroacetic acid for elution. Interaction of oligosaccharides with the graphitized carbon stationary phase is thought to occur mainly by hydrophobic interactions. Elution often seems to occur in the order of increasing oligosaccharide size, yet chain elongation may also lead to a decrease in retention, possibly by masking of hydrophobic faces of the oligosaccharide [53], and the elution orders seem to slightly resemble those found for 2-AP-labelled glycans in RP-HPLC [54]. Accordingly, graphitized carbon columns often allow the separation of structural isomers [49]. When analysing reducing oligosaccharides, the separation of anomeric species can complicate the obtained profiles. To overcome this problem, Fan et al. achieved a rapid conversion of anomeric species during the LC by doping the solvents with 10 mM ammonia, thus avoiding additional peaks [53]. Besides its unique retention properties, graphitized carbon exhibits excellent physical and chemical stability. Elution can be realised at low acetonitrile concentrations. Furthermore, by choosing additives the pH can be modified over a wide range [53], which makes this stationary phase suitable for sample preparation in MS of oligosaccharides [55]. The first LC–MS analyses of carbohydrates with a graphitized carbon stationary phase were by Kawasaki et al. [56–59], who used a 2.1 mm i.d. Hypercarb column coupled to a triple-stage quadrupole mass spectrometer. Eluents contained 5 mM ammonium acetate (slightly basic), an increasing acetonitrile concentration was used for elution, and the mass spectrometer was operated either in negative-ion or in positive-ion mode for the analysis of acidic or neutral oligosaccharides, respectively. Recently, Kawasaki and co-workers reported the use of microbore [60] and capillary-scale [61] graphitized carbon columns (1.0 and 0.2 mm i.d., respectively; Hypercarb, Thermo Electron Corporation). Using the latter system system, 50 ng of erythropoetin were sufficient to be able to generate the N-glycan profile in a single LC–MS run.

Karlsson and co-workers likewise used Hypercarb as stationary phase, eluting oligosaccharides with increasing acetonitrile concentrations in a 10 mM ammonium bicarbonate buffer, analysing the eluent on-line with electrospray MS in the negative ion mode [47,62]. They applied this method to analyze O-glycosylation [62–64] and N-glycosylation [64] of SDS-PAGE-separated glycoproteins. By scaling down column dimensions from 300 to 150 μm i.d., graphitized carbon LC, coupled on-line to an ion trap-MS resulted for sialylated N-glycan structures in detection limits of approximately 1 fmol in negative-ion mode [65,66].

Nano LC–MS of oligosaccharides with a graphitized carbon column has furthermore been described as a second separation dimension following HPAEC-PAD [67]. In addition, carbon nano-LC–MS has been applied for the mass spectrometric characterization of 2-AP-derivatized N-glycans from keyhole limpet hemocyanin (KLH) [68] and bovine neuronal cell-adhesion molecule (NCAM) [69], demonstrating that also oligosaccharides derivatized by reductive amination can be analyzed.

In conclusion, graphitized carbon LC–MS can be used for the sensitive analysis of native as well as derivatized glycans, both in the negative- and positive-ion mode. This stationary phase provides good resolutions with separation of isomeric glycans and is stable over a wide pH range.

3. Analysis of (glyco-)peptides

Although LC–MS analysis of released glycans may provide a detailed picture of the structure of the glycans derived from a protein or in fact any (complex) protein mixture, information on the original attachment sites of the glycans and the underlying proteins is lost. This often critical information can either be obtained by LC–MS analysis of the remaining peptides after glycan release (see Section 3.2) or, ideally, by the direct analysis of glycopeptides (see Sections 3.3 and 3.4; Fig. 1). Regarding the latter approach, significant progress has been made recently in the field of LC–MS based methods that support the seamless integration of glycosylation analysis in routine proteomics studies.

Since glycopeptides often only constitute a minor portion of a complex peptide mixture, e.g. the tryptic digest of a glycoprotein preparation, differentiation between glycosylated and non-glycosylated peptides prior to or during the LC–MS(/MS) analysis is essential. Different approaches are possible to achieve this. Several enrichment techniques have been developed for the selective purification of (classes of) glycopeptides from a complex peptide mixture (see Section 3.1). Alternatively, glycopeptides can be identified during LC–MS of the mixture in the MS mode, based on characteristic oxonium ions arising from in-source decay. These characteristic ions can be monitored by extracted ion chromatograms (EIC) of, for example, *m*/*z* 204, (protonated *N*-acetylhexosamine) and *m*/*z* 366 (protonated $HexNAc_1Hex_1$) [70–73]. Moreover, when running the MS in automatic MS/MS mode, the fragment ion spectra can be screened for similar, characteristic fragments by extracted ion chromatograms (see Fig. 3) or neutral loss chromatograms [74].

