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Utilizing Ion-Pairing Hydrophilic Interaction Chromatography Solid Phase Extraction for Efficient Glycopeptide Enrichment in Glycoproteomics

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Glycopeptide enrichment is a prerequisite to enable structural characterization of protein glycosylation in glycoproteomics. Here we present an improved method for glycopeptide enrichment based on zwitter-ionic hydrophilic interaction chromatography solid phase extraction (ZIC-HILIC SPE) in a microcolumn format. The method involves TFA ion pairing (IP) to increase the hydrophilicity difference between glycopeptides and nonglycosylated peptides. Three mobile phases were investigated, i.e., 2% formic acid (defined as $IP_{2\%\ FA}$ ZIC-HILIC SPE), 0.1% TFA and 1% TFA (defined as $IP_{0.1\%\ TFA}$ and $IP_{1\%\ TFA}$ ZIC-HILIC SPE) all containing 80% acetonitrile. Samples of increasing complexities, i.e., digests of single glycoproteins, a five-glycoprotein mixture, and depleted plasma, were used in the study. The presence of TFA in the mobile phase significantly improved the glycopeptide enrichment for all complexities, as evaluated by enhanced glycopeptide detection using MALDI-TOF MS and RP-LC−ESI-MS/MS, e.g., the glycopeptide ion signals were increased by up to 3.7-fold compared to $IP_{2\%\ FA}$ conditions. The enhanced glycopeptide detection was promoted by a substantial depletion of nonglycosylated peptides, offering an almost complete isolation of IgG glycopeptides using a single SPE enrichment step and a reduction from 711 nonglycosylated peptides observed in the $IP_{2\%\ FA}$ ZIC-HILIC SPE retained plasma fraction, to only 157 and 97 when 0.1% and 1% TFA was used in the mobile phase. In conclusion, this systematic study has shown that TFA-containing mobile phases increase glycopeptide enrichment efficiency considerably for a broad range of sample complexities when using ZIC-HILIC SPE.

Protein glycosylation is a common post-translational modification, and the attachment of glycans is known to affect the function of proteins.[1−5] Complete structural characterization of glycoproteins involves (i) identification of the glycoprotein, (ii) determination of the glycosylation site and the glycan occupancy, and (iii) characterization of the complete set of glycan structures including their monosaccharide composition, linkages, and branch points as well as their relative abundances. Because of the substantial heterogeneity of protein glycosylations, the full characterization of even purified glycoproteins can be challenging. However, the large-scale investigation of the glycoproteome, known as glycoproteomics, represents a much greater challenge. One of the most prominent challenges associated with glycoproteomics is the substoichiometry of the glycopeptides relative to nonglycosylated peptides as well as their relatively low ionization efficiency, resulting in glycopeptide suppression during MS.[6,7] This can be alleviated by selective isolation of glycopeptides from peptide mixtures prior to MS detection. Thus, there is a growing interest in designing robust glycopeptide enrichment techniques to advance the field of glycoproteomics.

Among the various techniques used for glycopeptide isolation, i.e., lectins,[8,9] graphitized carbon,[10,11] titanium dioxide,[12] and hydrazine chemistry,[13,14] which are described in a recent review,[15] hydrophilic interaction chromatography (HILIC) has been used

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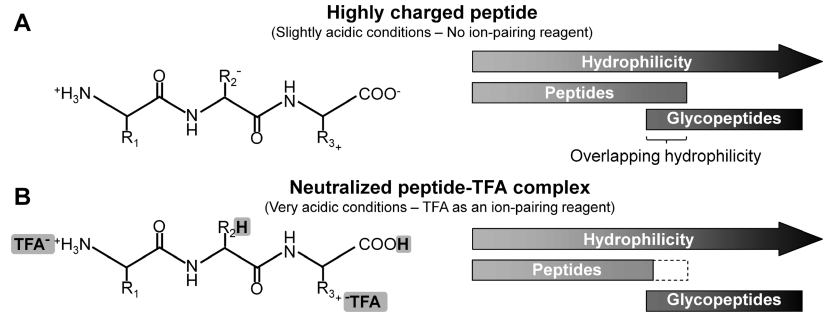

Figure 1. The proposed impact of TFA as an IPR on the hydrophilicity difference between glycopeptides and nonglycosylated peptides. Generally, glycopeptides represent a more hydrophilic population of protein digests due to the extensive hydrogen bonding potential enabled by the numerous hydroxyl groups of the attached glycans. (A) Without ion pairing, charged nonglycosylated peptides can be very hydrophilic and rival glycopeptides in terms of hydrophilicity. This overlapping subpopulation of nonglycosylated peptides will be coenriched with the glycopeptides in HILIC SPE. (B) Low-pH mobile phases containing IPRs such as TFA will neutralize charged peptides due to the formation of ion-pairs with positive charges of the peptides (N-termini, lysine and arginine residues), and the negative charges (C-termini, aspartate and glutamate residues) will be protonated. Charge removal inhibits the ability of peptide moieties to form electrostatic interactions with the stationary phase and generally reduces the hydrophilicity by lowering the hydrogen bonding potential. Because of their many hydroxyl groups, the glycopeptides are relatively less affected in terms of hydrophilicity compared to the nonglycosylated peptides, creating a larger hydrophilicity difference. In theory, this decreases the proportion of nonglycosylated peptides that rival glycopeptides with regard to hydrophilicity, resulting in fewer nonglycosylated peptides being coenriched when using HILIC SPE.

increasingly to separate and enrich for glycopeptides.[16−25] HILIC is a variant of normal phase (NP) LC in which aqueous/organic mobile phases and polar stationary phases are utilized. This creates roughly the opposite retention pattern of reversed phase (RP) LC.[26] In contrast to NP LC, where analytes are retained by adsorption to the stationary phase, the retention in HILIC is generally a result of hydrophilic partitioning of the analyte to the water enriched layer surrounding the polar stationary phase through hydrogen-bonding, and to a lesser extent due to contributions from electrostatic interactions.[27] However, the exact retention mechanisms of HILIC are largely determined by the individual stationary and mobile phases. The ability of HILIC to discriminate glycopeptides from nonglycosylated peptides in mixtures arises from the fact that glycopeptides in general are more hydrophilic due to the extensive hydrophilic contribution from the associated glycan moiety. In contrast to lectins, which have affinities for certain glycan determinants, structures, or families, HILIC is, in this sense, unbiased toward the glycoproteome, as it separates according to hydrophilicity and not affinity. However, it is expected that HILIC favors the N-glycoproteome over the O-glycoproteome, since N-linked glycans tend to be larger and consequently more hydrophilic than the O-linked glycans. Another significant advantage of using HILIC for glycopeptide purification is that it does not involve irreversible chemical/enzyme induced alterations of the peptide or glycan moieties, enabling the analysis of native glycopeptides as well as released glycans and deglycosylated peptides following enrichment. This favors HILIC over other enrichment techniques such as hydrazine chemistry.

We have previously reported the use of HILIC solid phase extraction (SPE) for the enrichment of glycopeptides from peptide mixtures.[21] In that study, zwitterionic (ZIC) HILIC resin was packed in a microcolumn format, and the mobile phase consisted of 80% acetonitrile (ACN) in 2% aqueous formic acid (FA). Although this proved quite efficient (reproducible, sensitive, and specific) for the enrichment of glycopeptides, hydrophilic nonglycosylated peptides were observed to be coenriched in some samples, in particular from complex peptide mixtures. This coenrichment, which arises from a hydrophilic overlap between glycopeptides and nonglycosylated peptides (Figure 1A), often results in suppression of the glycopeptide ionization during MS analysis.[6,7] It was recently reported that the addition of a more hydrophobic ion pairing reagent (IPR) than FA to the mobile phase such as trifluoroacetic acid (TFA) can act to decrease this overlap.[28,29] This was demonstrated by the improved separation of glycopeptides and nonglycosylated peptides using online HILIC ESI MS. Supposedly, ion pairing (IP) affects the proportional hydrophilicity of nonglycosylated peptides to a greater extent than the glycopeptides (hydroxyl groups of the glycan are not affected), separating the two groups in terms of hydrophilicity (Figure 1B).

This observation inspired us to investigate whether TFA in the mobile phase improves the glycopeptide enrichment efficiency of ZIC-HILIC SPE compared to the conditions previously used. The offline SPE enrichment using microcolumns has several advantages over online HILIC approaches, mainly due to the

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highly flexible nature of the microcolumns, the rapidity of the enrichment, and high-throughput potential through multiplexing, i.e., in quality control applications. Moreover, its compatibility with the traditional online RP-LC−ESI MS setups, which are used routinely in many proteomics laboratories, makes it fit readily into the existing workflows.

