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RESEARCH PAPER

Hydrophilic interaction liquid chromatography in the separation of glycopeptides and their isomers

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Abstract

The analysis of intact glycopeptides is a challenge because of the structural variety of the complex conjugates. In this work, we used separation involving hydrophilic interaction liquid chromatography using a superficially porous particle HALO® penta-HILIC column with tandem mass spectrometric detection for the analysis of *N*-glycopeptides of hemopexin. We tested the effect of the mobile phase composition on retention and separation of the glycopeptides. The results indicated that the retention of the glycopeptides was the combination of partitioning and adsorption processes. Under the optimized conditions, our HILIC method showed the ability to efficiently separate the glycoforms of the same peptide backbone including separation of the isobaric glycoforms. We achieved efficient separation of core and outer arm linked fucose of bi-antennary and tri-antennary glycoforms of the SWPAVGNCSSALR peptide and bi-antennary glycoform of the ALPQPQNVTSLLGCTH peptide, respectively. Moreover, we demonstrated the separation of antennary position of sialic acid linked via α2-6 linkage of the monosialylated glycopeptides. Glycopeptide isomers are often differentially associated with various biological processes. Therefore, chromatographic separation of the species without the need for an extensive sample preparation appears attractive for their identification, characterization, and reliable quantification.

Keywords Glycoproteomics · Glycopeptides · Hemopexin · Hydrophilic interaction liquid chromatography · LC-MS/MS

Introduction

Glycosylation plays an important role in many biological processes such as cell-cell recognition, cell adhesion, immune defense, and protein expression and folding [1]. Moreover, alteration of glycosylation has been found to be related to several diseases [1–4]. Despite the recent innovations of bioanalytical techniques [5], analysis of glycoproteins is still a challenge mainly because of their micro- and macro- heterogeneity [6]. Mass spectrometry (MS) is a standard technique of glycoproteomic analysis which can provide rich structural information. Specific fragmentation features have been reported for anionic glycans, enabling the distinction between isomeric structures [7, 8]. However, positive ionization mode of MS usually does not allow comprehensive characterization of isomeric glycopeptide structures due to similar fragmentation patterns using collision-induced dissociation [9, 10]. Therefore, it is essential to connect MS with chromatographic separation to achieve efficiently resolution of the analytes and to allow their quantification in complex samples [6, 11, 12]. Reversed-phase (RP) chromatography is the method of choice for proteomic experiments but typically does not adequately resolve isobaric glycoforms of glycopeptides [13]. An alternative chromatographic mode for separation of glycopeptides is hydrophilic interaction liquid chromatography (HILIC) which is able to separate glycopeptides that are inadequately resolved in RP-LC or those that may exhibit inadequate selectivity differences [14–16]. HILIC has also demonstrated the ability to separate glycans that differ only in branching and/or

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linkage position, both in their native and derivatized forms [11, 17]. Separation of these isomeric structures is important because changes in the abundance of these isomers can serve as a marker for monitoring of diseases [18]. Huang et al. showed HILIC separation of isomeric *N*-glycan structures, such as sialylated *N*-glycan isomers differing in α2-3 and α2-6 linkages, while these glycans remain attached to peptides [19]. Direct analysis of glycopeptides when glycans remain attached on peptides, which has the advantage that the glycosylation can be assigned to specific locations on the protein, is more difficult challenge than glycomic profiling [19–23].

In our recent study, we reported quantitative changes of the site-specific linkage isoforms of fucosylated hemopexin in liver disease by glycosidase assisted RP-LC/MS approach [24]. Moreover, we demonstrated that HILIC has the potential to separate fucose linkage isomers (core and outer arm) on bi-antennary glycoform of the SWPAVGDCSSALR peptide on HALO HILIC column [13]. In this paper, we investigated the separation potential of a relatively novel superficially porous particle (Fused-Core) Penta-HILIC column in the analysis of *N*-linked glycopeptides of hemopexin. We show that successful separation was achieved even in the nanoHILIC column format directly coupled to a mass spectrometer. We used exoglycosidase digestions to confirm the linkage of the separated *N*-glycopeptide isomers. Our results show that this stationary phase provides different selectivity; improved separation of *N*-linked glycopeptides and their isomeric structures are expected to enable analysis of hemopexin glycopeptide in the context of liver disease and other human pathologies.

