Published in final edited form as:

Rapid Commun Mass Spectrom. 2009 February ; 23(4): 495–505. doi:10.1002/rcm.3899.

Use of Activated Graphitized Carbon Chips for LC/MS and MSMS Analysis of Tryptic Glycopeptides

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Abstract

Protein glycosylation has a significant medical importance as changes in glycosylation patterns have been associated with a number of diseases. Therefore, monitoring potential changes in glycan profiles, and the microheterogeneities associated with glycosylation sites are becoming increasingly important in the search for disease biomarkers. Highly efficient separations and sensitive methods must be developed to effectively monitor changes in the glycoproteome. These methods must not discriminate against hydrophobic or hydrophilic analytes. The use of activated graphitized carbon as a desalting media and a stationary phase for the purification and the separation of glycans, and as a stationary phase for the separation of small glycopeptides has previously been reported. Here, we describe the use of activated graphitized carbon as a stationary phase for the separation of hydrophilic tryptic glycopeptides, employing a chip-based liquid chromatographic system. The capabilities of both activated graphitized carbon and C₁₈ LC chips for the characterization of the glycopeptides appeared to be comparable. Adequate retention time reproducibility was achieved for both packing types in the chip format. However, hydrophilic glycopeptides were preferentially retained on the activated graphitized carbon chip, thus allowing the identification of hydrophilic glycopeptides which were not effectively retained on C₁₈ chips. On the other hand, hydrophobic glycopeptides were better retained on C₁₈ chips. Characterization of the glycosylation sites of glycoproteins possessing both hydrophilic and hydrophobic glycopeptides is comprehensively achieved using both media. This is feasible considering the limited amount of sample required per analysis (< 1 pmol). The performance of both media was also appeared comparable when analyzing a 4-protein mixture. Similar sequence coverage and MASCOT ion scores were observed for all proteins when using either stationary phase.

Keywords

Activated graphitized carbon chip; Glycopeptides; LC/MSMS

Introduction

Recently, several studies have demonstrated that aberrant changes in glycosylation may be important in the diagnosis and prognosis of a number of diseases. Alterations in the glycomic profile have been associated with breast cancer,¹⁻³ prostate cancer,⁴ and ovarian cancer.⁵ However, it is not only important to monitor changes associated with the glycan profile, changes associated at the protein and peptide level must be also well characterized to

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better understand disease progression. Changes at the site (or sites) of glycosylation, both at the levels of glycans and the microheterogeneity, must be carefully monitored. Therefore, monitoring the changes in the glycan profiles of a glycoprotein and its glycosylation sites are becoming increasingly important in the search for disease biomarkers. To decipher the microheterogeneity of protein glycosylation sites, the separations of glycopeptides must be optimized to allow identification and characterization of all glycopeptides originating from the enzymatic digestion of a glycoprotein.

Activated graphitized carbon is an alternative stationary phase which has been used for the separation of hydrophilic species. Activated graphitized carbon consists of more or less perfect three-dimensional hexagonal crystalline order which is prepared from non-graphitic carbon by graphitization heat treatment. This type of media permits effective retention of hydrophilic compounds, including glycan samples. The mode of retention for activated graphitized carbon is thought to be based on adsorption.⁶ However, the charge and the size of the analyte may also influence the retention capabilities of activated graphitized carbon media.⁷ This media has a much broader working pH range than silica-based stationary phases. Activated graphitized carbon has been reported to be essentially inert to acids and bases, allowing separations to be performed across the entire pH range⁸, from highly acidic to highly basic. Thus separations requiring basic pH values may be optimized.⁶ It also offers high resolution, and is known to resolve isomers and other structurally related compounds.⁹ The separation of anomers of monosaccharides¹⁰ and branching isomers of oligosaccharides¹¹ have been demonstrated with activated graphitized carbon stationary phases.

Commonly, activated graphitized carbon has been utilized as a solid-phase media for desalting glycans¹² and peptides¹³ including phosphopeptides.¹⁴ It has also been used as a stationary phase for the liquid chromatographic separations of glycans, as well as glycopeptides⁷ and the products of pronase digests.⁸ It was observed that the products of pronase digestions of soybean agglutinin and ribonuclease B were not retained by C₁₈. However, when activated graphitized carbon was employed as the stationary phase, the glycan-containing analytes were retained and separated.⁸

Activated graphitized carbon stationary phases have been coupled to mass spectrometry for detection and characterization of glycans derived from ribonuclease B.¹⁵ More recently, the glycans, as well as glycopeptides derived from a Glu-C digestion of erythropoietin were studied using activated graphitized carbon as a stationary phase for LC/MS analysis.^{16;17} More recently, a dual column set-up has been described to profile N-glycans derived from human blood serum.¹⁸ In this configuration, the activated graphitized carbon column was set up in parallel with a C₁₈ column. This allowed for fast analysis of not only glycans, but also peptides, thus allowing high-throughput.

While the use of pronase is beneficial for glycan profiling,^{6;19} information pertaining to the glycans and their associated sites of glycosylation is lost. Therefore, complete characterization of a glycopeptide employing this approach may not be realized. In an attempt to further advance glycopeptide analysis, we have investigated the performance of both activated graphitized carbon and traditional C₁₈ stationary phases packed in chips for analyzing tryptic glycopeptides. By using tryptic peptides, a sufficiently long amino acid chain is present which allows the determination of both the amino acid sequence and subsequently, the glycosylation site, when employing an appropriate tandem MS fragmentation technique such as electron-transfer dissociation (ETD).