We show that the addition of 0.1% and 1% TFA as an IPR to the mobile phase dramatically improves the enrichment efficiency of glycopeptides when using ZIC-HILIC SPE microcolumns compared to the previously used mobile phase containing 2% FA. This was shown for enzymatically digested samples of different complexities, i.e., single glycoproteins, a five-glycoprotein mixture, and depleted plasma. Almost complete isolation of glycopeptides from single glycoprotein digests was obtained, as well as considerably fewer coenriched "contaminating" nonglycosylated peptides from more complex samples, resulting in greater glycopeptide ion intensity during analysis. The enrichment efficiencies were evaluated based on data obtained from MALDI-TOF MS and RP-LC−ESI-LTQ-Orbitrap MS/MS analyses.

EXPERIMENTAL SECTION

Materials and Reagents. The following glycoproteins were purchased from Sigma: ribonuclease B (RNase B) (catalog no. R-7884, P61823), bovine fetuin (catalog no. 2379, P12763), human serotransferrin (catalog no. T-4382, P02787), chicken ovalbumin (catalog no. A-5503, P01012), and bovine α-1-acid glycoprotein (catalog no. G-3643, Q3SZR3). Murine immunoglobulin G-1 (IgG1, P01868) was purchased from Antibody Solutions (Mountain View, CA). Plasma samples were kindly provided by Professor Søren Cold (Odense University Hospital, Odense, Denmark). Trifluoroacetic acid (TFA), formic acid (FA), 1,4-dithiothretiol (DTT), and iodoacetamide (IAA), all of highest possible grade, were from Sigma. Sequence-grade trypsin was purchased from Promega (Madison, WI). ZIC-HILIC SPE resin (10 μm, 200 Å) was kindly provided by Merck SeQuant (Umeå, Sweden), and polyhydroxyethyl aspartamide (PolyHE A) (12 μm, 100 Å) was purchased from PolyLC (Columbia, MD). Ultrahigh-quality water was used for all experiments.

Reduction, Alkylation, and Tryptic Digestion. Glycoproteins were dissolved in water (1 nmol/μL), mixed in equimolar amounts (for the five glycoprotein mixture), and reduced by addition of DTT to a final concentration of 40 mM and incubation for 30 min at 56 °C. The glycoproteins were alkylated prior to digestion by the addition of IAA to a final concentration of 100 mM and incubation for 30 min in the dark at room temperature. To quench the reaction, 1 μL of 1 M DTT was added after which NH_4HCO_3 (pH 7.8) was added to a final concentration of 50 mM. Trypsin (5%, w/w) was added, and the mixture incubated for at least 12 h at 37 °C.

A volume of 37.5 μL of plasma (pooled from four healthy individuals) was depleted for the six most abundant proteins (albumin, IgG, IgA, transferrin, haptoglobulin, and α-1 antitrypsin) using a Multiple Affinity Removal LC Column (Hu-6HC) and Starter Reagent Kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's specifications. The depleted plasma was resuspended in 100 μL of 50 mM triethylammonium bicarbonate (TEAB) using a Microcon Ultracel YM-10 spin filter (10 kDa cutoff) (Millipore, Billerica, MA). While still in the spin filter, the plasma sample was reduced by addition of DTT to a final concentration of 10 mM and incubation for 40 min at 56 °C. Subsequently, alkylation was performed by the addition of IAA to a final concentration of 50 mM and incubation for 40 min in the dark at room temperature. The plasma protein concentration was determined using the Quant-iT protein assay kit (Molecular Probes/Invitrogen, Eugene, OR) on a Qubit fluorometer (Invitrogen) according to the manufacturer's specifications. For digestion, 2% (w/w) trypsin was added and the mixture incubated for 16 h at 37 °C. To remove trypsin, the resulting digest was spun through the filter using an additional 500 μL of 50 mM TEAB. The plasma digest was treated with alkaline phosphatase (1 U/20 μg) (Roche, Basel, Switzerland) for 2 h at 37 °C to dephosphorylate the digest. All digests were dried in a vacuum centrifuge, resuspended in the various HILIC mobile phases (see below for details on volumes, mobile phases, and sample amounts loaded), and applied to HILIC SPE microcolumns except for a fraction of the plasma sample, which was resuspended in 5% aqueous FA and applied to a Poros R3 microcolumn.

HILIC SPE Glycopeptide Enrichment and Sample Preparation. HILIC SPE microcolumns were prepared as previously described.[21] In brief, GELoader tips (Eppendorf, Hamburg, Germany) were constricted and HILIC SPE resin applied to generate column lengths of 20−25 mm for enrichments from digests of single glycoproteins (10 pmol of glycoprotein applied), 30−35 mm for enrichments from digests of the five glycoprotein mixture (5 × 10 pmol of glycoprotein applied), and 40−45 mm for enrichments from the plasma digest (5 μg applied). For column length to column volume conversion, please consult Thaysen-Andersen et al.[30] ZIC-HILIC or PolyHE A-HILIC resin was used to pack microcolumns. Three mobile phases (for sample loading and washing) were prepared (all v/v): (a) 2% FA, 18% H_2O, 80% ACN (pH 2.89), (b) 0.1% TFA, 19.9% H_2O, 80% ACN (pH 2.11), and (c) 1% TFA, 19% H_2O, 80% ACN (pH 1.61). All pHs reported were measured in ACN/H_2O with electrodes calibrated in water.[31] Following loading in a 10 μL sample volume, the microcolumns were washed twice with 10 μL of mobile phase. Elutions (the eluate is defined as the retained fraction) from the HILIC SPE microcolumns were performed with 10 μL of 5% aqueous FA and dried in a vacuum centrifuge. For MALDI-TOF MS, the dried fractions were resuspended in 2 μL of 5% aqueous FA and applied to a MALDI target. Subsequently, 1 μL of 2,5-dihydroxybenzoic acid (10 mg/mL in 0.1% TFA, 29.9% H_2O, and 70% ACN) was added and the mixture dried. For RP-LC−ESI-LTQ-Orbitrap MS/MS, the dried fractions were redissolved in 5 μL of 5% aqueous FA.

Microcolumns containing Poros R3 resin (hydrophobic material; particle size, 30 μm; pore size not declared; Applied Biosystems, Framingham, MA) were packed essentially as above. A fraction of the plasma digest was loaded onto an R3 microcolumn (5 μg for a 35 mm microcolumn length), washed twice in 10 μL of 5% aqueous FA, and eluted with 10 μL of 100% ACN. The eluate was dried and redissolved in 5 μL of 5% aqueous FA.

Mass Spectrometry. MALDI-TOF MS was performed using a Bruker Ultraflex (Bruker Daltonics, Bremen, Germany). Spectra

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were obtained in positive ionization mode using linear and reflector detection.

Peptide mixtures were separated using online RP nanoscale capillary LC and analyzed by ESI MS/MS. The experiments were performed with an EasyLC nanoflow HPLC (Proxeon Biosystems, Odense, Denmark) connected to an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with a nanoESI ion source (Proxeon Biosystems, Odense, Denmark). The chromatographic separation was performed on a 15 cm fused silica emitter (100 μm inner diameter) in-house packed with RP ReproSil-Pur C_{18}-AQ 3 μm resin (Dr Maisch GmbH, Ammerbuch-Entringen, Germany). Peptide mixtures were injected onto the column with a flow of 550 nL/min and subsequently separated with a flow of 250 nL/min. The gradient increased from 2% to 40% ACN in 0.1% FA in 78 min. The mass spectrometer was operated in data dependent mode to automatically switch between MS and MS/MS acquisition. Survey full scan MS spectra (from m/z 300 to 1800) were acquired in the Orbitrap with resolution $R = 60\,000$ at m/z 400 (after accumulation to a target value of 500 000 in the linear ion trap). The five most intense ions were sequentially isolated and fragmented in the linear ion trap using collision-induced dissociation at a target value of 10 000. Former target ions selected for MS/MS were dynamically excluded for 30 s. The general mass spectrometric conditions were as follows: spray voltage, 2.3 kV; no sheath and auxiliary gas flow; ion transfer tube temperature, 200 °C; collision gas pressure, 1.3 mTorr; normalized collision energy using wide-band activation mode; 35% for MS/MS. Ion-selection thresholds were 50 000 counts for MS/MS. An activation $Q = 0.25$ and activation time of 30 ms were applied in MS/MS acquisitions. Multiple enrichments of the digests were performed and analyzed using MALDI-TOF MS to ensure that high analytical reproducibility of the enrichment method could be obtained.