Materials and methods

Chemicals

Acetonitrile (ACN, LC-MS grade), acetonitrile with 0.1% formic acid (LC-MS grade), water (LC-MS grade), water with 0.1% formic acid (LC-MS grade), ammonium bicarbonate (purity ≥ 99%, LC-MS grade), and iodoacetamide (purity ≥ 99%) were supplied by Sigma-Aldrich (St. Louis, MO). Dithiothreitol (ultrapure grade) and ammonium formate (LC-MS grade) were purchased from Thermo-Fischer Scientific (Waltham, MA). Trypsin Gold for MS was obtained from Promega (Madison, WI). α2-3,6,8,9 Neuraminidase, α2-3 neuraminidase, and α1-2 and α1-3,4 fucosidase with GlycoBuffer 1 (5 mM calcium chloride, 50 mM sodium acetate, pH 5.5) were purchased from New England BioLabs (Ipswich, MA). Hemopexin from human plasma was supplied by Athens Research and Technology (Athens, GA).

Sample preparation

Tryptic digestion of hemopexin was performed as follows: 20 μL (200 μg) of hemopexin dissolved in water was diluted in 180 μL of 50 mM ammonium bicarbonate. Disulfide bonds were reduced with 5 mM dithiothreitol at 60 °C for 60 min. Alkylation was carried out with 15 mM iodoacetamide for 30 min in the dark and excess iodoacetamide was neutralized with 5 mM dithiothreitol. Trypsin was added to the sample at an enzyme/protein ratio of 1:25 *w*/w and the sample was digested at 37 °C in Barocycler NEP2320 (Pressure BioSciences, South Easton, MA) for 1 h. The enzymatic digestion was stopped by heating at 99 °C for 10 min. Trypsin digest was desalted using solid phase extraction (SPE) on a Sep-Pak Vac C18 cartridge (Waters. Milford). SPE procedure was as follows: condition step, 2 mL of 100% acetonitrile and 2 mL of 1% acetic acid; loading step; washing step, 1 mL of 1% acetic acid; and elution step, 0.5 mL of 65% acetonitrile. Eluate was evaporated using a vacuum concentrator (Labconco, Kansas City, MO) and reconstituted in 100 μL of 0.1% formic acid in 2% acetonitrile. Fifty microliters of the sample was evaporated and desialylated by the addition of 82 μL of water, 10 μL of GlycoBuffer 1, and 8 μL of α2-3,6,8,9 neuraminidase overnight at 37 °C and then desalted by SPE, evaporated, and reconstituted in a solution of 0.1% formic acid in 2% acetonitrile to the final concentration of 1 μg/μL. Fifty microliters of the desialylated sample was evaporated and reconstituted in 80 μL of water, mixed with 10 μL of GlycoBuffer 1, and digested with 5 μL of each fucosidase overnight at 37 °C and then cleaned by SPE, evaporated, and reconstituted in a solution of 0.1% formic acid in 2% acetonitrile to the concentration of 0.5 μg/μL. Two aliquots (20 μL) of the tryptic digest were desialylated by α2-3,6,8,9 neuraminidase and α2-3 neuraminidase, respectively, to distinguish sialic acid linkages. An aliquot was evaporated and reconstituted in 86 μL of water, mixed with 10 μL of GlycoBuffer 1, and digested with 4 μL of neuraminidase overnight at 37 °C and then cleaned by SPE, evaporated, and reconstituted in a solution of 0.1% formic acid in 2% acetonitrile to the concentration of 0.1 μg/μL. Samples were diluted to the concentration of 0.02 μg/μL by sample solvent consisted of 0.1% formic acid in 80% acetonitrile.

Instrumentation and the experimental conditions

Quality control of the hemopexin digests was carried out on an Orbitrap Fusion Lumos (Thermo Scientific) coupled to Dionex 3500 RSLC-nano-LC System (Thermo Scientific). The mobile phases consisted of 0.1% formic acid in 2% acetonitrile (solvent A) and 0.1% formic acid in 100% acetonitrile (solvent B). Digests were separated using a 90-min gradient ([(min)/% B] was 0/1, 60/50, 70/99, 75/99, 80/1, 90/1) on 150 mm × 75 μm C18 pepmap column at a flow rate of