3.1. Enrichment techniques

Lectin affinity enrichment is a strong tool in glycoproteomics. In the approach of Hirabayashi and co-workers, glycoproteins are first enriched using a lectin (for example, concanavalin A) column. After proteolytic digestion of the eluted glycoproteins, glycopeptides are selectively purified from the digest via the same lectin column [75,76]. Glycopeptides are then analyzed by LC–MS, after enzymatic deglycosylation using PNGase F, to obtain the identity of the glycoproteins, as well as to determine the N-glycosylation sites (see Section 3.2).

An alternative protocol for the isolation and identification of N-glycosylated proteins has recently been presented by Zhang et al. [77]. This approach involves (i) glycoprotein

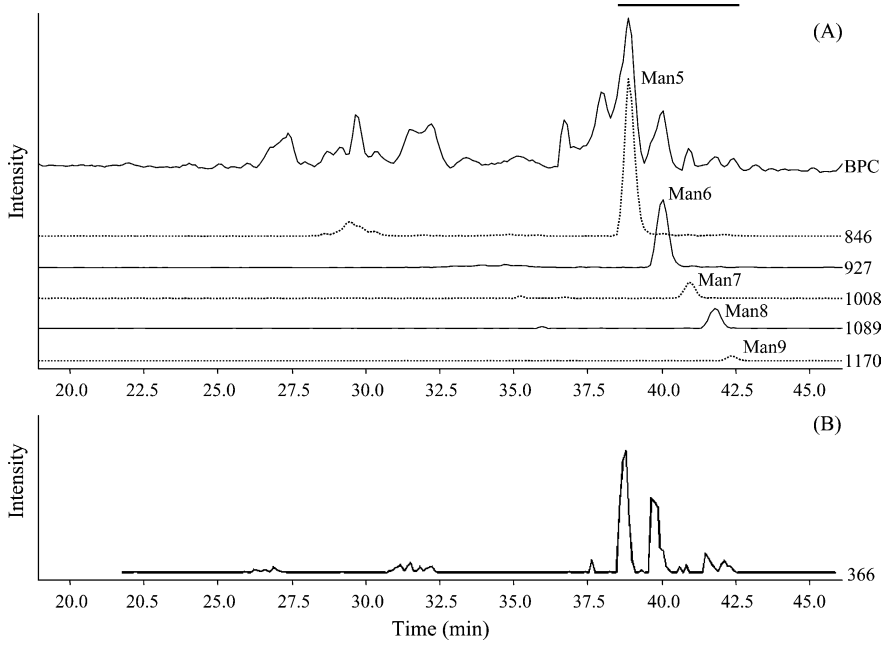

Fig. 3. NP-LC of N-glycosylated peptide(s) from an RNase B tryptic digest. RNAse B was digested with trypsin, and a 5 ng aliquot was analyzed by normal phase-nano-LC–MS using the conditions described elsewhere [74]. The ion trap-mass spectrometer performed up to three cycles of automatic precursor isolation and fragmentation. A screening of fragment ion spectra for the reporter ion of 366 Da ($[HexNAc_1Hex_1 + H]^+$) (B) indicated glycosylated species in the region of 37–43 min. MS data (Fig. 4) indicated these species to be tryptic peptides carrying the oligomannosidic Man5-Man9 N-glycan species known to occur on RNase B. Extracted ion chromatograms representing the double-protonated glycopeptide species are given in (A). BPC, base peak chromatogram. A mass spectrum of the glycopeptide region as indicated by a horizontal bar is given in Fig. 4A.

oxidation by periodate to introduce aldehyde groups on the glycans; (ii) immobilization of the glycoproteins via the aldehyde groups to a hydrazine-decorated solid phase; (iii) proteolysis; (iv) isotope labelling of the immobilized glycopeptides; (v) release of the peptide moiety by PNGase F; and (vi) analysis of the deglycosylated, isotope-labelled peptides by LC–MS/MS. Due to the introduction of a mass tag at the α-aminogroups of the peptides, this method should allow the semi-quantitative analysis of N-glycosylation of samples processed in parallel [77].