Peptide Identification and Calculation of Their Hydrophilicity. LTQ-Orbitrap raw data files were converted to Mascot generic format (MGF) files using Proteome Discoverer, version 1.1 (Thermo Scientific, Franklin, MA) at default settings. These files were then searched against the Swiss-Prot 57.9 database for either bony vertebrates (80 308 sequences) (for the five glycoprotein mixture) or humans (20 407 sequences) (for the plasma sample) using Mascot, version 2.2 (Matrix Science, http://www.matrixscience.com/). For all samples, Mascot search parameters were as follows: trypsin, two missed cleavages, fixed carbamidomethylated cysteine residues, variable oxidation of methionine residues, monoisotopic, unrestricted protein mass, 10 ppm peptide mass tolerance, 0.8 Da fragment mass tolerance, and ESI-TRAP instrumentation. For the five-glycoprotein mixture, peptide identifications belonging to the five known glycoproteins were considered to be true identifications. For plasma samples, peptides belonging to proteins with a Mascot score >100 were considered to be true identifications. The relatively high cutoff of the Mascot score was used to increase the confidence of the peptide identification and eliminate false positive identifications. From these identifications, the sum of queried peptide matches, unique peptide identifications, and the protein emPAI were determined. The hydrophilicities of the identified peptides were manually calculated using the Whimley−White hydrophilicity scale.[32] The Gibbs free energies of the transfer from *n*-octanol to water (ΔG_{octw}) were determined for the individual peptides. For the calculation, the neutral values for aspartate and glutamate residues (Asp^0 and Glu^0) and the charged values for histidine, lysine, and arginine residues (His^+, Lys^+, and Arg^+) were used. A value of −7.9 kcal/mol was used for the C- and N-termini of each peptide.[29,33,34] The hydrophilic average of the peptides was determined by calculating the mean of their individual hydrophilicities.

Glycopeptide Identification and Quantitation. Glycopeptide precursor MS/MS spectra were identified by manual inspection of the LTQ-Orbitrap raw data using the Xcalibur 2.1 qualitative browser (Thermo Scientific, Franklin, MA). Glycopeptide precursors were identified by the presence of at least one of the following oxonium ions in the MS/MS spectra: m/z 204.1 ($HexNAc_1$), m/z 292.1 ($NeuAc_1$), m/z 366.1 ($Hex_1HexNAc_1$), m/z 454.2 (Hex_1NeuAc_1), m/z 528.2 ($Hex_2HexNAc_1$), or m/z 657.2 ($Hex_1HexNAc_1NeuAc_1$) and a glycan sequence ladder consisting of at least two monosaccharides (singly, doubly, triply, or quadruply charged Hex, HexNAc, or NeuAc residues). The precursors were distributed evenly through the entire LC−MS run. Extracted ion chromatograms (XIC) were created for the entire set of glycopeptide precursors (217 precursors for the mixture of five glycoproteins and 600 precursors for plasma). In practice, this was done by making XICs of grouped precursors (50 precursors in each group of increasing m/z values) and summing the area from the groupings. XICs were created in the Xcalibur 2.1 qualitative browser from the Fourier transformed mass spectra based on base peak intensities for precursor intervals of $\pm m/z$ 0.01, using the Genesis peak algorithm. Precursors deviating less than this were grouped into the same interval to avoid an overlap. For each precursor value, the XIC was manually checked to ensure that the m/z predominantly corresponded to a glycopeptide. If significant additional chromatographic peaks corresponding to nonglycosylated peptides appeared in the XIC, the precursor m/z value was discarded. The chromatographic peak area from both the XIC and the total ion current (TIC) was determined by summing the signal from retention time 30−80 min for the plasma sample and 40−90 min for the five-glycoprotein mixture.

RESULTS

Design of Experiment. The glycopeptide enrichment using ZIC-HILIC SPE microcolumns was investigated using three mobile phases, all containing 80% ACN and an acidic modifier in an aqueous solvent: (a) 2% FA (defined as $IP_{2\%\ FA}$ ZIC-HILIC SPE), (b) 0.1% TFA, and (c) 1% TFA (defined as $IP_{0.1\%\ TFA}$ and $IP_{1\%\ TFA}$ ZIC-HILIC SPE, respectively). The enrichment efficiencies were determined using peptide mixtures of increasing complexities, i.e., digests of a single glycoprotein (murine IgG1), digests of a mixture of five glycoproteins (bovine pancreatic RNase B, chicken ovalbumin, human serotransferrin, bovine fetuin, and human α-1-acid glycoprotein), and a digest of plasma depleted for the six most abundant proteins, Figure 2.

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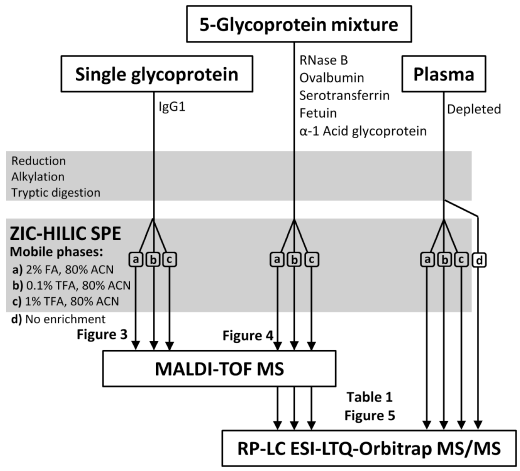

Figure 2. Experimental workflow. The impact of including an IPR in the mobile phase on the glycopeptide enrichment efficiency of ZIC-HILIC SPE was investigated in this study. Three mobile phases all containing 80% ACN were tested in parallel: (a) 2% FA (defined as $IP_{2\%\ FA}$ ZIC-HILIC SPE), (b) 0.1% TFA, and (c) 1% TFA (defined as $IP_{0.1\%\ TFA}$ and $IP_{1\%\ TFA}$ ZIC-HILIC SPE, respectively). The glycopeptide enrichments were tested on peptide mixtures of increasing complexity, i.e., digests of single glycoproteins, digests of a mixture of five glycoproteins, and a digest of depleted plasma. The enrichment efficiencies were evaluated qualitatively using MALDI-TOF MS, whereas quantitative measurements of the enrichment efficiencies were obtained using RP-LC−ESI-LTQ-Orbitrap MS/MS. (d) For comparison, a nonenriched (desalted with Poros R3) plasma peptide mixture was included in the ESI-LTQ-Orbitrap MS/MS analysis.

The glycopeptide enrichment efficiencies were evaluated qualitatively for the single glycoprotein and the mixture of five glycoproteins using MALDI-TOF MS and quantitatively for the five glycoprotein mixture and the plasma sample using RP-LC−ESI-LTQ-Orbitrap MS/MS. The glycopeptide ion intensities and the depletion of nonglycosylated peptides were used as measurements to evaluate the enrichment efficiencies.

Enrichment of Glycopeptides Derived from Single Glycoproteins. To enable easy interpretation, the initial enrichment experiments were performed on a peptide mixture derived from a single glycoprotein using MALDI-TOF MS detection to characterize the composition of the enriched fraction. IgG was chosen as the model glycoprotein, because of its limited number of well-characterized glycan structures attached to a known glycosylation site. Although the glycopeptide enrichment was made from a single glycoprotein digest, the resulting peptide mixture was relatively complex due to the large size of the glycoprotein. The tryptic IgG1 peptide mixture was split in three equal fractions, dried, and redissolved in the three mobile phases and applied to ZIC-HILIC SPE microcolumns. The retained fractions were eluted and analyzed by MALDI-TOF MS, Figure 3.

A considerable number of nonglycosylated peptides were coenriched in the $IP_{2\%\ FA}$ ZIC-HILIC SPE retained fractions, and the glycopeptide signals appeared with weak intensities in the mass spectrum, Figure 3A. In contrast, a marked increase in the glycopeptide intensities was observed in the $IP_{0.1\%\ TFA}$ and $IP_{1\%\ TFA}$ ZIC-HILIC SPE retained fractions, Figure 3B,C. This increase in glycopeptide detection, which was most prominent when using 1% TFA, was likely induced by the depletion of nonglycosylated peptides. This was substantiated by the presence of only a few coenriched nonglycosylated peptides in the $IP_{0.1\%\ TFA}$ and $IP_{1\%\ TFA}$ ZIC-HILIC SPE retained fractions.