Experimental Methods

Materials

Bovine ribonuclease B, bovine fetuin, horseradish peroxidase, human haptoglobin, TPCK-treated trypsin, formic acid (95-97%), guanidine hydrochloride, and ammonium bicarbonate were purchased from Sigma Chemical Co. (St. Louis, MO). Dithiothreitol (DTT) and iodoacetamide (IAA) were acquired from Bio-Rad Laboratories (Hercules, CA). High-purity water (18 M Ω) and acetonitrile were products of EMD Laboratories (Gibbstown, NJ).

Tryptic Digestion

Model glycoproteins (2.0-5.0 μ g) were simultaneously denatured and reduced at 50° C for 1 hr, using 6 M guanidine hydrochloride and 10 mM DTT prepared in 50 mM ammonium bicarbonate buffer. Glycoprotein samples were then allowed to cool to room temperature prior to the addition of iodoacetamide to a final concentration of 25 mM. Reduced cysteine residues were alkylated for 1 hr at room temperature in the dark. The final digestion volume was adjusted to 300 μ l by adding 50 mM ammonium bicarbonate buffer. Trypsin was added at a trypsin-to-protein ratio of 1:30 by mass. Digestion was allowed to proceed for 18 hrs at 37° C. The digestion reaction was quenched by adjusting the pH of the resulting peptide solution to ca. 3, using formic acid.

LC-MS/MS

The Agilent ChipCube LC/MS interface (Agilent Technologies, Inc. Palo Alto, CA) was used in this study. Liquid chromatographic separations were accomplished by utilizing an Agilent 1200 Series nano-pump system (Agilent Technologies, Inc., Palo Alto, CA). The system included a binary capillary-flow pump, used for loading samples, and a nano-flow gradient pump for nano-flow separations. Mobile phases were degassed with a vacuum degasser (Agilent Technologies, Inc. Palo Alto, CA), while samples were injected via an auto-sampler. Liquid chromatographic chips containing either activated graphitized carbon or C₁₈ (5 μ m StableBond Zorbax) as both trapping and separation media were used. Each chip contained a 40 nl trapping column and a 75 μ m $\times$ 43 mm separation column. Liquid chromatographic separation was performed using a linear gradient from 3% B to 45% B over 45 min at a flow rate of 300 nl/min. Mobile phase A consisted of 97%/3%/0.1% water/acetonitrile/formic acid, while mobile phase B was composed of 97%/3%/0.1% acetonitrile/water/formic acid. The same mobile phases were used for both the capillary- and nano-flow pumps. Tryptic peptide samples of 1 μ l (500 fmol to 1 pmol) were injected using the capillary-flow pump operating at a 2 μ l/min flow rate. The total volume for the injection/wash cycle was 4 μ l (1 μ l of injected peptides, followed by 3 μ l of mobile phase A).

Separated peptides/glycopeptides were detected and identified using a Bruker Daltonics (Bruker Daltonik GmbH, Bremen, Germany) ultra-high capacity ion-trap mass spectrometer equipped with a negative-chemical ionization source for electron-transfer dissociation (ETD). Electrospray ionization was accomplished with spray voltages of 1825 V (activated graphitized carbon chip) or 1975 V (C₁₈ chip). Standard “enhanced” mode was used for MS analyses, while “ultrascan” mode was used for tandem MS analyses. The *m/z* scanning range was set to 200-3000. For most experiments, the identities of glycopeptides were confirmed by accurate mass and collision-induced dissociation (CID) data. However, in the case of ineffective CID fragmentation, ETD was employed to fragment the peptide backbone. In the case of the latter, the conditions employed were optimum as we have recently described.²⁰ Briefly, flouranthene radicals were generated internally, using a reactant temperature of 65° C, an ionization energy of 65 eV, and an emission current of 5.0 μ A. Radicals were accumulated in the trap for 7.5 ms and allowed to react with glycopeptide ions for 150 ms.

Results and Discussion

ChipCube Interface

Recently, Agilent Technologies introduced a new commercially-available separation system based on polyimide chips. A chip consisted of two valve rotors (an inner and an outer rotor) as well as trapping and separation columns. Only the inner rotor is currently used. Connected to the valve is a 40-nl trap column packed with the same material as the separation column. Also attached to the column is a nanoelectrospray emitter. By integrating all of the components to a single chip, manual connections and dead volumes are minimized. The fabrication and applications of the chip have been recently described in detail.²¹

Reproducibility and Repeatability

The reproducibility and repeatability of the activated graphitized carbon stationary phase for tryptic glycoprotein analysis were examined using two glycopeptides: the Man₅-SRNLTK glycopeptide derived from bovine ribonuclease B, and GlcNAc₂(Fuc)Man₃(Xyl)-NVGLNR derived from horseradish peroxidase. Results are depicted in Figure 1a and 1b for the ribonuclease B-derived glycopeptide and the horseradish peroxidase-derived glycopeptide, respectively. Each analysis was conducted 10 times for each glycopeptide. For ribonuclease B, all analyses were conducted in a single sequence using a 500-fmol aliquot of the tryptic digest per injection. The horseradish peroxidase analyses were conducted in two sequences over two days to monitor the day-to-day reproducibility and repeatability of the chip. Each sequence for this glycopeptide consisted of 5 analyses, utilizing 500 fmol of tryptic digest per injection.