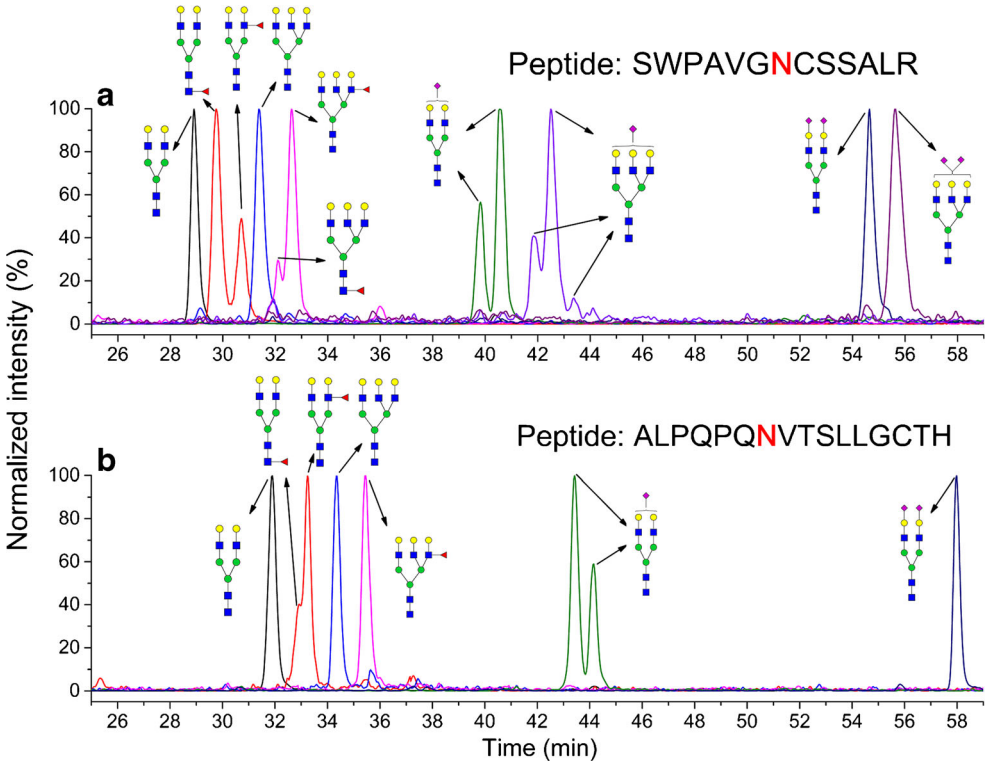

Fig. 1 Overlay of the normalized SRM chromatograms of all the analyzed glycoforms of the SWPAVGDCSSALR (**a**) and ALPQPQNVTSLLGCTH (**b**) peptides of hemopexin under the optimized HILIC conditions. Symbols: blue square, *N*-acetylglucosamine (GlcNAc); yellow circle, galactose (Gal); red right-pointing triangle, fucose (Fuc); green circle, mannose (Man); purple diamond, sialic acid (SA)

300 nL/min and analyzed in a data-dependent mode. All subsequent chromatographic measurements were carried out on a Tempo Capillary LC (Eksigent, Framingham, MA) interfaced with 6500 Q-TRAP (AB Sciex, Framingham, MA). The Analyst software (Sciex, Framingham, MA) was used for data acquisition. PeakView software (Sciex, Framingham, MA) was used to process LC-MS data. Glycopeptides were separated using a 150 mm × 75 μm HALO® penta-HILIC column packed with 2.7-μm-diameter superficially porous particles (Advanced Materials Technology, Wilmington, DE, USA). The flow rate of the mobile phase was maintained at 400 nL/min. The injection volume was 1 μL, samples were thermostated at 15 °C, and column temperature was at a laboratory temperature of 20 °C due to the absence of a column oven on this nanoLC-MS system. Several gradient programs were tested for the separation of glycopeptides. The optimized gradient program [(min)/% B] was 0/85, 40/60, 60/30, 65/30, 67/85, and 85/85. MS analysis was performed in the selected reaction monitoring (SRM) mode. The SRM transitions of targeted glycopeptides with collision energies and declustering potentials are listed in the Electronic Supplementary Material (ESM) Table S1. The ion source was set as follows: curtain gas, 20; ion spray voltage, 2300 V; ion source gas, 11; interface heater temperature, 150 °C; entrance potential, 10 V; and collision exit potential, 13.

For this paper, glycans are named using the following nomenclature: A2G2 means bi-antennary glycan terminated with two galactoses, A3G3 tri-antennary glycan terminated with three galactoses, F fucose, and S sialic acid. Gaussian smoothed SRM chromatograms were exported as text files to OriginPro 8.5.0 (OriginLab Corporation, Northampton, MA) for the visualization presented in figures. Each SRM chromatogram was normalized to the target glycopeptide and all target glycopeptide SRM chromatograms were overlaid and zoomed to the appropriate retention time window.