The same trend was observed for other purified glycoproteins such as RNase B, which is a considerably smaller glycoprotein. Here, $IP_{0.1\%\ TFA}$ and $IP_{1\%\ TFA}$ ZIC-HILIC SPE generated an almost complete isolation of glycopeptides, whereas a number of nonglycosylated peptides were copurified using the $IP_{2\%\ FA}$ ZIC HILIC SPE (data not shown). Together, these data indicated that the addition of a relatively hydrophobic IPR such as TFA to the mobile phase was very beneficial for the glycopeptide enrichment.

Enrichment of Glycopeptides Derived from a Mixture of Five Glycoproteins. To increase sample complexity, a peptide mixture derived from the digestion of five glycoproteins, i.e., RNase B, ovalbumin, serotransferrin, fetuin, and α-1-acid glycoprotein was analyzed. Glycopeptide enrichments were performed with $IP_{2\%\ FA}$, $IP_{0.1\%\ TFA}$, and $IP_{1\%\ TFA}$ ZIC-HILIC SPE employing the same mobile phases as described above and using MALDI-TOF MS detection for the qualitative evaluation of the enrichment, Figure 4.

Numerous signals in the low mass region (m/z 1000−2500) were observed for the $IP_{2\%\ FA}$ ZIC-HILIC SPE retained fraction, Figure 4A, indicating the presence of nonglycosylated peptides. In contrast, these signals were depleted in the $IP_{0.1\%\ TFA}$ and $IP_{1\%\ TFA}$ ZIC-HILIC SPE retained fractions, where a collection of signals in the high mass region (m/z 3000−5000) appeared, Figure 4B,C. The majority of these signals corresponded to glycopeptides, which generally have higher molecular masses due to the mass contributions from the attached glycans. Since the molecular mass of the glycopeptides exceeded the limit for allowing MS/MS, the assignment of the individual signals were based on their molecular mass only as derived from the MALDI-MS detection. However, as the assigned species were identified in the subsequent RP-LC−ESI-LTQ-Orbitrap MS/MS experiment, the confidence in their presence was high. As expected, not all of the glycopeptides derived from the five-glycoprotein mixture were present in the MALDI-MS spectrum. For example, the RNase B glycopeptides were not detectable in the MALDI-MS analysis, although they were clearly present in the enriched mixture, as shown by the LC−ESI-MS analysis (see Figure S1 in the Supporting Information). This is due to differential ionization efficiencies and suppression effects between the enriched glycopeptides, which represent an inherent problem when using MALDI-MS to analyze mixtures of analytes. Taken together, these results yield another line of evidence of increased glycopeptide enrichment efficiency using $IP_{0.1\%\ TFA}$ and $IP_{1\%\ TFA}$ ZIC-HILIC SPE.

Reproducibility of IP ZIC-HILIC SPE. The reproducibility of the IP ZIC-HILIC SPE was addressed by performing multiple technical replicates of the enrichments with subsequent MALDI-MS detection. Specifically, three technical replicates were performed for $IP_{2\%\ FA}$, $IP_{0.1\%\ TFA}$, and $IP_{1\%\ TFA}$ ZIC-HILIC SPE enrichments from the digested IgG sample and five-glycoprotein mixture. Figures S2 and S3 in the Supporting Information present the technical replicates of the $IP_{1\%\ TFA}$ ZIC-HILIC SPE enrichments from IgG and the five-glycoprotein mixture, respectively, to allow visual comparison between the resulting mass spectra. The similarity between the spectra originating from the replicas confirmed the high reproducibility of the ZIC-

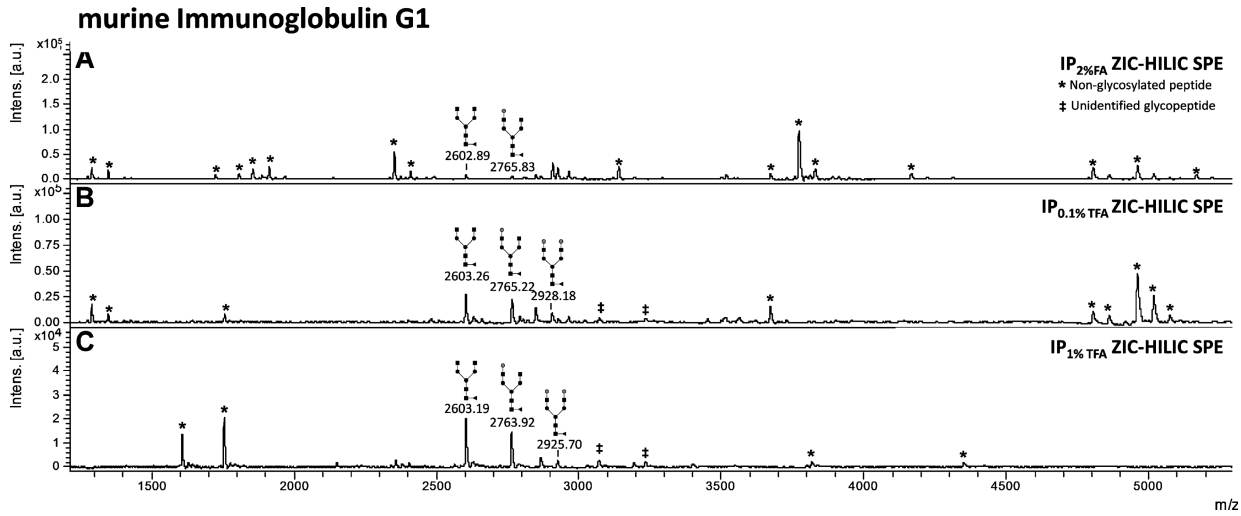

Figure 3. Glycopeptide enrichment from a peptide mixture derived from murine IgG1. A tryptic digest of murine IgG1 was split in three equal fractions and applied to $IP_{2\%\ FA}$, $IP_{0.1\%\ TFA}$, and $IP_{1\%\ TFA}$ ZIC-HILIC SPE and the retained fractions analyzed using MALDI-TOF MS (A−C). The assigned glycopeptides correspond to the [170−178] tryptic peptide of the IgG1 heavy chain Fc region (P01868) linked with biantennary glycan structures of the G0f, G1f, and G2f glycoforms. The assignments were based on the agreement of the molecular masses to the theoretical values predicted from this well-characterized glycoprotein. Signals assigned as unknown glycopeptides (‡) have a mass difference of 162.1 Da indicating that these analytes are glycosylated but could not be assigned as IgG1 glycopeptides with tryptic missed cleavage sites or with any known adducts. Signals for nonglycosylated peptides are assigned with asterisks (*). The identities of a subset of these were obtained based on their molecular masses. The remaining signals could not be identified, as only the sequence of the murine IgG1 Fc region was available, but these were expected to be nonglycosylated peptides. Spectra were recorded in positive ionization mode with linear (shown) and reflector detection.