For the 10 analyses of the ribonuclease B glycopeptide, the average retention time was 19.2 min $\pm$ 0.1 min (Figure 1a). The shortest retention time was 19.1 min, while the longest retention time was 19.4 min (2 analyses). These results suggest that the injection-to-injection repeatability of activated graphitized carbon is excellent. We also examined the repeatability of injection-to-injection over two days. This was evaluated utilizing the tryptic digest of horseradish peroxidase. In this case, 10 injections were performed over two days (five injections per day). The average retention time attained in the first day was 12.1 min $\pm$ 0.1 min (Figure 1b). The shortest retention time was 12.0 min, while the longest retention time was 12.3 min. The injections of the second day were also very repeatable with an average retention time of 11.8 min $\pm$ 0.1 min. Here, the shortest retention time was 11.6 min and the longest was 11.9 min. However, there was a half-minute change in the average retention time between the two days which might be attributed to temperature fluctuation. Separations attained on activated graphitized carbon media appeared to be highly temperature dependent. Nevertheless, the average retention time over the two days considering all 10 injections was 11.8 min $\pm$ 0.1 min. Thus, not only is the injection-to-injection retention time repeatability appeared to be excellent, but the day-to-day reproducibility was also excellent.

Injection-to-injection carry-over of the activated graphitized carbon chips was determined to be very low, while the efficiency of recovery of the same chip was very high. These were deduced from the high repeatability of the intensity of the extracted-ion chromatograms illustrated in Figure 1. For the glycopeptide derived from ribonuclease B, the average intensity was $(6.5 \pm 0.4) \times 10^7$ counts as determined from the integration of the extracted-ion chromatograms. The lowest observed value was 5.9×10^7 counts, while the highest was 7.1×10^7 counts. The average intensity for the horseradish peroxidase glycopeptide for the first sequence was $(3.5 \pm 0.3) \times 10^7$ counts, based on the extracted-ion chromatograms.

Analysis of Bovine Ribonuclease B

Ribonuclease B is a glycoprotein with a single site of N-glycosylation at N₆₀ with 5 high-mannose glycan structures (GlcNAc₂Man₅₋₉). Tryptic digestion of this protein resulted in small, hydrophilic glycopeptides with an amino acid sequence of SRNLTK (1 missed cleavage). Because of its hydrophilicity, this glycopeptide was not trapped by the C₁₈ chip, and was flushed out the waste line (data not shown). However, when the analysis was performed using an activated graphitized carbon chip, all 5 glycoforms of the peptide backbone were detected (Figure 2). Interestingly, the Man₅ glycopeptide was the most retained. The Man₆ glycopeptide was the second most retained and was well separated from the Man₅ glycopeptide. The remaining three glycopeptides were not well resolved, but did elute before the Man₆ glycopeptide. (See Figure 2.) This elution order of ribonuclease B glycopeptides is in agreement with previously published data,⁸ where pronase ribonuclease B glycopeptides with N or NL amino acid sequences were separated on a 4.6 × 100 mm Hypercarb column. This work showed that both the glycan structures and the peptide backbone interacted with activated graphitized carbon stationary phase. It was reported that GlcNAc₂-Man₆-N eluted before GlcNAc₂-Man₅-N and GlcNAc₂-Man₆-NL eluted before GlcNAc₂-Man₅-NL. The two amino acid-peptides were more strongly retained. Only the Man₅ and Man₆ glycopeptides, the two most abundant, were reported; nevertheless, the elution order reported agreed with the elution order observed here. The elution profile depicted in Figure 2 suggested that the separation shown was the result of hydrophilic interaction between the glycan moieties and the activated graphitized carbon stationary phase. Moreover, the results also indicate that the peptide backbone was not substantially involved in the interaction, since the separation was mainly based on the size of the glycan moiety attached to the peptide backbone.

Collision-induced dissociation of ribonuclease B glycopeptides provided sufficient information to confirm the identity of the glycopeptide. The inset of Figure 2 depicts the CID spectrum of the triply-protonated Man₅ glycopeptide. Several diagnostic ions were present in the tandem mass spectrum, thus confirming the identity of the Man₅ glycopeptide. Electrospray ionization produced triply- and doubly- protonated ions possessing *m/z* values of 645.9 and 968.0, respectively. In the CID spectrum, the product ion at *m/z* 204.5 is a glycan oxonium ion corresponding to GlcNAc residue. Loss of all 5 mannose residues, leaving only the core 2 GlcNAc residues attached to the peptide backbone is evidenced by the triply-protonated product ion at *m/z* 375.9. A triply-protonated ion series with *m/z* values of 429.2, 483.7, 537.7, and 591.7 are all separated by 54 corresponding to the sequential loss of 4, 3, 2, or 1 mannose residues, respectively. A peptide backbone with only a single GlcNAc residue is observed at *m/z* 464.4 as a doubly-protonated product ion. A doubly-protonated product ion series with *m/z* values of 724.9, 806.3, and 886.4 is also observed in the CID spectrum. Each ion is separated by 81 *m/z* units. These ions result from the loss of 3, 2, or 1 mannose residues respectively. A singly-protonated product ion at *m/z* 921.6 is that of the peptide backbone to which a GlcNAc residue is attached. Similar CID data were also observed for the Man₆₋₉ glycopeptides (data not shown), thus allowing the identification of the glycopeptides shown in Figure 2.

