Journal of Chromatography A, 1299 (2013) 18–24

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

# Application of a strong anion exchange material in electrostatic repulsion–hydrophilic interaction chromatography for selective enrichment of glycopeptides

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ARTICLE INFO

*Article history:*
Received 10 April 2013
Received in revised form 15 May 2013
Accepted 15 May 2013
Available online 23 May 2013

*Keywords:*
SAX
ERLIC
Glycopeptides
Enrichment
Mass spectrometry

ABSTRACT

Glycoproteins are involved in various cellular activities, including inter- and extracellular signaling. However, glycopeptide signals are significantly suppressed by coeluting non-glycosylated peptides in mass spectrometry-based analysis. For detailed elucidation of the biological functions of glycoproteins, selective enrichment of glycopeptides from non-glycosylated peptides is crucial. In the present study, a SAX material, XCharge SAX, was used in a column in the ERLIC mode with the aim of specifically enriching glycopeptides. Enrichment conditions were initially optimized, and selectivity, glycosylation heterogeneity coverage and detection sensitivity of XCharge SAX were subsequently assessed. In the selectivity assessment, glycopeptides were effectively isolated from a peptide mixture (human serum immunoglobulin G (IgG) and human serum albumin digests) and a tryptic digest of human serum using XCharge SAX. In the evaluation of glycosylation heterogeneity coverage, five glycosites and eleven glycopeptides from horseradish peroxidase were identified after enrichment with XCharge SAX. In detection sensitivity assessment, glycopeptides within four orders of magnitude were identified after enrichment with XCharge SAX. In addition, volatile solvents were used in the loading and eluting buffers so that desalting was not necessary for ERLIC fractions. Our results collectively support the utility of XCharge SAX as a suitable chromatographic material for global glycosylation site analysis.



## 1. Introduction

Glycoproteins are involved in various extracellular activities, such as cell adhesion [1,2], recognition [3], and signal transduction [4], and additionally influence protein folding, degradation, as well as subcellular localization [5]. Nearly 50% of mammalian proteins are estimated to be glycosylated [6]. Nevertheless, the glycopeptide content in peptide mixtures of biological samples is extremely low. A study by Zhang et al. [7] showed that only 7% of peptides in a serum tryptic digest contain the N-X-T/S motif, a potential N-linked glycosite. Furthermore, the ionization efficiency of glycopeptides is significantly lower than that of non-glycosylated peptides. As a result, glycopeptide signals are rarely detected in direct mass spectrometry (MS) analysis of peptide mixtures from biological samples. The development of efficient methods for glycopeptide enrichment is therefore crucial. Over the decades, several strategies have been developed, including hydrazide chemistry [8–11], lectin affinity chromatography [12–17], boronic acid affinity-based method [18–20], and hydrophilic interaction (HILIC) method [21–26]. In particular, the HILIC method has attracted increasing attention, owing to its global affinity for different types of glycans. However, a major limitation of this method is low selectivity [27–31]. Moreover, strong retention of basic peptides on specific hydrophilic materials, particularly those carrying zwitterion or amide groups, often leads to ready co-elution with the glycopeptide fraction.

Recently, a novel mode of chromatography, designated electrostatic repulsion–hydrophilic interaction chromatography (ERLIC), based on electrostatic and hydrophilic properties, was introduced by Alpert for separation of biomolecules and phosphopeptide enrichment [32]. This method has been extended to fractionation of N-linked glycopeptides from biological samples [25,33,34]. Lewandrowski and colleagues were the first to use the ERLIC mode to separate glycopeptides from non-glycosylated peptides [33]. Subsequently, Zhang et al. [25] developed an improved protocol using ERLIC for the simultaneous characterization of glyco- and phosphopeptides from a mouse brain membrane preparation. The results showed that the ERLIC approach is more efficient in the identification of glycopeptides and glycoproteins than the hydrazide chemistry method. However, in the above studies, non-volatile salts were used in the mobile phase, so fractions subsequently

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http://dx.doi.org/10.1016/j.chroma.2013.05.037

required desalting prior to MS analysis. Hao et al. further optimized the ERLIC method to facilitate separation of glyco- and phosphopeptides from rat kidney tissue [34]. With the low pH and high organic content of the mobile phase, most tryptic peptides carried a positive net charge due to amino protonation of the C-terminal arginine/lysine and N-terminal residues and uncharging of carboxyl-groups. Therefore, retention of non-glycosylated (including basic) peptides on polyWAX was weak due to electrostatic repulsion.

In the present study, a SAX material, XCharge SAX, was applied to selectively enrich glycopeptides from a peptide mixture in the ERLIC mode. The effectiveness of XCharge SAX in glycopeptide enrichment was assessed based on selectivity, glycosylation heterogeneity coverage and detection sensitivity. Peptide mixtures (human serum immunoglobulin G (IgG) and human serum albumin (HSA) digests) and a tryptic digest of human serum were used to evaluate selectivity. For glycosylation heterogeneity coverage assessment, horseradish peroxidase (HRP) containing eight known glycosites was selected as the model glycoprotein. Notably, good selectivity and glycosylation heterogeneity coverage were obtained with XCharge SAX. Detection sensitivity was additionally assessed using different amounts of IgG digest. Our results collectively support the utility of XCharge SAX as a potential chromatographic material for ERLIC in glycopeptide isolation from biological samples.