Results and discussion

HILIC separation of glycopeptides

Different HILIC stationary phases can provide different selectivity and efficiency in the separation of polar analytes [14]. Our previous work has shown a good potential of the bare silica stationary phase of HALO HILIC column in glycopeptide separation [13]. Encouraged by these results, we tested in the present study the separation ability of HALO® penta-HILIC stationary phase containing five hydroxyl groups on the bonded ligand. We selected two glycopeptides from hemopexin, SWPAVGNCSSALR and ALPQPQNVTSLLGCTH, occupied by different *N*-glycans [24]. Figure 1 shows the HILIC separation of *N*-glycoforms of the studied peptides under the optimized gradient conditions. Glycans attached to the peptides have a significant effect to HILIC retention. Glycopeptides show stronger HILIC retention with an increasing number of sugar units (increasing

Table 1 Chromatographic parameters (t_R, retention time; R_s, resolution) for HILIC separation of all the studied glycoforms of the SWPAVGNCSSALR and ALPQPQNVTSLLGCTH peptide of hemopexin under the optimized gradient elution with different composition of mobile phase

Analyte	MP1 t_R/R_s	MP2 t_R/R_s	MP3 t_R/R_s	MP4 t_R/R_s
SWPAVGNCSSALR				
A2G2	28.89/0.82	48.35/0.68	49.33/0.69	48.46/0.62
A2G2F1 (core)	29.75/0.70	49.29/0.32		49.37/0.29
A2G2F1 (outer arm)	30.71/0.64	49.74/0.49	50.40/–[a]	49.92/0.42
A3G3	31.38/0.63	50.27/–	ND	50.36/–
A3G3F1 (core)	32.11/0.37	ND	ND	ND
A3G3F1 (outer arm)	32.62/5.31	ND	ND	ND
A2G2S1 (isomer 1)	39.84/0.71			
A2G2S1 (isomer 2)	40.57/1.03	52.01/0.69[b]	51.98/0.66[b]	52.25/0.70[b]
A3G3S1 (isomer 1)	41.86/0.44			
A3G3S1 (isomer 2)	42.50/0.78			
A3G3S1 (isomer 3)	43.37/11.46	53.23/1.06[b]	53.13/0.95[b]	53.36/0.99[b]
A2G2S2	54.66/0.69	55.30/–[c]	54.17/–[c]	55.42/–[c]
A3G3S2	55.64/–	54.81/0.32[c]	53.66/0.33[c]	54.88/0.30[c]
ALPQPQNVTSLLGCTH				
A2G2	31.89/0.90	47.97/0.83	48.88/–	48.03/0.78
A2G2F1 (core)	32.90/0.26		ND	
A2G2F1 (outer arm)	33.24/0.98	49.28/0.74[a]	ND	49.31/0.80[a]
A3G3	34.36/1.02	50.27/–	51.01/–	50.39/–
A3G3F1	35.45/7.01	ND	ND	ND
A2G2S1 (isomer 1)	43.44/0.67			
A2G2S1 (isomer 2)	44.15/12.28	51.42/2.48[b]	51.36/2.19[b]	51.59/2.53[b]
A2G2S2	57.97/–	54.81/–	53.62/–	54.82/–

MP1 solvent A, 0.1% HCOOH in 2% ACN, solvent B, 0.1% HCOOH in 100% ACN; *MP2* solvent A, 20 mM $HCOONH_4$, pH 4.00 in 2% ACN, solvent B 100% ACN; *MP3* solvent A, 40 mM $HCOONH_4$, pH 4.00 in 2% ACN, solvent B 100% ACN; *MP4* solvent A, 20 mM $HCOONH_4$, pH 6.50 in 2% ACN, solvent B, 100% ACN, *ND* not detected analyte