Analysis of Horseradish Peroxidase

Horseradish peroxidase is a glycoprotein with 8 sites of glycosylation. The major glycan associated with this protein has been reported to be GlcNAc₂(Fuc)Man₃(Xyl), existing at 70% abundance relative to the other known glycan structures.²²

The analysis of horseradish peroxidase tryptic digest on an activated graphitized carbon chip resulted in the successful identification of 5 of the 8 sites of glycosylation from 4 glycopeptides. One of the identified glycopeptides contained two sites of glycosylation. The

C₁₈ chip was only able to retain a single glycopeptide, which was also observed with the activated graphitized carbon chip in addition to the other 3 glycopeptides. Of the glycopeptides identified using activated graphitized carbon, we were able to confirm the identity of only one through CID, while the others resulted in poor CID data. Therefore, ETD was employed here to verify the amino acid sequences of these glycopeptides.

The extracted-ion chromatogram of the glycopeptide NVGLNR with GlcNAc₂(Fuc)Man₃(Xyl) glycan structure using the activated graphitized carbon chip is depicted in Figure 3. This glycopeptide possesses a very short peptide backbone (6 amino acids) and is very hydrophilic. The associated glycan can only increase the overall hydrophilicity of the glycopeptide. Therefore, this glycopeptide was effectively retained by activated graphitized carbon chip (Figure 3), but was not retained by C₁₈ chip (data not shown).

The CID spectrum of this glycopeptide acquired for m/z value of 922.1 (theoretical m/z is 922.4) is depicted in the inset of Figure 3. A product ion at m/z 366.1 represents a glycan oxonium ion corresponding to GlcNAc-Man, while product ions at m/z values of 528.2 and 690.2 are representative of GlcNAc-Man₂ and GlcNAc-Man₃ structures, respectively. The ion at m/z 822.3 represents GlcNAc-Man₃-Xyl, while a series of doubly-protonated ions at m/z values of 539.7, 620.9, 701.9, and 782.9 corresponds to the peptide with GlcNAc₂ core, and the sequential addition of 1, 2, or 3 mannose residues, respectively. The ion at m/z 767.5 represents the loss of the fucose and 1 mannose unit. The ion present at m/z 848.3 results from the loss of the core fucose from the glycopeptide. A product ion observed at m/z 1078.6 originates from the peptide backbone retaining the core GlcNAc residues, while that at m/z 875.5 represents the peptide backbone with a single GlcNAc residue. The entire loss of the carbohydrate structure from the peptide backbone is represented in the CID spectrum by the product ion observed at m/z 672.3. The CID spectrum resulting from the chip-based analysis is nearly identical to previously published results for the same glycopeptide analyzed with a traditional nano-LC column and nanospray source.²²⁻²⁴

Two additional horseradish peroxidase glycopeptides were only observed using the activated graphitized carbon stationary phase. Figure 4 illustrates the extracted-ion chromatogram of the doubly-glycosylated glycopeptide (LYNFSNTGLPDPTLNTTYLQTLR) to which the GlcNAc₂(Xyl)Man₃(Fuc) glycan structure is attached at N₂₁₆ and N₂₂₈. The retention time of this glycopeptide was *ca.* 49 min, and was only eluted from the column during the 85% mobile phase B wash. This particular glycopeptide produced a CID spectrum that was difficult to interpret, most likely due to its two glycosylation sites. The presence of two glycan structures on this peptide could lead to complex fragmentation pathways, producing a difficult-to-interpret spectrum (data not shown). The observed m/z value for this glycopeptide was 1274.1, while the theoretical value was 1274.3. To verify the amino acid sequence of this glycopeptide, ETD was employed. The ETD tandem MS spectra of this glycopeptide resulted in a series of z' ions from z'_{4-8} . A series of c' ions was also present from c'_{3-8} and c'_{10} . Due to the presence of a proline in the ninth position of this peptide, the c'_9 ion was not observed. Enough information is present to accurately deduce the amino acid sequence of this peptide. Due to the m/z of the c'_3 ion, 1579.5, the first glycosylation site is determined. Unfortunately, this data alone does not allow the second site of glycosylation of this peptide to be determined. To unequivocally locate the second site associated with this glycopeptide, the z'_9 ion must be identified which was not in this case.

The second glycopeptide observed in the activated graphitized carbon analysis (Figure 5a) and not in C₁₈ (Figure 5b) has an amino acid sequence of LHFHDCFVNGCDASILLDNTTSFR to which GlcNAc₂(Fuc)Man₃(Xyl) glycan structure is attached. This glycopeptide did not generate informative CID or ETD spectra. Thus,

accurate mass was used to identify this glycopeptide. The observed m/z value for this glycopeptide was 1299.9, while the theoretical value is 1300.2. The variation between the observed and the theoretical m/z value is within the mass accuracy used here.

Analysis of Bovine Fetuin

Bovine fetuin is a glycoprotein with 3 sites of N-glycosylation. Tryptic digestion results in large glycopeptides with 15, 28, and 32 amino acids. Glycans associated with these glycosylation sites are complex type mono-, di-, tri- and tetrasialylated. When 500 fmol of tryptic digest were injected onto the activated graphitized carbon chip, none of the glycopeptides were detected (data not shown). These large glycopeptides were apparently too hydrophobic to be effectively eluted from the activated graphitized carbon stationary phase. Only when the C_{18} chip was employed, the 3 sites of N-glycosylation and 4 sites of O-glycosylation were detected (Figure 6). Glycopeptides were observed with a diverse microheterogeneity leading to the identification of 16 different glycopeptides. Moreover, the CID spectra of these fetuin glycopeptides confirmed glycosylation (see inset of Figure 6). The glycopeptide at m/z 1470.1 has a trisialylated triantennary glycan structure associated with VVHAVEVALATFNAESNGSYLQLVEISR amino acid sequence. For this glycopeptide, the product ion at m/z 657.3 is a common oxonium ion corresponding to GlcNAc-Gal-NeuNAc residue. The loss of this oxonium ion produces the triply-protonated product ion observed at m/z 1741.5. The loss of one antenna and an additional sialic acid results the formation of a triply-protonated product ion with an m/z value of 1644.4. The loss of two antennas results in the detection of triply-protonated ion at m/z 1522.2. Finally, when an antenna, a sialic acid residue, and a mannose residue are lost a triply-protonated ion with an m/z value of 1590.4 is observed.