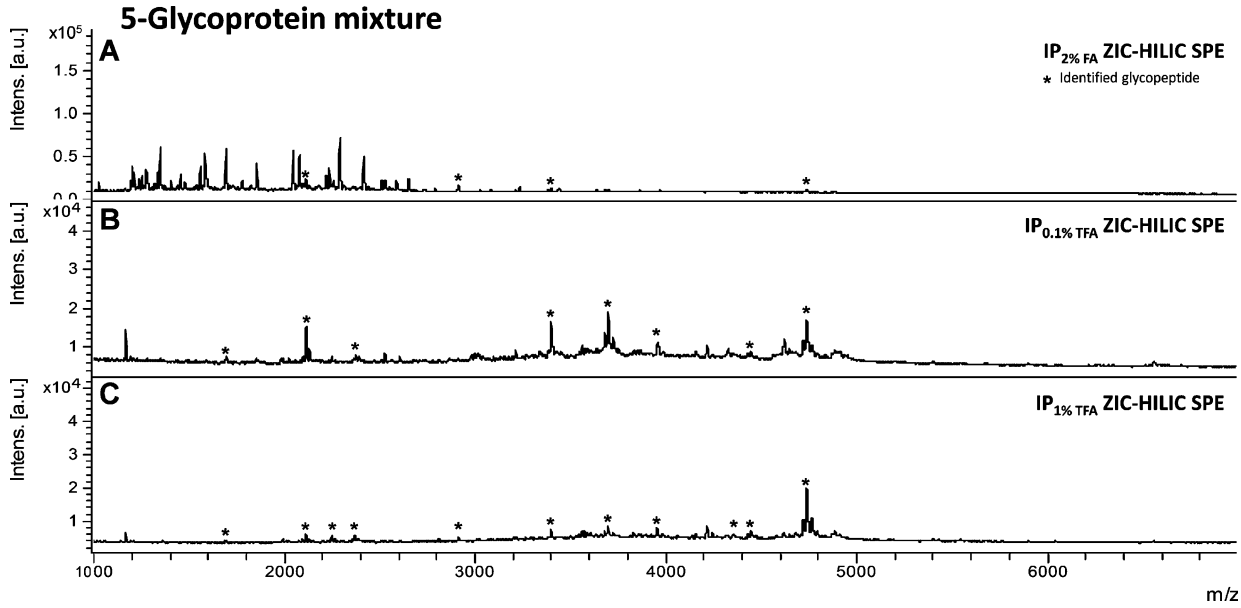

Figure 4. Glycopeptide enrichment from a peptide mixture derived from a tryptic digest of five glycoproteins (RNase B, ovalbumin, serotransferrin, fetuin, and α-1-acid glycoprotein). The digest was divided in three equal fractions and applied to $IP_{2\%\ FA}$, $IP_{0.1\%\ TFA}$, and $IP_{1\%\ TFA}$ ZIC-HILIC SPE and the retained fractions analyzed using MALDI-TOF MS (A−C). Numerous signals in the low *m*/*z* region (*m*/*z* 1000−2500) were observed for the $IP_{2\%\ FA}$ ZIC-HILIC SPE retained fraction, which most likely corresponded to nonglycosylated peptides. These analytes were depleted in the $IP_{0.1\%\ TFA}$ and $IP_{1\%\ TFA}$ ZIC-HILIC SPE retained fraction, and a number of signals in the higher *m*/*z* region were observed (*m*/*z* 3000−5000) corresponding to glycopeptides (assigned with asterisks (*)). This mixture was analyzed further in the subsequent ESI-LTQ-Orbitrap MS/MS experiment. Spectra were recorded in positive ionization mode with linear detection.

HILIC SPE enrichments, which agrees well with our previous observations.[20,21,30]

Glycopeptide Enrichment from Complex Mixtures and Detection with ESI-LTQ-Orbitrap MS/MS. The improved glycopeptide enrichment efficiency of $IP_{0.1\%\ TFA}$ and $IP_{1\%\ TFA}$ ZIC-HILIC SPE, indicated by the qualitative MALDI-MS studies on relatively simple peptide mixtures, was further investigated using RP-LC−ESI-LTQ-Orbitrap MS/MS detection for enrichments from the five-glycoprotein mixture and depleted plasma. To enable a better understanding of the glycopeptide binding mechanism to HILIC, the plasma sample was dephosphorylated prior to enrichment. This precaution was taken due to the hydrophilic nature of phosphopeptides, giving them a propensity for being coenriched in HILIC and thereby complicating

Table 1. Presence of Nonglycosylated Peptides and Glycopeptide Quantitation of the ZIC-HILIC SPE Retained Fractions[a]

	nonglycosylated peptide presence (Mascot data)			glycopeptide quantitation (Ion chromatogram data)		
	queries matched	unique peptide IDs	emPAI sum	glycopeptide XIC (area, 10^6)	TIC (area, 10^9)	XIC/TIC (10^{-3})[b]
five glycoprotein mixture−2% FA	913	131	72.82	7971.25	948.85	8.40 [1.00]
five glycoprotein mixture−0.1% TFA	395	80	16.51	4609.47	157.59	29.25 [3.48]
five glycoprotein mixture−1% TFA	190	49	9.68	7836.12	230.93	33.93 [4.04]
plasma−2% FA	1363	711	83.45	14154.25	2874.20	4.92 [1.00]
plasma−0.1% TFA	489	157	10.19	7320.35	743.32	9.85 [2.00]
plasma−1% TFA	249	97	7.83	6367.82	351.21	18.13 [3.68]

[a] The table highlights results extracted from ESI-LTQ-Orbitrap MS/MS data acquired from $IP_{2\%\ FA}$, $IP_{0.1\%\ TFA}$, and $IP_{1\%\ TFA}$ ZIC-HILIC SPE enrichments from digests of the five glycoprotein mixture and plasma. The presence of nonglycosylated peptides identified by Mascot was evaluated by the queries matched, sum of their individual emPAI scores, and the number of unique nonglycosylated peptides, whereas the glycopeptide quantities were evaluated based on the XIC/TIC ratio. The glycopeptide XIC for the "plasma−2% FA" is significantly higher than the XICs from the enrichments using the 0.1% and 1% TFA. This is likely to be an artificial high value caused by m/z interference of nonglycosylated peptides in the very complex plasma sample rather than the presence of a higher number of glycopeptides. [b] Values presented in brackets are normalized. Please consult the Experimental Section and Results sections for further details on the specific calculations.

the interpretation of the data. Again, parallel experiments were performed using the same three mobile phases. The glycopeptide enrichment efficiencies were evaluated by measuring the presence of coenriched nonglycosylated peptides and the glycopeptide ion intensities in the ZIC-HILIC SPE retained fractions, Table 1.

The presence of nonglycosylated peptides was evaluated based on identifications made using standard database-driven proteomics approaches. Specifically, the sum of queries matched, the sum of unique peptides identified, and the sum of emPAI scores, which is a semiquantitative value for the peptide presence,[35] were determined. These values consistently indicated that the nonglycosylated peptides were significantly depleted in the $IP_{0.1\%\ TFA}$ and $IP_{1\%\ TFA}$ ZIC-HILIC SPE enrichments compared to experiments where 2% FA was present in the mobile phase. This was observed for both the mixture of five glycoproteins, i.e., the emPAI sums were 72.82 ($IP_{2\%\ FA}$), 16.51 ($IP_{0.1\%\ TFA}$), and 9.68 ($IP_{1\%\ TFA}$), and the much more complex plasma sample, i.e., the emPAI sums were 83.45 ($IP_{2\%\ FA}$), 10.19 ($IP_{0.1\%\ TFA}$), and 7.83 ($IP_{1\%\ TFA}$). The number of unique (nonglycosylated) peptides identified was also much lower in the ZIC-HILIC SPE experiment containing TFA in the mobile phase, e.g., 711 peptides were identified for the $IP_{2\%\ FA}$ enrichment from the plasma digest, whereas only 157 and 97 peptides were observed in the $IP_{0.1\%\ TFA}$ and $IP_{1\%\ TFA}$ experiments, respectively.

In addition, the detection of glycopeptide ions was dramatically increased relative to the total ion current (TIC) in the $IP_{0.1\%\ TFA}$ and $IP_{1\%\ TFA}$ ZIC-HILIC SPE retained fractions compared to the $IP_{2\%\ FA}$ ZIC-HILIC SPE enrichments. The glycopeptide quantitation was based on the relative glycopeptide ion intensities, which were measured by the sum of chromatographic peak areas from extracted ion chromatograms (XIC) belonging to a vast number of glycopeptide precursors relative to the chromatogram area of the TIC, Table 1. XICs from 217 and 600 glycopeptide precursors were created for the five-glycoprotein mixture and plasma, respectively. The glycopeptide precursors were identified by manual interpretation of MS/MS spectra and based on the presence of abundant oxonium ions from the glycan (e.g., m/z 366.1 and 657.2) and monosaccharide sequence ladders in the MS/MS spectra. It is well-known that the glycan moiety of glycopeptides fragments preferentially when collision-induced dissociation is used for fragmentation.[15] The proportion of glycopeptides present in each enrichment were quantified by the XIC/TIC ratio and normalized (the $IP_{2\%\ FA}$ ZIC-HILIC SPE enrichment was set to equal 1). Enhanced detection of glycopeptides was observed for the mixture of five glycoproteins and the plasma sample in the $IP_{0.1\%\ TFA}$ and $IP_{1\%\ TFA}$ ZIC-HILIC SPE experiments. This improvement of glycopeptide detection was observed for both the 0.1% and 1% TFA concentration but clearly higher in the latter, i.e., $XIC/TIC_{normalized\ to\ 2\%\ FA} = 2.00$ (0.1% TFA) and 3.68 (1% TFA) for the plasma sample. Similarly, the glycopeptide ion intensity was increased 3.48- and 4.04-fold for the 0.1% and 1% TFA IP enrichments relative to the $IP_{2\%\ FA}$ enrichments for the mixture of five glycoproteins. The combination of increased glycopeptide detection and the dramatic reduction of nonglycosylated peptides when using TFA in the mobile phase represent strong evidence for a significant improvement of the glycopeptide enrichment efficiency of ZIC-HILIC SPE.