[a] Coelution of glycopeptides containing core and outer arm fucose in one peak

[b] Coelution of isomers in one peak

[c] Reversed elution order of A2G2S2 and A3G3S2

hydrophilicity). This retention behavior is typical for HILIC separation of glycans and glycopeptides [21–23, 25]. Compared with HALO HILIC column [13], HALO® penta-HILIC column provides higher selectivity and better resolution for most glycopeptides (selectivities and resolutions are shown in ESM Table S2). Interestingly, sialic acid in glycopeptide structure significantly prolonged retention. We speculate that five hydroxyl groups on the bonded ligand of HALO® penta-HILIC column could provide stronger hydrogen bonding with carboxylic acid group of sialic acids resulting in higher retention. Although the retention of studied glycopeptides is mainly driven by the composition of glycans, peptide backbone also plays an important role in HILIC retention. Glycoforms of SWPAVGNCSSALR peptide elute slightly earlier than glycoforms of ALPQPQNVTSLLGCTH peptide, which is in agreement with the estimation of the retention order of peptides. The retention was computed based on amino acid coefficients in a peptide retention model using HALO® penta-HILIC column proposed recently by Badgett et al. [26]. Figure 1 shows that our HILIC method was not able to baseline separate the glycoforms of both peptides. Using the proposed gradient HILIC method, A3G3 and A3G3F1 glycoforms of the SWPAVGNCSSALR peptide partially co-eluted with A2G2 and A2G2F1 glycoforms of the ALPQPQNVTSLLGCTH peptide.

Characterization of glycopeptide isomers by exoglycosidase digestion

As shown in Fig. 1, we obtained multiple isobaric peaks for SRM chromatograms of fucosylated and monosialylated glycoforms. A possible explanation of multiple peaks with the same SRM transitions is that our HILIC method can resolve isomeric structures of the studied glycopeptides. All

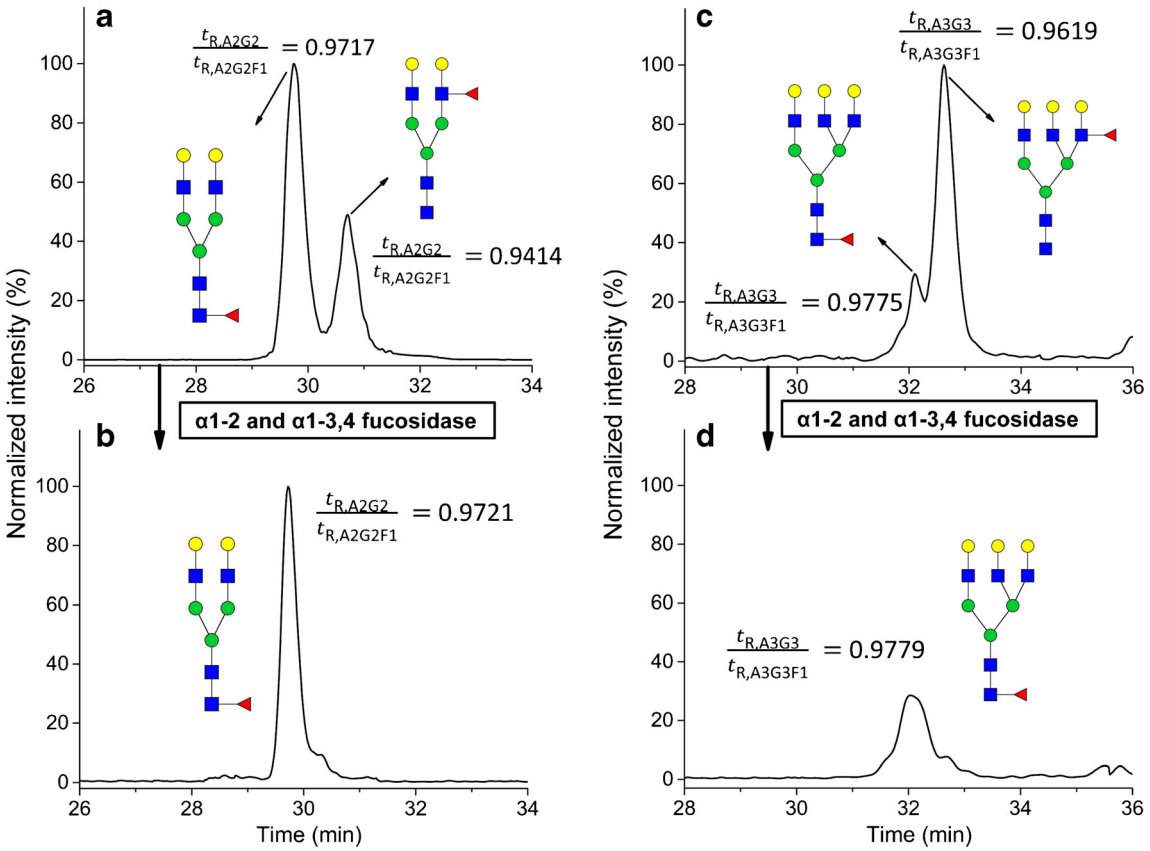

Fig. 2 Normalized SRM chromatograms of A2G2F1 (**a**, **b**) and A3G3F1 (**c**, **d**) glycoforms of the SWPAVGDCSSALR peptide of hemopexin under the optimized HILIC conditions before (**a**, **c**) and after (**b**, **d**) α1-2 and α1-3, 4 fucosidase treatment. For symbols, see Fig. 1