It has been previously reported that glycopeptides originating from the tryptic digestion of fetuin could be separated on an activated graphitized carbon,⁷ but these glycopeptides possessed missed cleavages. Using the method described in that publication, the hydrophobicity/hydrophilicity index was calculated for all glycopeptides analyzed here (Table 1). According to the hydrophobicity/hydrophilicity index, peptides with more negative values are more hydrophobic, while those with more positive values are more hydrophilic or less hydrophobic. Hydrophobicity/hydrophilicity index only accounts for the peptide backbone, and does not consider what is attributed by the glycan moiety.

When comparing the hydrophobicity/hydrophilicity index values for horseradish peroxidase and fetuin, three glycopeptide backbones from horseradish peroxidase, separated using an activated graphite chip, possess lower hydrophobicity/hydrophilicity index values (−232, −102, and −83) than the fetuin glycopeptide with a peptide sequence of RPTGEVYDIEIDTLETTCHVLDPTPLANCSVR (hydrophobicity/hydrophilicity index value of −55). According to this hydrophobicity/hydrophilicity index, the fetuin-derived glycopeptide should be separated on an activated graphitized carbon chip. However, this glycopeptide was not detected using the activated graphitized carbon chip. Therefore, we believe the glycans attached to the peptide attributes to the retention of the glycopeptide on the stationary phase. Horseradish peroxidase possess only small, non-sialylated structures, while much larger, sialylated glycans are attached to the fetuin backbone. Under our mobile phase conditions (0.1% formic acid, Davies *et al.* used 0.1 trifluoroacetic acid, which is not ESI compatible), the carboxylate groups of the sialic acids will not possess any charge, and therefore their overall hydrophobicity will be increased. This is evidenced by reversed-phase C_{18} chromatography where the same glycopeptide will elute according to the number of sialic acid groups associated with its glycan; a glycopeptide possessing a mono-sialylated glycan will elute before a glycopeptide possessing a di-sialylated glycan, which will elute before glycopeptide possessing a tri-sialylated glycan. (Data not shown.) Therefore, we believe the presence of sialic acid groups contribute significantly to the hydrophobicity of a

glycopeptide in acidic conditions utilized here, thus preventing fetuin glycopeptides elution from the activated graphitized carbon chip under the LC conditions used in this work.

Thus far, results from these model glycoproteins, summarized in Table 1, suggest that glycopeptides with short peptide backbones (6 amino acids long) are well retained by activated graphitized carbon chips, but not by C₁₈. Moreover, glycopeptides with moderately long peptide backbones (23-24 amino acids, with the exception a 14-amino acid long fetuin derived glycopeptide that was only detected when a C₁₈ stationary phase was employed) were also observed by activated graphitized carbon chips, but could also be retained by C₁₈. However, glycopeptides with long peptide backbones (28-32 amino acids long) and sialylated glycan structures were not identified by activated graphitized carbon and could only be detected and identified using C₁₈ chip. This suggests that to achieve a comprehensive analysis of a complex glycopeptide sample, two analyses should be conducted utilizing two different media (activated graphitized carbon and C₁₈), allowing for the retention of both hydrophilic and hydrophobic glycopeptides.

Analysis of Peptide Mixture: Activated Graphitized Carbon v. C₁₈

While smaller, more hydrophilic glycopeptides can be characterized using the activated graphitized carbon chip, and may not be achieved with C₁₈, many other peptides were also identified using activated graphitized carbon chip. To compare the two stationary phases, a 4-protein mixture composed of human haptoglobin, bovine fetuin, horseradish peroxidase, and bovine ribonuclease B was prepared. The concentration of each protein in the mixture was 100 fmol/μl, while a 1-μl aliquot of this mixture was injected and analyzed. Peptides were identified using MASCOT; therefore, glycopeptides were not identified. They are however, included in the calculation of sequence coverage. The MASCOT results for the 4-protein mixture are summarized in Table 2.

Interestingly, in many cases, the two different stationary phases produced very similar results. In many cases, differences between observed sequences coverages and MASCOT total-ion scores do not appear to be significant. These observations were evidenced by the results obtained from the 4-protein mixture analyzed by both stationary phases. From the mixture, a total of 13 peptide backbones originating from haptoglobin were identified using the activated graphitized carbon chip (11 identified by MASCOT and 2 glycopeptide backbones identified manually), while a total of 12 were identified using the C₁₈ chip (10 identified by MASCOT and the same 2 glycopeptide backbones identified manually). The two phases had a total of 9 peptide backbones in common of which 7 were identified via MASCOT while the remaining two peptide backbones of glycopeptides were manually identified. The activated graphitized carbon chip was able to identify 4 peptide backbones not observed with C₁₈, while C₁₈ was able to identify 3 unique peptide backbones. Both chips were able to identify 3 of the 4 sites of glycosylation for haptoglobin. In both phases, a doubly-glycosylated peptide backbone containing N₂₀₇ and N₂₁₁ was observed with multiple glycoforms. In addition, both chips were also capable of identifying the glycopeptide possessing N₂₄₁, with multiple glycoforms. Neither chip resulted in the identification of the N-glycosylation site at N₁₈₄. When all identified peptide backbones of haptoglobin are considered, including those identified by MASCOT and the manually identified glycopeptides, the activated graphitized carbon chip yielded a 41% sequence coverage, while 40% sequence coverage was observed using the C₁₈ chip. The peptides identified by MASCOT using the activated graphitized carbon chip resulted in a total-ion score of 779, while that attained using the C₁₈ chip was slightly lower at 680.