Investigating for Bias of ZIC-HILIC SPE. The enrichments from the five-glycoprotein peptide mixture were examined to ensure that both neutral and sialylated N-linked glycopeptides could be enriched in an unbiased manner using ZIC-HILIC SPE. For this purpose, the RNase B and fetuin Asn_{138} glycoprofiles were determined from the LC−ESI-MS data obtained from the ZIC-HILIC SPE enrichments (2% FA, 0.1% TFA, and 1% TFA). The relative glycoform abundance was determined using the area of XICs produced for the 2+ ion species (RNase B) or 3+ and 4+ ion species (fetuin) for each of the glycopeptides. As presented in Figure 5, the RNase B and fetuin Asn_{138} glycoprofiles obtained from all enrichments performed using the three mobile phases agree very well with the previously published reference glycoprofiles,[30] indicating that no bias between individual glycoforms had been introduced by switching to TFA ion-pairing conditions. Importantly, the correct sialylated fetuin Asn_{138} glycoprofile also demonstrated that no hydrolysis of the terminal sialic acids occurred when employing

(35) Ishihama, Y.; Oda, Y.; Tabata, T.; Sato, T.; Nagasu, T.; Rappsilber, J.; Mann, M. *Mol. Cell. Proteomics* **2005**, *4*, 1265–1272.

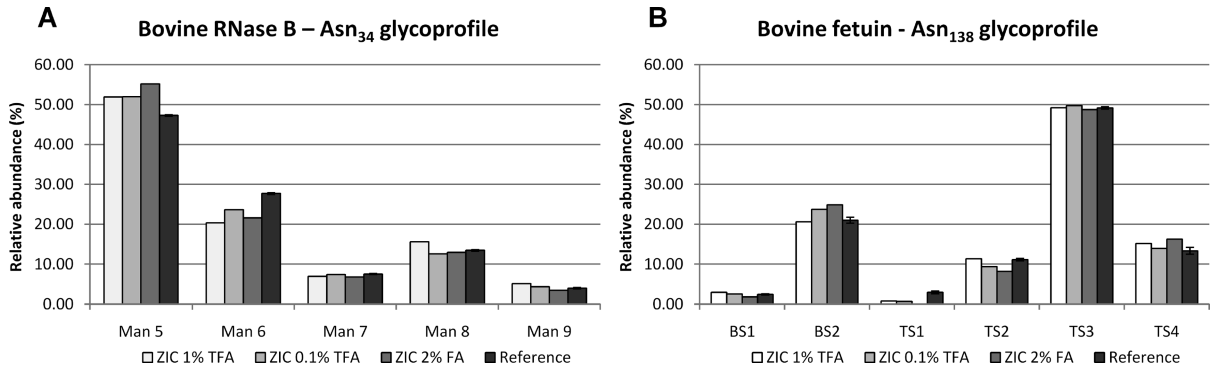

Figure 5. Comparison of RNase B Asn_{34} (A) and bovine fetuin Asn_{138} (B) glycoprofiles obtained from the RP-LC−ESI-MS analysis of $IP_{1\%\ TFA}$, $IP_{0.1\%\ TFA}$, and $IP_{2\%\ FA}$ ZIC-HILIC SPE enrichments from the digested five-glycoprotein mixture. The glycoprofiles are compared to reference glycoprofiles.[30] The RNase B glycopeptides have a neutral glycan population attached while the sialylated fetuin glycopeptides are linked with relatively labile, charged glycan species. The relative abundances were determined using the area of XICs produced for the 2+ ion species (RNase B) or 3+ and 4+ ion species (fetuin) of each glycopeptide. In both cases, XIC mass intervals of ±0.01 Da from the most abundant isotope were used.

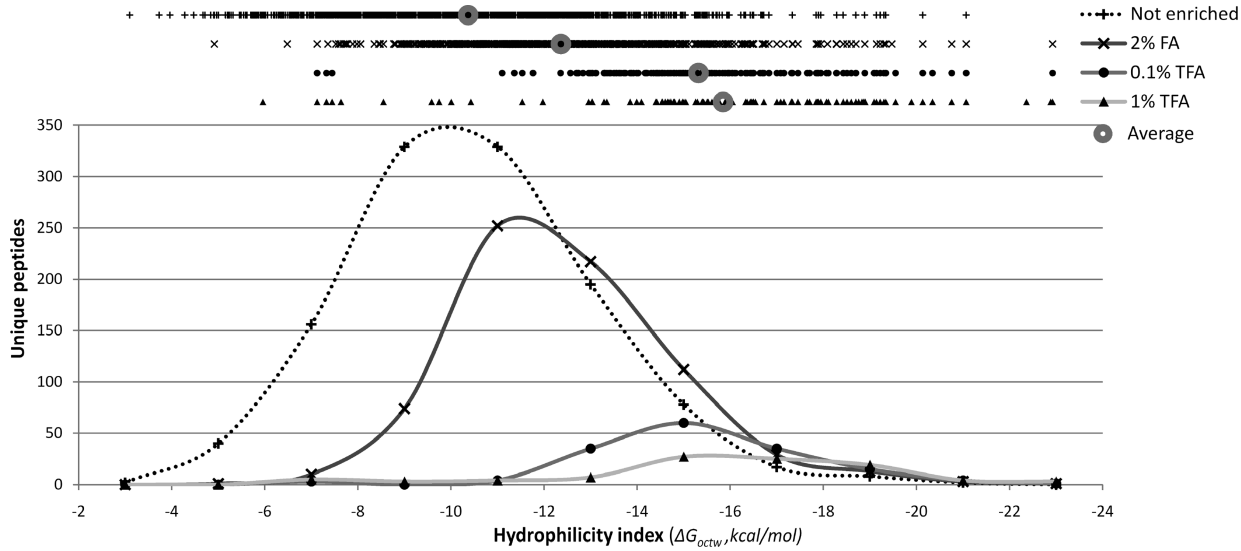

Figure 6. Hydrophilic distribution of retained nonglycosylated peptides. The hydrophilicities of the nonglycosylated peptides identified in the retained fractions of the $IP_{2\%\ FA}$, $IP_{0.1\%\ TFA}$, and $IP_{1\%\ TFA}$ ZIC-HILIC SPE enrichments of the plasma digest were manually calculated using the Wimley−White hydrophilicity scale. The Gibbs free energy with respect to the transfer from octanol to water was calculated meaning that lower ΔG_{octw} values (more negative values) indicate more hydrophilic peptides. For the calculation, the neutral values for aspartate and glutamate residues (Asp^0 and Glu^0) and the charged values for histidine, lysine, and arginine residues (His^+, Lys^+, and Arg^+) were used. A value of −7.9 kcal/mol was used for the C- and N-termini of each peptide.[29,33,34] For comparison, the hydrophilicities of peptides identified from a nonenriched plasma peptide mixture (desalted using Poros R3) were also calculated. The hydrophilicities of the individual peptides are plotted onto a hydrophilicity scale (upper part) and their hydrophilic average indicated. The number of unique peptides was also plotted as a function of their hydrophilicity (lower part). Here, hydrophilicity intervals were made ($\Delta(\Delta G_{octw}) = 2$) and the number of unique peptides within each interval were determined and plotted.

the TFA containing solvent. This was further substantiated by the fact that the completely desialylated Asn_{138} glycopeptides could not be observed (data not shown).

To further investigate whether the improved glycopeptide enrichment efficiency of IP ZIC-HILIC SPE was biased toward certain subgroups of the glycoproteome, the XIC/TIC ratio of a number of grouped glycopeptide precursors (50 precursors per group according to their m/z values) were plotted and compared for the three mobile phases, Figure S4 in the Supporting Information. This was performed for both the mixture of five glycoproteins and the plasma sample. The glycopeptide detection was consistently higher for all m/z intervals when using $IP_{0.1\%\ TFA}$ and $IP_{1\%\ TFA}$ ZIC-HILIC SPE compared to $IP_{2\%\ FA}$. This indicated that the improved enrichment efficiency with TFA as an IPR was uniformly distributed among the complete set of glycopeptides and not only beneficial for the enrichment of a subset of the glycosylated species.