For the analysis of O-GlcNAc, a dynamic, regulatory modification of intracellular proteins, a chemoenzymatic approach has recently been introduced, which allows specific enrichment of O-GlcNAc-modified peptides and their identification by RP-LC–MS/MS [78,79]. An engineered galactosyltransferase is used to introduce a ketone-labelled galactose to O-GlcNAc-containing proteins, and the ketone group is subsequently used to specifically attach a biotin tag. After proteolytic cleavage, biotin-tagged glycopeptides are purified by avidin-affinity chromatography and then analyzed by RP-LC–MS/MS. This approach has been applied successfully to the analysis of O-GlcNAc-containing proteins from rat brain [78].

3.2. Analysis of deglycosylated peptides

Many strategies used in glycoprotein analysis include an enzymatic or chemical deglycosylation step at the level of (purified) glycopeptides obtained from a glycoprotein sample. Removal of N-glycans is mostly performed by PNGase F or PNGase A treatment. With these enzymes deglycosylation is achieved by enzymatic cleavage of the side-chain amide bond of a glycosylated asparagine, resulting in the enzymatic release of the oligosaccharide as a glycosylamine, which spontaneously converts to a reducing-end glycan. The deglycosylated peptide moiety now contains an aspartic acid at the position which was formerly N-glycosylated. Thus, deglycosylated sites (aspartate; increment of 115 Da) can be distinguished from unglycosylated sites (asparagine; 114 Da) by LC–MS/MS [80-82] to determine where N-glycans were originally attached. Unfortunately, due to the lack of a useful enzymatic approach, no similar method is available for O-glycans.

3.3. Glycopeptide analyses on reverse-phase systems

RP-LC–MS(/MS) analysis of glycopeptides obtained by enzymatic cleavage of glycoprotein samples is a widely used technique, because it can employ the standard peptide LC–MS setup present in many laboratories active in the proteomics field (RP pre-column, capillary or nano-scale RP column and an acetonitrile/formic acid solvent system, which is compatible with online-coupling to electrospray MS). Analysis of glycopeptides may be performed either with [71,73,82–85] or without [70,80,86,87] acquisition of MS/MS spectra. Under the usual conditions for RP-LC–MS (formic acid or low concentrations of trifluoroacidic acid, gradient of increasing acetonitrile concentration),

glycopeptides elute slightly earlier than a non-glycosylated peptide of the same sequence, and variations in size and composition of the attached glycan result in a mostly partial separation of different glycoforms of one peptide. By modifying the separation conditions, Ohta et al. obtained an optimized protocol for glycopeptide analysis by RP-LC–MS [86,87]. Elution was achieved with an acetonitrile gradient in combination with low salt concentration (1 mM ammonium acetate, pH 6.8), instead of the conventional solvents for (glyco-)peptide RP-LC–MS, which are acidic (formic acid or low concentrations of trifluoroacidic acid [57]). For the analysis of an enzymatic digest of recombinant erythropoietin, this solvent system exhibited several advantages: (1) it allowed the selective elution of glycopeptides at relatively low acetonitrile concentrations, while non-glycosylated peptides eluted later; (2) different glycoforms with identical peptide noieties could be nicely separated; (3) even isomers resulting from differences in glycan branching could be resolved by this system. The low concentration of ammonium acetate (1 mM) still allowed sensitive detection by electrospray MS, while higher concentrations of salt were found to interfere with MS detection. This promising method certainly deserves evaluation with other glycoproteins.

When analyzing glycopeptides by collision induced dissociation, obtained spectra are usually dominated by cleavages of the glycosidic linkages [71,73,82–84], and peptide cleavages, if observed at all, result in low-intensity ions [85,88]. In order to generate information on the peptide moiety (peptide sequence, glycan attachment site), the following approaches may be followed:

1. With an ion trap instrument, glycopeptides can be analyzed by repetitive ion isolation/low-energy CID steps. MS^2 spectra contain mainly glycan sequence information while MS^3 spectra of the truncated, single core-GlcNAc containing peptides are indicative for the peptide sequence. This approach has been demonstrated without LC-coupling by Demelbauer et al. [89], and has recently been incorporated by us in the nano-LC–MS analysis of tryptic digests (see Section 3.4).
2. In nano-LC of a glycoprotein digest, glycopeptide detection can alternatively be performed by MALDI-TOF-MS after automatic fraction deposition onto a MALDI target plate [90]. This allows the analysis of glycopeptides by MALDI-TOF/TOF-MS, which readily provides information on both the glycan structure, peptide sequence, and glycan attachment site [91,92].

Taken together RP-nano-LC–MS of glycopeptides is an extremely useful and sensitive technique for the analysis of glycopeptides in a fashion that is readily compatible with general proteomics-type setups.