## 2. Experimental

### 2.1. Materials and reagents

IgG, HRP, HSA, acetic acid, dithiothreitol (DTT), iodoacetic acid (IAA), and ammonium bicarbonate ($NH_4HCO_3$) were ordered from Sigma (St. Louis, MO). Acetonitrile (ACN) and ammonium acetate ($CH_3COONH_4$) were products of Merck (Darmstadt, Germany). GELoader tips were purchased from Eppendorf (Hamburg, Germany). Sequencing-grade-modified trypsin was obtained from Promega (Madison, WI). Formic acid (FA) was ordered from Acros (Geel, Belgium). XCharge SAX (5 μm, 100 Å) and XAqua $C_{18}$ (5 μm, 100 Å) were obtained from Acchrom (Acchrom Ltd., Beijing). Water used in this study was purified by the Milli-Q system (Millipore, Bedford, MA). Human normal serum ($n = 1$, female, age 49) was provided by the Second Affiliated Hospitals of Dalian Medical University according to the Institutional Review Broad (IRB) approval.

### 2.2. Protein digestion

About 1 mg protein (model protein or human serum protein) was denatured in urea (8 M) in $NH_4HCO_3$ solution (50 mM, 100 μl) for 3 h. The denatured protein was reduced by DTT (50 mM, 4 μl) for 2 h at 37 °C and subsequently carboxymethylated with IAA (50 mM, 5 μl). The obtained solution was incubated in the dark for 30 min and then diluted tenfold with $NH_4HCO_3$ (50 mM) buffer. Finally, the diluted solution was mixed with trypsin at an enzyme/substrate ratio of 1:25 (w/w) and incubated for 16 h at 37 °C.

### 2.3. Purification of glycopeptides using XCharge SAX

An ACN slurry containing XCharge SAX (about 1 mg) was pushed into the GELoader tip. The packed microcolumn was washed with 10 mM $CH_3COONH_4$ in ACN/$H_2O$/acetic acid (50/50/0.1, 90 μl) and equilibrated with 10 mM $CH_3COONH_4$ in ACN/$H_2O$/acetic acid (70/30/0.1, 90 μl). Tryptic digest was subsequently redissolved in 10 mM $CH_3COONH_4$ in ACN/$H_2O$/acetic acid (70/30/0.1) and loaded onto the microcolumn. Non-glycosylated peptides were washed out with 10 mM $CH_3COONH_4$ in ACN/$H_2O$/acetic acid (70/30/0.1, 100 μl) and 10 mM $CH_3COONH_4$ in ACN/$H_2O$/acetic acid (60/40/0.1, 80 μl). The glycopeptide fraction was eluted with 10 mM $CH_3COONH_4$ in ACN/$H_2O$/acetic acid (50/50/0.1) and directly analyzed on a mass spectrometer. The enrichment conditions described above were the optimal ones, an alternative can be found in the caption of Fig. 1.

### 2.4. Desalting

ACN slurry containing $C_{18}$ material was packed into the GELoader tip. The obtained microcolumn was washed with ACN/$H_2O$/FA (50/50/0.1, 90 μl) and equilibrated with $H_2O$/FA (100/0.1, 90 μl). Then tryptic digest was loaded onto the microcolumn. The column was washed with $H_2O$/FA (100/0.1, 45 μl) to remove salt. Peptides were eluted with ACN/$H_2O$/FA (50/50/0.1).

### 2.5. Mass spectrometric analysis

The quadruple time-of-flight (Q-TOF) mass spectrometer (Waters, Manchester, UK) used in this study was coupled to a nanoACQUITY$^{TM}$ UltraPerformance LC (Waters, Manchester, UK). The nano-ESI source was operated under positive ion mode with nanospray voltage at 2.3 kV. MS data was acquired at $m/z$ 500–2000 for MS analysis and $m/z$ 100–2000 for MS/MS analysis. The collision energy in MS/MS analysis was 40 eV.

The Axima MALDI-QIT-TOF mass spectrometer (Shimadzu Corp., Kyoto, Japan) used in this study was operated in the positive ion and reflectron mode, and argon was used as the collision gas. One microlitre of analyte was spotted onto the MALDI plate and dried at room temperature. Then one microlitre of 2,5-dihydroxybenzoic acid (10 mg mL$^{-1}$ in 0.1% TFA, 49.9% $H_2O$ and 50% ACN) was applied to the target and dried.

## 3. Results

### 3.1. Optimization of enrichment conditions

Selection of the appropriate acid is crucial to adjust electrostatic interactions. In the present study, formic and acetic acid were added as mobile phase modifiers, respectively. The well-characterized glycoprotein, IgG, was used as the model glycoprotein. All loading buffers contained 70% ACN to maintain the hydrophilic interaction property of XCharge SAX. Following the addition of 0.1% formic acid to the mobile phase, peptides from the tryptic digest of IgG were not effectively retained on XCharge SAX, and glycopeptides co-eluted with non-glycosylated peptides (data not shown). This result is attributed to the fact that electrostatic repulsion is significantly stronger than hydrophilic interactions under such conditions. To reduce electrostatic repulsion, formic acid was replaced with 0.1% acetic acid. However, retention of peptides remained weak (data not shown). Next, ammonium acetate was added to the mobile phase for shielding the quaternary amine on the XCharge SAX surface, with the aim of further reducing electrostatic repulsion. When the mobile phase contained 10 mmol ammonium acetate, some or most non-glycosylated peptides were efficiently separated from the glycopeptide fraction (Fig. 1A). In total, 22 IgG glycopeptides were detected from mass spectra based on calculated $m/z$ values (SI Table 1). The results obtained with other concentrations of ammonium acetate (20 and 50 mmol) were analogous to those shown in Fig. 1B. Accordingly, 10 mmol ammonium acetate was selected as the optimal salt concentration. Optimization of acetic acid concentrations in the mobile phase was carried out to adjust the ionization state of peptides at a fixed salt concentration of 10 mmol. Upon addition of 1% acetic acid to the mobile phase, non-glycosylated peptides, e.g., 1219.5543 (2+), 1273.1611 (2+), dominated the mass spectra, and only sixteen glycopeptides were identified (Fig. 1B). A