Hydrophilicity of ZIC-HILIC SPE Retained Peptides. To advance the understanding of the retention mechanisms of ZIC-HILIC SPE using the various mobile phases, the hydrophilicities of the total set of identified nonglycosylated peptides in the ZIC-HILIC SPE retained plasma fractions were determined, Figure 6. These values were manually calculated using the Wimley−White hydrophilicity scale of the common amino acid residues.[32] Specifically, the Gibbs free energies for the transfer of analytes from *n*-octanol to water (ΔG_{octw}) were determined for the individual peptides. Thus, lower ΔG_{octw} values (more negative values) indicate more hydrophilic peptides. Unfortunately, the glyco-

peptides could not be included in this analysis as the hydrophilic contributions from the various glycan structures were unknown. However, it is expected that the hydrophilic contribution from the glycan moiety is significant, in particular for the larger N-glycan structures.[29]

The hydrophilic distribution and the average hydrophilicity of the tryptic plasma peptides identified in each of the parallel experiments are shown in the upper panel of Figure 6. The lower panel graphically illustrates the number of unique peptides as a function of their hydrophilicity. As expected, the plasma peptides retained by ZIC-HILIC SPE were on average more hydrophilic than the peptides identified when no enrichment had been performed (Poros R3 desalting).

In addition, the amount and hydrophilicity of the nonglycosylated peptides retained by ZIC-HILIC SPE proved to be very dependent on the mobile phase. The hydrophilic average of the retained peptides increased from $\Delta G_{octw} = -12.4$ ($IP_{2\%\ FA}$) to $\Delta G_{octw} = -15.4$ and -15.9 for $IP_{0.1\%\ TFA}$ and $IP_{1\%\ TFA}$ ZIC-HILIC SPE, respectively. When the number of identified peptides and their distribution on the hydrophilicity scale are examined, this shift in hydrophilic average was a consequence of the depletion of more hydrophobic (less hydrophilic) peptides rather than retention (and detection) of a higher number of more hydrophilic species with $IP_{0.1\%\ TFA}$ and $IP_{1\%\ TFA}$ ZIC-HILIC SPE. This was supported by the fact that the majority of the 97 and 157 nonglycosylated peptides detected in the IP ZIC-HILIC SPE retained fractions (using 1% TFA and 0.1% TFA in the mobile phase, respectively) were also identified among the 711 nonglycosylated peptides observed in the $IP_{2\%\ FA}$ ZIC-HILIC SPE retained fraction (data not shown).

In summary, efficient, reproducible, and unbiased glycopeptide enrichment from a wide range of sample complexities was demonstrated using $IP_{0.1\%\ TFA}$ and $IP_{1\%\ TFA}$ ZIC-HILIC SPE microcolumns. The improvements were consistently most distinct when 1% TFA was used in the mobile phase, although 0.1% TFA also improved the glycopeptide enrichment efficiency relative to the mobile phases containing 2% FA. The robustness of the technique was also recently shown by the efficient glycopeptide enrichment from a digest of a complex mixture of myocardial membrane proteins using TFA IP in a different ZIC-HILIC SPE format (i.e., commercial available ZIC-HILIC SPE mini-columns, Merck SeQuant, Umeå, Sweden) (data not shown). This was performed with larger amounts of starting material and significantly larger loading volumes than for the microcolumns and showed a similar increase in enrichment efficiency comparing $IP_{2\%\ FA}$ to $IP_{0.1\%\ TFA}$ and $IP_{1\%\ TFA}$ ZIC-HILIC SPE.

DISCUSSION

We have previously reported that glycopeptides can be enriched from peptide mixtures using ZIC-HILIC SPE resin packed in microcolumn format.[21] Recently, Ding et al. showed that TFA and other more hydrophobic IPRs, compared to FA, can improve the separation of glycopeptides from nonglycosylated peptides using HILIC HPLC coupled online with MS detection.[29] For glycoproteomics, offline glycopeptide enrichment in microcolumns is an ideal approach, since it is compatible with the existing proteomic workflows. This inspired us to investigate whether TFA can improve the enrichment of glycopeptides using IP ZIC-HILIC SPE and thereby create a method, which retains the efficiency of IP HILIC, while ensuring that detection can still be performed with the RP-LC−ESI-MS/MS setups, which are already implemented in many laboratories and commonly used to separate analytes with a higher resolution than HILIC HPLC. Additionally, enrichments using SPE microcolumns can be performed more rapidly and in multiplexed setups, making them preferable when high-throughput enrichments are desired. Furthermore, TFA can be used in relatively high concentrations throughout the entire chromatographic separation, in contrast to online HILIC HPLC−MS, where the presence of TFA reduces the MS sensitivity.[36] This enables easy control of the TFA concentration in the mobile phase. Moreover, the silica-based HILIC resin is unstable and can be degraded over time when using very acidic mobile phases (the long-term stability pH range of ZIC-HILIC is recommended to be 3−8, and the pH of the mobile phase containing 1% TFA is 1.6) However, since the low pH conditions are only applied to the column for 10−15 min, the typical duration of a HILIC SPE enrichment including microcolumn equilibration, sample load, wash, and elution and the fact that each microcolumn is only used once, the influence of the pH-related degradation of HILIC column material on the enrichment is negligible. Finally, the SPE microcolumns are advantageous over HILIC HPLC due to the fact that a variety of stationary phases can readily be used while the column lengths and capacities are easily adjusted for the specific application.

The initial MALDI-TOF MS analyses of enrichments from single glycoprotein digests and digests obtained from a mixture of five glycoproteins indicated that $IP_{0.1\%\ TFA}$ and $IP_{1\%\ TFA}$ ZIC-HILIC SPE increased the glycopeptide enrichment efficiency, enabling a close to complete glycopeptide isolation from the digest of IgG and five-glycoprotein mixture by employing a single SPE enrichment step (Figures 3 and 4). Considerable increases in the relative glycopeptide ion intensities were observed as the presence of nonglycosylated peptides was reduced. This is in good agreement with previous reports of glycopeptide suppression by nonglycosylated peptides,[6,7] highlighting the need for efficient depletion of nonglycosylated peptides when performing glycosylation analyses using MALDI-TOF MS.

In agreement with the MALDI-MS analyses of the more simple mixtures, $IP_{0.1\%\ TFA}$ and $IP_{1\%\ TFA}$ ZIC-HILIC SPE clearly improved the glycopeptide enrichment efficiency from the complex mixtures as shown by the enhanced detection of glycopeptides using RP-LC−ESI-LTQ-Orbitrap MS/MS (Table 1). Similarly, the increased glycopeptide detection was here promoted by the depletion of nonglycosylated peptides rather than the retention of a higher number of glycopeptides. As previously observed, the depletion of nonglycosylated peptides enhanced the detection of glycopeptides and other hydrophilic analytes due to their reduced suppression in the mass spectrometer. Taken together, uniform and reproducible improvements of the glycopeptide enrichment from a great span of sample complexities were demonstrated when using TFA in the mobile phase for ZIC-HILIC SPE.

The presented method for glycopeptide quantitation, which is based on the calculated XIC of the observed glycopeptides relative to the TIC, is not an accurate quantitation method but rather a

(36) Naidong, W. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2003**, *796*, 209–224.

rough estimate of the glycopeptide abundance in the sample. This is due to possible m/z interference or overlap of glycopeptide signals with signals originating from nonglycosylated peptides, which are often more intense than the glycopeptide signals. As a result, the XIC sum is expected to be somewhat overestimated, in particular from very complex samples. This is exemplified in the "2% FA−plasma" sample, Table 1, where the XIC value ($14\,154.25 \times 10^6$) is significantly higher than the XIC for the "0.1% TFA−plasma" (7320.35×10^6) and "1% TFA−plasma" (6367.82×10^6) due to the much higher complexity of the enriched fraction obtained when using the mobile phase containing 2% FA. It should however be noted that the XIC/TIC values for the TFA enrichments still clearly indicate that the proportion of glycopeptides in the sample is increased, even if the XIC values are over-represented in the less enriched samples. An alternative method for glycopeptide quantitation would involve deglycosylation after the HILIC SPE enrichment but prior to the LC−ESI-MS detection. This way the deamidated and deglycosylated glycopeptide could be identified using existing database search engines, making the data analysis less labor-intensive. Although this would reduce the glycopeptide quantitation to be based on the number of identified deglycosylated glycopeptides and not their relative abundance, which is roughly estimated by the XIC/TIC, this method would avoid the interference of nonglycosylated peptides and possibly be more accurate in terms of quantitation. However, it has to be mentioned that naturally occurring deamidation should be taken into account in such an approach.