Similar to haptoglobin, a large overlap of fetuin peptide backbones identified by MASCOT was observed using both phases. When activated graphitized carbon was used as a stationary phase media, 7 peptide backbones were identified, while 8 were identified when C₁₈ was

employed. All peptide backbones identified for analysis using activated graphitized carbon as the stationary phase were also identified when C₁₈ was employed. Though the MASCOT results were very similar for native peptides, significantly different results were obtained for the analysis of glycopeptides. None of the N- or O-glycosylation sites were identified utilizing the activated graphitized carbon chip. By utilizing the C₁₈ chip, glycopeptides possessing the 3 sites of N-glycosylation at N₉₉, N₁₅₆, and N₁₇₆ were identified with multiple glycoforms by manual inspection. In addition to the N-glycosylation sites, the 4 sites of O-glycosylation at O₂₇₁, O₂₈₀, O₂₈₂, and O₃₄₁ were also manually identified. O-glycosylation sites were observed on 2 different peptide backbones; one O-glycopeptide contained 3 sites of O-glycosylation and was observed with multiple glycoforms. Overall, a sequence coverage of 27% and a MASCOT total-ion score of 505 resulted from the separation performed with an activated graphitized carbon chip. Because of the addition of the glycopeptide backbones, the total sequence coverage increased to 71% when C₁₈ was utilized. However, because these peptides were not identified by MASCOT, only a modest gain in the total-ion score resulted (*ca.* 513).

Similarly, horseradish peroxidase analyses with both stationary-phases were comparable. MASCOT searching of the tandem MS data identified 5 common peptide backbones and 3 unique peptide backbones for each of the stationary phases. As before, 4 additional glycopeptide backbones were manually identified from the activated graphitized carbon analysis. Only one of these glycopeptide backbones could be observed for the C₁₈ analysis. Overall, when all peptide backbones identified are considered, the sequence coverage produced by the activated graphitized carbon chip was 55%, while that of the C₁₈ chip was 32%. The MASCOT total-ion scores were 528 and 370 for the activated graphitized carbon and C₁₈ chips, respectively.

Ribonuclease B is a much smaller protein and a total of 3 peptides were identified by each chip. For the analysis using activated graphitized carbon, MASCOT identified two peptides. The 5 glycoforms corresponding to a single site of glycosylation were identified through manual inspection. The two peptides identified by MASCOT for the activated graphitized carbon analysis were also identified in the C₁₈ analysis, along with a single unique peptide. Overall, a sequence coverage of 40% resulted from the analysis of with the activated graphitized carbon chip. This value increased to 52% when the C₁₈ chip was employed. MASCOT total-ion scores were 148 and 244 for activated graphitized carbon and C₁₈ respectively.

Several chromatographic differences existed between the two phases. Figure 7a depicts the base-peak chromatogram for the analysis conducted using activated graphitized carbon, while the C₁₈ analysis is shown in Figure 7b. Activated graphitized carbon produced chromatographic peaks that were significantly broader. While peak widths as narrow as 0.6 min were observed, most peaks had a width of *ca.* 1 min. Some peaks had widths of over 1.2 min, with the broadest peak being 1.6 min for QHMDSSTSAASSSNYCQMMK derived from ribonuclease B. When C₁₈ was employed, peak widths ranged from 0.4 min to 0.8 min. Peptides analyzed with C₁₈ had an average peak width of 0.5 min.

In most cases, peptides identified by MASCOT were retained longer by activated graphitized carbon, by an average of nearly 6 minutes. For peptides more retained by activated graphitized carbon, retention times increased by as little as 1.7 min, to as much as 18 min. Only two of the common peptides identified, HTLNQIDSVK from bovine fetuin and TPTIFDNK from horseradish peroxidase, eluted earlier in activated graphitized carbon by 1.0 and 3.9 min, respectively. Comparison of chromatographic peak widths and retention times are included in Table 2.