parent masses in multiple peak SRM chromatograms were confirmed using HR/MS analysis. SRM transitions of A2G2F1 and A3G3F1 glycoforms of the SWPAVGNCSSALR peptide have two peaks with the resolution of 0.70 and 0.37 (Fig. 1a and Table 1), respectively. SRM transition of A2G2F1 of the ALPQPQNVTSLLGCTH peptide shows two peaks with the resolution of 0.26 (Fig. 1b and Table 1). We assumed that the two peaks of monofucosylated glycopeptides correspond to the core and outer arm linked fucose. To confirm our hypothesis, we conducted the treatment with α1-2 and α1-3,4 fucosidase which cleave outer arm fucose of indicated linkage and leave intact the α1-6-linked core fucose. To avoid experimental variability in retention times, we used ratio of retention times (A2G2 or A3G3 glycoform/targeted peak) for reliable assignment of the peaks. The A2G2F1 glycoform of the SWPAVGNCSSALR peptide remained at the same retention time ratio and covered practically the same area following the α1-2 and α1-3,4 fucosidase digests (Fig. 2a, b). These facts revealed that the first peak corresponded to the core fucosylated glycopeptide and the second peak to the glycopeptide with outer arm fucose. With respect to A3G3F1 glycoform of the SWPAVGNCSSALR peptide, the first eluting peak was the core fucosylated glycopeptide and the second peak was the outer arm fucosylated glycopeptide (Fig. 2c, d). In the case of glycoforms of the ALPQPQNVTSLLGCTH peptide, we found that fucose resided on a core in the first eluting peak of the A2G2F1 glycoform, while the second peak corresponded to a glycan structure with outer arm fucosylation (ESM Fig. S1a, b). Our results further showed that fucose on A3G3F1 glycoform of the ALPQPQNVTSLLGCTH peptide was outer arm fucose (ESM Fig. S1c, d).

The SRM chromatograms shown in Fig. 1a depict two peaks for the A2G2S1 glycoform with the resolution of 0.71 and three peaks for the A3G3S1 glycoform with the resolution of 0.44 and 0.78. Figure 1b shows two peaks for the A2G2S1 glycoform with the resolution of 0.67. We expected that the main differences between multiple peaks of monosialylated glycoforms would be the linkage of sialic acid (α2-3 and α2-6). To confirm the separation of sialic acid linkage isomers, we carried out digestion using α2-3 neuraminidase which cleaves specifically α2-3-linked sialic acids. As shown in Fig. 3b–h, we did not observe any effect of α2-3 neuraminidase on the monosialylated glycoforms. After the treatment with α2-3,6,8,9 neuraminidase, all peaks disappeared from the chromatograms (Fig. 3c–i), except for A2G2S1 glycoform of the ALPQPQNVTSLLGCTH peptide because of incomplete