3.4. Glycopeptide analysis on a normal-phase system

NP-LC can be used for the separation of labelled and unlabelled glycans (see Section 2.2), and has also been applied to the separation of peptides [93–96]. It has been applied for the enrichment of glycopeptides from (glyco-)protein digests for subsequent analysis by MALDI-TOF-MS [97]. In a recent study, we showed that glycopeptides can be separated on an amide column in the context of a sensitive nano-LC–MS system [74]. The running solvent contained high concentrations of acetonitrile and 0.5% formic acid, for the sensitive registration of glycopeptide species, as demonstrated in Figs. 3 and 4. On injecting 5 ng of an RNase B tryptic digest, the profile of the N- glycans (oligomannosidic structures with five to nine mannose residues) linked to the tryptic peptide Asn_{60}-Lys_{63} could be registered, with the minor nonamannosidic glycoform being detected at low femtomol sensitivity. The different glycoforms of the peptide are clearly separated on the nano-NP column according to their increasing numbers of monosaccharide residues. With the coupled ion trap-MS, glycopeptides were analyzed by several consecutive ion isolation/fragmentation cycles. This extensive MS analysis provided detailed information on both glycan structure and peptide sequence, as demonstrated for the peptide with the pentamannosidic N-glycan structure (Fig. 4). The MS^2 spectrum was dominated by fragmentation of glycosidic linkages, and did not show ions arising from peptide bond cleavages (Fig. 4B). Analysis of the peptide moiety retaining a single GlcNAc residue by two further isolation/fragmentation cycles, however, provided MS^3 (Fig. 4C) and MS^4 spectra (Fig. 4D) which allowed the deduction of the peptide sequence.

NP-nano-LC–MS was found to be of special benefit when applied to pronase-treated glycoprotein samples. Pronase treatment results in glycopeptides which retain a peptide moiety of several amino acids due to the glycans that inhibit the complete action of pronase by steric hindrance. An et al., have reported the use of MALDI-FT-ICR-MS to deduce site-specific glycosylation patterns from highly accurately registered masses of pronase-generated glycopeptides [98]. We could recently demonstrate the following advantages of analysing pronase-generated glycopeptides by NP-nano-LC–MS(/MS) [74]: (i) pronase digests can be directly analyzed without further work-up; (ii) glycopeptides elute later than the other degradation products like single amino acids; (iii) different glycoforms of one peptide moiety are resolved, allowing to make a distinction between real glycopeptide species and those arising from in-source decay (e.g. loss of fucoses). Although the peptide tags remaining on the glycopeptide are relatively short, this LC–MS approach could provide information on glycan structure and attachment site for *Dolichos biflorus* lectin, a protein of which the glycosylation had not been studied before [74].

In conclusion, NP-LC–MS of glycopeptides is a sensitive technique to obtain structural information of the glycan as well as the peptide moiety of glycopeptides in a complex mixture. The NP-fractionation provides an alternative and complementary dimension to RP-fractionation.