Conclusions

Several tryptic glycopeptides from model glycoproteins were analyzed using a chip-based LC/ESI interface. Chips were packed with either activated graphitized carbon, or C₁₈. The same media was used for both the trapping and separation. Glycopeptides possessing short to moderate length peptide backbones, like those derived from ribonuclease B and horseradish peroxidase, were better retained and separated on activated graphitized carbon media. When tryptically-digested ribonuclease B was analyzed using a C₁₈ chip, none of the 5 glycoforms associated with the site of glycosylation were detected. All were successfully detected and identified using an activated graphitized carbon chip. Similarly, when horseradish peroxidase glycopeptides were analyzed with a C₁₈ chip, 1 glycopeptide was detected and identified. Three additional glycopeptides were only detected and identified when an activated graphitized carbon chip was employed. While activated graphitized carbon appeared to be effective at retaining and separating smaller, more hydrophilic glycopeptides, it was not an appropriate stationary phase for all glycopeptide analyses. When tryptically-digested bovine fetuin was analyzed with an activated graphitized carbon chip, none of the larger, more hydrophobic glycopeptides were detected or identified. These glycopeptides could only be detected and identified using the C₁₈ chip. Moreover, it was also found that gradients similar to those used for C₁₈ reversed-phase separations were appropriate for the activated graphitized carbon stationary phase. Therefore, both stationary phases must be employed to comprehensively characterize glycosylation of a protein. This requires 2 separate analyses, but is equivalent to performing the more traditional multi-dimensional chromatographic separations employing cation-exchange, followed by reversed-phase LC/MS. Moreover, many of the same peptide backbones identified with C₁₈ were also identified when using activated graphitized carbon. Similar MASCOT total ion scores were observed, along with comparable sequence coverage when only considering those peptides identified through MASCOT. In general, peptides were more retained by activated graphitized carbon, by an average of 6 min, and resulted in broader chromatographic peaks.

Acknowledgments

This work was primarily supported by the METACyt Biochemical Analysis Center, a component of the Indiana Metabolomics and Cytomics Initiative (METACyt), funded by a grant from Eli Lilly Endowment. Also, this work was partially supported by grant No. GM24349 from the National Institute of General Medical Sciences, U.S. Department of Health and Human Services, and grant No. RR018942 from the National Center for Research Resources, a component of the National Health Institute (NIH-NCRR) for the National Center for Glycomics and Glycoproteomics (NCGG).

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Figure 1.

Extracted-ion chromatograms depicting the retention time reproducibility and repeatability of activated graphitized carbon for (a) the glycopeptide SRNLTK-GlcNAc₂Man₅ derived from the tryptic digestion of bovine ribonuclease B, and (b) the glycopeptide NVGLNR-GlcNAc₂(Fuc)Man₃(Xyl) derived from tryptic digestion of horseradish peroxidase. Ribonuclease B tryptic digest (500 fmol) was injected 10 times in one day, while horseradish peroxidase tryptic digest (500 fmol) was injected 10 times over two days (5 injections per day).

Figure 2. Extracted-ion chromatogram of glycopeptides derived from tryptic digestion of bovine ribonuclease B and analyzed using the activated graphitized carbon chip. Inset, CID spectrum of the glycopeptide to which Man₅ is attached.

Figure 3. Extracted-ion chromatogram of the tryptic glycopeptide NVGLNR-GlcNAc₂(Fuc)Man₃(Xyl) derived from horseradish peroxidase and analyzed with an activated graphitized carbon chip. Inset, CID spectrum of the glycopeptide.

Figure 4.

Extracted-ion chromatogram for the horseradish peroxidase tryptic glycopeptide LYNFSNTGLPDPTLNTTYLQTLR to which GlcNAc₂(Fuc)Man₃(Xyl) glycan structure is attached. Inset, ETD tandem mass spectrum of the glycopeptide.

Figure 5. Extracted-ion chromatogram of the tryptic glycopeptide LHFHDCFVNGCDASILLDNTTSFR-GlcNAc₂(Fuc)Man₃(Xyl) derived from horseradish peroxidase and analyzed using activated graphitized carbon (a) and C₁₈ (b) chips. This peptide was identified by accurate mass matching.

Figure 6. Extracted-ion chromatogram of the tryptic glycopeptide GLIQSDQELFSSPNATDTIPLVR-GlcNAc₂(Fuc)Man₃(Xyl) derived from horseradish peroxidase and analyzed using C₁₈ chip. Inset, CID spectrum of the glycopeptide.

Figure 7. Base-peak chromatogram of a 4-protein mixture attained using activated graphitized carbon (a) and C₁₈ (b) chips. The concentration of each protein was 100 fmol/ μ l, while 1 μ l was injected in each case.

Tryptic glycopeptides derived from model glycopeptides analyzed by LC-MS/MS utilizing activated graphitized carbon and C₁₈ LC chips.

Table 1

Protein	Observed <i>m/z</i> activated graphitized carbon	Observed <i>m/z</i> C ₁₈	Hydrophilicity/ Hydrophobicity Index Value	Retention Time (min) C ₁₈	Retention Time (min) activated graphitized carbon
Ribonuclease B					
SRNTLK-GlcNAc ₂ Man ₅	645.5 (+3), 968.3 (+2)	ND	183	ND	16.3
SRNLTK-GlcNAc ₂ Man ₆	699.2 (+3), 1049.2(+2)	ND	183	ND	14.8
SRNLTK-GlcNAc ₂ Man ₇	753.3 (+3), 1130.2(+2)	ND	183	ND	13.8
SRNLTK-GlcNAc ₂ Man ₈	807.3 (+3), 1211.3(+2)	ND	183	ND	13.8
SRNLTK-GlcNAc ₂ Man ₉	861.3 (+3), 1292.3(+2)	ND	183	ND	13.7
Horseradish Peroxidase					
NVGLNR-GlcNAc ₂ (Fuc)Man ₃ (Xyl)	922.3 (+2)	ND	146	ND	9.9
LYNFSNTGLPDPTLTNTTYLQTLR- GlcNAc ₂ (Fuc)Man ₃ (Xyl)	1247.7 (+4)	ND	-233	ND	10.6
LHFHDCFVNGCDASILLDNITTSFR- GlcNAc ₂ (Fuc)Man ₃ (Xyl)	1299.9 (+3)	ND	-102	ND	49.5
GLIQSDQELFSSPNATDTIPLVR- GlcNAc ₂ (Fuc)Man ₃ (Xyl)	1274.4 (+3)	1274.3 (+3)	-85	15.7	23.5
Fetuin					
LCPDCLLAPLNDNR-T3S ^a	ND	1534.6 (+3)	-170	30.5	ND
VVHAVEVALATFNAESNGSYLQLVEISR-T3S ^a	ND	1470.1 (+4)	-94	36.4	ND
RPTGEVYDIEIDTLETTCVHLDPDTPLANCSVR-T3S ^a	ND	1634.1 (+4)	-53	38.0	ND