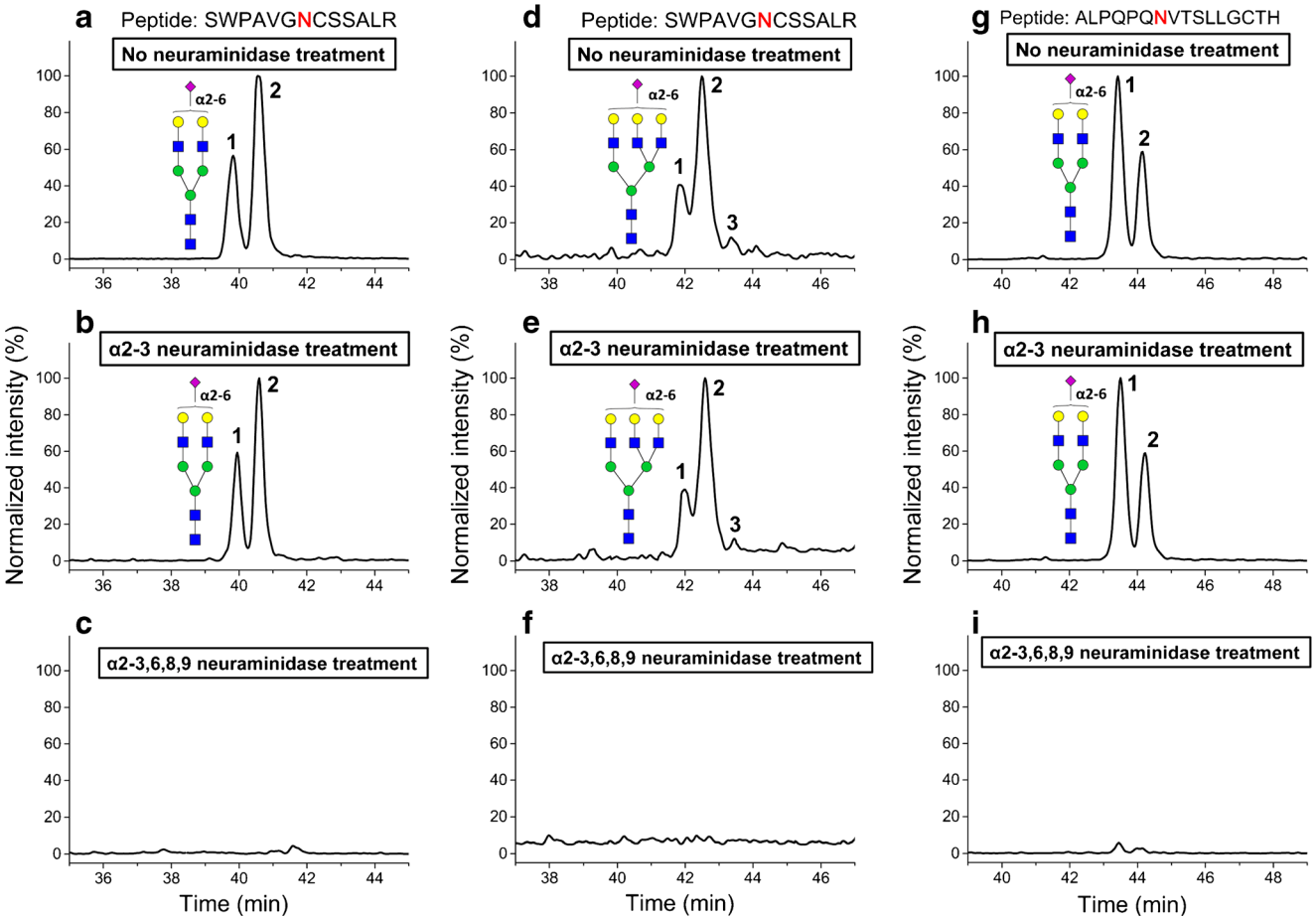

Fig. 3 Normalized SRM chromatograms of A2G2S1 (**a**–**c**) and A3G3S1 (**d**–**f**) glycoforms of the SWPAVGDCSSALR peptide and A2G2S1 (**g**–**i**) glycoform of the ALPQPQNVTSLLGCTH peptide of hemopexin under the optimized HILIC conditions before neuraminidase (**a**, **d**, **g**) after α2-3 neuraminidase (**b**, **e**, **h**) and after α2-3,6,8,9 neuraminidase (**c**, **f**, **i**) treatment. For symbols, see Fig. 1

digestion (Fig. 3i). Nonetheless, the results indicated that all sialic acids are attached via α2-6 linkage. We assume that separation of the A2G2S1 glycoform is caused by isomers that differ in a localization of sialic acid on different arms (the six-antenna or the three-antenna). Although the abundance of A3G3S1 glycoform of the SWPAVGNCSSALR peptide was very low, we observed three isomers of the α2-6 sialic acid (likely the unbranched six-antenna or the branched three-antenna with two- or four-branching). However, we cannot distinguish which peak corresponds to which isomer at this point. The separation of branch position of sialic acid of released glycans from glycopeptides using porous graphitized carbon as stationary phase was reported previously [8, 27]. To our knowledge, this is the first description of HILIC separation of the sialylated branches of glycopeptides. In addition, digestions with specific neuraminidase revealed that desialylated glycopeptides contain sialic acids linked through α2-6 linkage because α2-3 neuraminidase had no effect on the chromatographic peaks, whereas after α2-3,6,8,9 neuraminidase treatment, relevant chromatographic peaks disappeared.