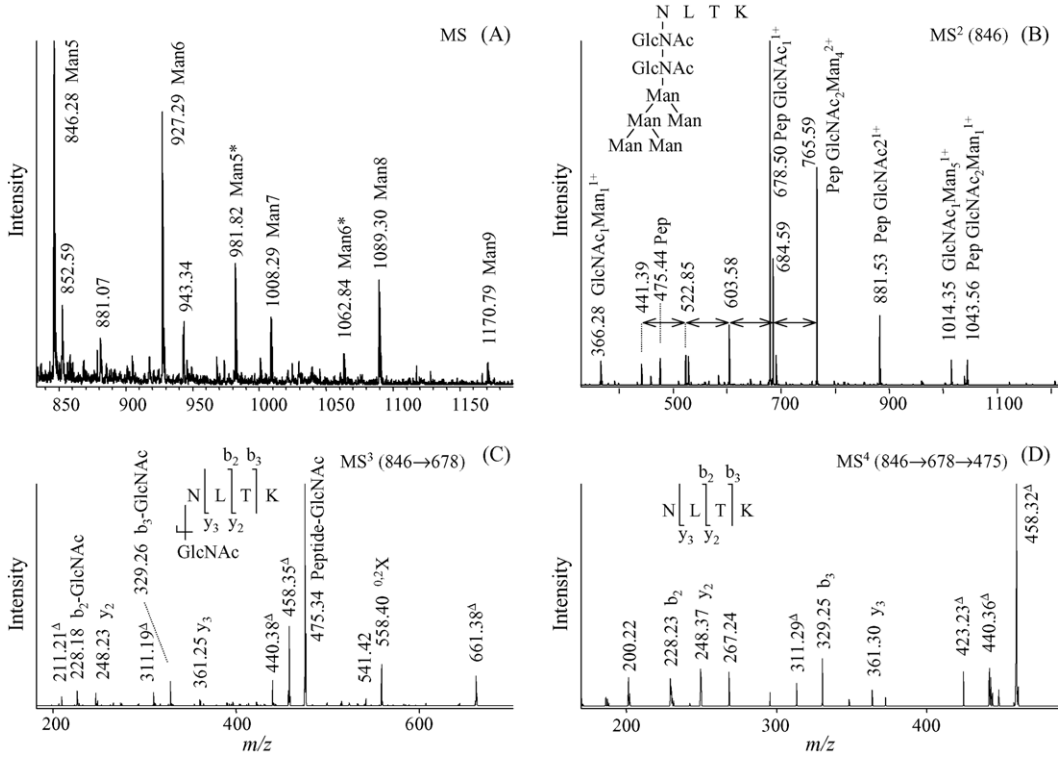

Fig. 4. Mass spectra of RNase B glycopeptides. (A) Sum spectrum over the whole range of detected glycopeptides as indicated in Fig. 3. All assigned ions correspond to double-protonated glycopeptide species. The shared peptide moiety is the tryptic peptide Asn_{60}-Lys_{63} of RNase B, except for species labelled with an asterisk, which exhibited an Asn_{60}-Arg_{65} peptide moiety due to a missed tryptic cleavage site. Man5, glycopeptide carrying a pentamannosidic N-glycan structure, etc. (B) Fragment ion spectrum (MS^2) of the glycopeptide with a pentamannosidic N-glycan (*m/z* 846). Heterogeneity in mannose content is indicated by double-headed arrows. Pep, peptide moiety. (C and D) Automatic, repetitive ion isolation/fragmentation cycles result in MS^3 and MS^4 spectra, respectively. $^{0,2}X$, ring fragmentation of the GlcNAc residue (nomenclature by Domon and Costello [31]). Ions formed by additional loss of water and/or ammonia are labelled with Δ.

4. Perspectives

Clearly, in recent years significant improvements have been achieved that have carried LC–MS technology for protein glycosylation analysis to a higher level, more in line with the demands for modern proteomics approaches. Further developments can be expected in the near future concerning both new MS equipment as well as modification of the LC system.

First of all, an additional gain in sensitivity should be possible with a further miniaturisation of the LC system. Experimental systems using RP-columns of 15 μm i.d. have already been developed and applied to LC–MS/MS analysis of complex peptide mixtures with unprecedented sensitivity [99]. Additionally, modification of solvent compositions (e.g. by addition of salts), spray conditions and ionisation parameters in existing setups may still result in major improvements.

A technology that should more extensively be explored for the analysis of glycopeptides is nano-LC coupled on-line to FT-ICR-MS. The extremely accurate, high-resolution mass determination inherent to the FT-ICR system is effective in glycopeptide identification [98]. Moreover, the combination of electron capture dissociation (ECD) and infrared multiphoton dissociation (IRMPD) that is supported by some FT-ICR-MS systems has been reported to be very useful for the sequence analysis of both the peptide and the glycan part of glycopeptides [100,101].

Application of targeted affinity approaches to select for glycoproteomes still deserves more attention. Lectins are very effective tools to target glycoproteins and glycopeptides in proteomics research but so far they have mainly been applied for the study of N-glycosylation. Apart from procedures for the general targeting of N-glycoconjugates, general procedures aimed at O-glycoconjugates, as well as specific glycoprotein/glycopeptide sup-populations could open new venues. For this last objective, more specific lectins that recognize, e.g. only fucosylated or sialylated glycoproteins and glycopeptides could be used. In addition, anti-carbohydrate antibodies, which bind to specific carbohydrate epitopes (e.g. the Lewis antigens, Tn-antigen, so-called core-xylose and -fucose residues) might prove valuable tools in glycoproteomics methodology.

Although a quantitative picture of protein glycosylation can routinely be produced on the basis of released, fluorescently labelled glycans, it will be a challenge to develop novel ways of quantitative analyses at the glycopeptide level. In an

ideal situation, glycosylation can be analysed, including site occupation and (partial) glycan structure, in a quantitative fashion, directly from a glycopeptide/peptide mixture.

Finally, as a general note, software used for the evaluation of LC–MS data could be further improved by the incorporation of more tools that support the interpretation of glycoconjugate derived (fragmentation) spectra. This would help to further integrate LC–MS(/MS) based glycosylation analysis as an essential routine in proteomics studies.

Acknowledgement

The authors thank Carolien A.M. Koeleman for expert technical assistance.

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