^aT3S- trisialo-triantennary complex glycan

Table 2

Peptides analyzed by activated graphitized carbon and C₁₈ chips, and identified by MASCOT from a 4-protein mixture (100 fmol/μl each protein, 1 μl injected). Shown are MASCOT ion scores for peptides identified and total-ion scores for each protein. Chromatographic data (retention time and peak width) are provided only for those peptides common between the two stationary phases.

Obs	Mr(exp)	Mr(cal)	Peptide	Ion Score Graphite	Ion Score C18	Retention Time Graphite (min)	Peak Width Graphite	Retention Time C18 (min)	Peak Width C18
Human Haptoglobin									
462.31	922.61	922.52	ILGGHLDK	ND	46				
490.85	979.69	979.49	VGYVSGWGR	58	54	43.6	1.6	25.6	0.6
522.86	1043.7	1043.6	VMPICLPSK	31	ND				
573.84	1145.7	1145.5	HYEGSTVPEK	48	ND				
602.39	1202.8	1202.6	VTSIQDWVQK	81	81	35.5	0.7	28.2	0.5
645.89	1289.8	1289.7	DIAPTLTLVVGK	78	ND				
673.43	1344.9	1344.6	SCAVAEYGVYVK	61	56	39	1.0	26.7	0.7
463.32	1386.9	1386.7	LPECEAVCGKPK	ND	33				
720.39	1438.8	1438.7	TEGDGVYTLNNEK	ND	83				
854.58	1707.2	1706.8	YVMLPVADQDQCIR	107	92	44.4	1.2	30.3	0.6
599.4	1795.2	1794	VVLHPNYSQVDIGLIK	80	ND				
612.61	1834.8	1833.9	VMPICLPSKDYAEVGR	41	41	35.6	0.7	29.8	0.5
620.33	1858	1856.9	AVGDKLPECEAVCGKPK	83	85	22.8	0.8	21.1	0.4
1087.12	2172.2	2171.1	SPVGVQPILNEHTFCAGMSK	111	109	33.3	1.0	30.5	0.5
			total-ion score	779	680				
Bovine Fetuin									
577.9	1153.8	1153.6	HTLNQIDSVK	69	49	19.1	1.3	20.1	0.5
635.5	1269	1268.6	QDQGFSLFTK	70	70	34.2	1.1	31.9	0.5
738.28	1474.6	1473.8	TPVGGPSIPGGPVR	84	51	31.1	1.8	27.5	0.7
501.78	1502.3	1501.8	GYKHTLNQIDSVK	50	52	23.9	0.8	21	0.4
565.28	1692.8	1691.9	HTLNQIDSVKVPWR	66	69	37.8	1.2	27.4	0.5
660.41	1978.2	1976.9	QQTQHAIVEGDCDIHVLK	53	62	25.8	1	22.4	0.6

Obs	Mr(exp)	Mr(cal)	Peptide	Ion Score Graphite	Ion Score C18	Retention Time Graphite (min)	Peak Width Graphite	Retention Time C18 (min)	Peak Width C18
707.61	2119.8	2119	HTFSGVASVESSSGEAFHV GK	113	95	37.4	1.5	26.8	0.5
840.96	2519.9	2518.3	AQFVPLPVSVSVEFAVAATDCIAK	ND	65				
			total-ion score	505	513				
Horseradish Peroxidase									
468.31	934.61	934.48	TPTIFDNK	41	46	20.7	0.6	24.6	0.5
480.3	958.59	958.51	DTIVNELR	63	49	25.3	0.9	20.2	0.4
511.78	1021.6	1021.5	DAFGNANSAR	65	ND				
593.36	1184.7	1184.6	YYVNLEEQK	66	66	31	0.9	24.6	0.6
691.37	1380.7	1380.7	STIQAFQSDTR	ND	94				
690.91	1379.8	1379.6	TEKDAFGNANSAR	64	ND				
738.84	1475.7	1474.7	SSDLVALSGGHTFGK	73	66	28.9	0.6	27.5	0.7
793.95	1585.9	1585.8	MGNITPLTGTGGQIR	111	99	29.4	0.8	27.2	0.4
701.85	2102.5	2101	TPTIFDNKYYVNLEEQK	45	ND				
749.95	2246.8	2246.1	FSAVGLNTNDLVALSGAHTFGR	ND	81				
868.71	2603.1	2601.3	TVSCADLLTIAAQQSVTLAGGPSWR	ND	44				
			total-ion score	528	545				
Bovine Ribonuclease B									
742.42	2224.2	2223.1	HIIVACEGNPYVPVHFDASV	ND	104				
789.41	2365.2	2363.9	QHMDSSTSAASSSNYCQMMIK	66	59	34.7	1.6	21.4	0.8
840.31	2517.9	2516.2	CKPVTFTVHESLADVQAVCSQK	82	81	31.7	1.4	30.1	0.5
			total-ion score	148	244				