The effect of mobile phase composition on the separation of glycopeptides

We tested the effect of different mobile phase composition on retention and separation of studied glycopeptides. We used ammonium formate buffer compatible with MS detection and soluble at high percentage of organic solvents. We tested two ionic strengths (20 and 40 mM) and two pH values (4.0 and 6.5 pH). Table 1 shows retentions and resolutions of the studied glycopeptides for four compositions of the mobile phase under the same gradient program (see "Instrumentation and the experimental conditions" section). We observed a significant increase of the retention times for neutral glycoforms with decreasing resolution for 20 mM ammonium formate buffer pH 4.0 in the mobile phase. For instance, we obtained increased retention times of A2G2 glycoform of the SWPAVGNCSSALR peptide from 28.89 to 48.35 min. We also observed suppressed ionization of all the analytes, thereby some minor glycoforms were not detected, e.g., A3G3F1 glycoform of both peptides. The obtained retention behavior correlates to observations showed in the

analysis of different polar analytes in HILIC mode when buffer present in the mobile phase of high organic solvent could increase water-rich layer adsorbed on the stationary phase resulting in stronger retention [28–30]. Accordingly, the retention of monosialylated glycoforms increased. However, the increase was not as dramatic as in the case of the neutral glycoforms. Surprisingly, we observed only slightly increased retention time of A2G2S2 glycoform and slightly decreased retention time of A3G3S2 glycoform of the SWPAVGNCSSALR peptide. Decrease of retention time was observed also for A2G2S2 glycoform of the ALPQPQNVTSLLGCTH peptide. Further, we observed the change in the elution order of A2G2S2 and A3G3S2 glycoforms of the SWPAVGNCSSALR peptide in comparison with 0.1% formic acid in the mobile phase. Ammonium formate in the mobile phase also changed the selectivity of separation as evidenced by glycoforms of the ALPQPQNVTSLLGCTH peptide that started to elute moderately before the same glycoforms of the SWPAVGNCSSALR peptide. Moreover, buffer in mobile phase caused approximately two times decreased resolution between the core and outer arm fucosylated glycoforms (A2G2F1) of the SWPAVGNCSSALR peptide and co-elution of the A2G2F1 positional isomers of ALPQPQNVTSLLGCTH peptide in one peak. All branched position isomers of glycoforms with sialic acid (A2G2S1 and A3G3S1), which were partially separated in mobile phase containing 0.1% formic acid, were not resolved and co-eluted as one peak. Higher ionic strength of the buffer (40 mM) slightly increased retention times of the neutral glycoforms and moderately decreased retention times of the sialylated glycoforms. In addition, it caused co-elution of all isomeric structures which were partially separated in 0.1% formic acid. No significant effect of pH on glycopeptide retention and separation was observed on HALO® penta-HILIC column. Although it is generally accepted that the retention in HILIC is mainly driven by partitioning, we assume that the retention behavior mentioned above is caused by a combination of partitioning and adsorption processes. These processes can play an important role in retention and separation of glycopeptides in HILIC, especially for the separation of isomeric structures. As a future plan, we plan to perform a detailed study to clarify the retention mechanism of glycopeptides in the HILIC mode.

Conclusion

This work expands the application of HILIC separation of glycopeptides. We optimized the separation of intact glycopeptides of hemopexin using a novel HALO® penta-HILIC stationary phase containing five hydroxyl groups on the bonded ligand. Under the optimized conditions, we obtained high separation efficiency of the glycopeptides and we documented increasing retention of glycopeptides with increasing number of monosaccharide units of the glycan moiety. Sialic acid addition in the glycan structure increased glycopeptide retention times significantly. The effect of mobile phase composition on retention and separation of the glycopeptides were tested. The retention of glycopeptides was affected mainly by the replacement of 0.1% formic acid by ammonium formate. The obtained results indicate a multimodal retention mechanism. The composition of mobile phase offers the opportunity to efficiently tune the separation for an optimal retention and separation of glycopeptides. Our HILIC method showed the ability to separate some but not all isomeric glycopeptides; we carried out exoglycosidase digestions to elucidate in part the *N*-glycopeptide isomers. We observed the separation of the core and outer arm linked fucose to A2G2 and A3G3 glycoforms of the SWPAVGNCSSALR peptide and A2G2 glycoform of the ALPQPQNVTSLLGCTH peptide, respectively. The separation of α2-6 sialylated *N*-glycan branches was obtained for mosialylated glycopeptides. Therefore, our HILIC method provides a suitable way of direct analysis of the glycopeptides of hemopexin which could assist with linkage resolution in the context of monitoring of human diseases like liver fibrosis.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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