We have previously reported that HILIC SPE using FA containing mobile phases is not biased toward specific N-glycoforms when enriching glycopeptides from purified or semi-purified glycoproteins and ensuring sufficient column capacity.[20,21,30] In this present study, the unbiased nature of HILIC SPE when using TFA containing solvents was demonstrated, Figure 5 and Figure S1 in the Supporting Information. Both neutral (RNase B) and sialylated (fetuin) glycopeptides were stoichiometrically retained, allowing for glycoprofiles which fit previously published profiles. However, it is still likely that a fraction of the O-linked glycopeptides was under-represented or even absent in the HILIC SPE retained fraction due to their lower hydrophilicity. Manual interpretation of the LC−ESI MS data from the five-glycoprotein mixture revealed indications of O-linked glycopeptides, e.g., from bovine fetuin (data not shown), but with the current data, it is not possible to conclude whether (or to what extent) HILIC SPE is discriminating against the O-glycoproteome. Importantly, the fetuin glycoprofile and the lack of desialylated fetuin glycopeptides proved that the sialylated glycopeptides are not hydrolyzed in the TFA containing solvent.

We observed that mobile phases containing 1% TFA consistently performed better than those containing 0.1% TFA in terms of enrichment efficiency. Presumably, the 10-fold increase in TFA concentration resulted in more ion-pairs being formed in the mobile phase. Specifically, the positively charged functional groups of the peptides (N-termini and lysine residues, amino groups; arginine residues, guanidinium groups) ion pair with the dissociated (deprotonated) TFA to form $-NH_3^+CF_3COO^-$ and $-HC(NH_2)_2^+CF_3COO^-$, which are significantly more hydrophobic than their free counterparts due to the reduced hydrogen-bonding potential.[37] Similarly, but of less importance for the overall hydrophobicity, the carboxylic acid groups ($-COO^-$) of the peptides (C-termini, aspartate and glutamate residues) ion pair with the hydronium ion in the low-pH mobile phase and form the neutral −COOH, which has been shown to be more hydrophobic relative to its charged counterpart.[33,34] Taken together, the use of TFA to IP with cationic and anionic charges make the peptides markedly more hydrophobic. Glycopeptides have a great number of hydrophilic hydroxyl groups (−OH), which will not be affected by the IP. Thus, the overall hydrophilicity of the glycopeptides will not be influenced to the same degree by the IP, reducing the hydrophilic overlap between glycopeptides and nonglycosylated peptides (see Figure 1).[28,29] Presumably, this is the mechanism which enables a more efficient glycopeptide enrichment using TFA in the mobile phase, in particular for the $IP_{1\%\ TFA}$ ZIC-HILIC SPE.

The retention mechanisms of HILIC were investigated by analyzing the depletion of nonglycosylated peptides. Thus, the hydrophilicities of the nonglycosylated peptides observed in the parallel plasma experiments were calculated, Figure 6. The selectivity for hydrophilic analytes using HILIC was clearly shown by comparing the hydrophilicities of the HILIC retained peptides and the peptides observed in the Poros R3 desalted fraction. This confirms that the HILIC retention mechanism is truly based on hydrophilicity. In addition, it was observed that the peptides retained by the ZIC-HILIC SPE were very dependent on the mobile phase. Both a reduced number and an increased hydrophilicity of the retained peptides were observed for the $IP_{0.1\%\ TFA}$ and $IP_{1\%\ TFA}$ ZIC-HILIC SPE enrichments. The difference in hydrophilicity relates primarily to the depletion of peptides of lower hydrophilicity ($\Delta G_{octw} > -14$ kcal/mol) but also to the depletion of some relatively hydrophilic peptides ($\Delta G_{octw} < -14$ kcal/mol). Meanwhile, the glycopeptides are not expected to be depleted from the samples to a similar degree, as evaluated by the increased proportion of glycopeptide ion signals present. This indicates that the hydrophilic overlap between glycopeptides and nonglycosylated peptides was decreased when using $IP_{0.1\%\ TFA}$ and $IP_{1\%\ TFA}$ ZIC-HILIC SPE. For simplicity, the global peptide hydrophilicities were calculated and presented in this study. However, for longer peptides the local hydrophilicity is probably more important for the HILIC retention than their overall hydrophilicity. Further studies will need to be performed to shed light on this aspect.

In a complex tryptic peptide mixture there is a high propensity for the presence of some peptides that are significantly more hydrophilic than the least hydrophilic glycopeptides. Even with relative hydrophobic IPRs, these peptides can end up being more hydrophilic than some glycopeptides and can thus not be separated from the glycopeptide population using HILIC. With an RP-LC separation step following the ZIC-HILIC SPE, this should not constitute a problem, as the hydrophobicity of the peptide moiety will largely determine the RP-LC elution time and thereby separate the analytes. With MALDI-TOF MS detection, however, the presence of hydrophilic nonglycosylated species represents a larger problem due to the substoichiometry of the glycopeptides. Thus, the use of online RP-LC ESI-MS/MS is ideal for glycopro-

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teomics following the offline enrichment of glycopeptides using IP ZIC-HILIC SPE.

We have previously reported that the capacity of ZIC-HILIC SPE microcolumns is important for the unbiased purification and that low-capacity columns generate a large degree of competition between the analytes, where the most hydrophilic species will be preferentially retained.[30] Thus, in this present study, a greater depletion of nonglycosylated peptides could likely have been achieved using the $IP_{2\%\ FA}$ ZIC-HILIC SPE by increasing the binding competition. This was supported by an experiment where the five-glycoprotein mixture was enriched using reduced lengths of $IP_{2\%\ FA}$ HILIC SPE microcolumns (22, 15, and 7 mm compared to the normal 35 mm). These shorter $IP_{2\%\ FA}$ HILIC SPE microcolumns reduced the coenrichment of nonglycosylated peptides but also depleted some less hydrophilic glycopeptides (data not shown). These results indicate that the HILIC column capacity plays a significant role in determining the degree of depletion of nonglycosylated peptides but also shows that the hydrophilic overlap between the glycopeptides and the nonglycosylated peptides, which is the true challenge in glycopeptide enrichment, needs to be reduced by other means, in order to improve the glycopeptide enrichment. The addition of TFA as an IPR to the mobile phase provided an advancement toward reducing this hydrophilic overlap. With regard to this, it would be interesting to test other IPRs, including ones that have a more hydrophobic tail. Heptafluorobutyric acid and pentafluoropropionic acid are potential candidates for this, as it is expected that their greater hydrophobic contribution would separate glycopeptides and nonglycosylated peptides further in terms of hydrophilicity. Ideally, this would enable a complete separation of glycopeptides and nonglycosylated peptides when using HILIC SPE strategies and possibly facilitate the enrichment of glycopeptides bearing very small glycans, e.g., O-linked glycopeptides with two to four monosaccharide units attached.

ZIC-HILIC is a silica-based zwitterionic stationary phase having both positive (quaternary amine) and negative charges (sulfonic acid). In addition, it can be expected that any residual nonderivatized silica will contribute with additional negative charges to the resin. The ZIC-HILIC retention mechanisms are therefore determined both by the hydrophilic partitioning of analytes from the mobile phase into the water-enriched layer around the stationary phase and by a minor, but significant, electrostatic interaction (repulsion and/or attraction) from analytes to the charged functional groups of the stationary phase. It is anticipated that TFA in the mobile phase will ion pair with the stationary phase as well as the analytes. Hence, in terms of the HILIC retention mechanism, it is expected that the contribution from electrostatic interactions will be negligible under IP conditions and the retention based solely on hydrophilic partitioning. In theory, this would be similar to the use of neutral HILIC stationary phases such as the TSK Amide-80 or the polyhydroxyethyl aspartamide (polyHE A) and result in a more true-HILIC retention mechanism for ZIC-HILIC, where the retention is based primarily on the hydrophilicity of the analyte.

To test the effect of IP mobile phases on neutral HILIC stationary phases, the same set of experiments presented in Figure 2 was performed using PolyHE A-HILIC SPE microcolumns (only mobile phases containing 2% FA and 1% TFA were compared). MALDI-TOF analyses of $IP_{2\%\ FA}$ and $IP_{1\%\ TFA}$ polyHE A-HILIC SPE retained fractions indicated that TFA also increased the enrichment efficiency of this neutral HILIC stationary phase. Enrichment efficiencies similar to the ZIC-HILIC SPE experiments were achieved when using MALDI-MS detection. However, as shown by the ESI-LTQ-Orbitrap MS/MS data, the presence of TFA did not increase the glycopeptide ion intensities, even though the presence of nonglycosylated peptides was reduced by two-thirds when using $IP_{1\%\ TFA}$ polyHE A-HILIC SPE (Table S1 in the Supporting Information). These findings indicated that the TFA containing mobile phase increased the depletion of nonglycopeptides in the sample but that the amount of nonglycosylated peptides was already depleted sufficiently when using $IP_{2\%\ FA}$ polyHE A-HILIC SPE, allowing the glycopeptides to ionize optimally when separated in the RP-LC prior to ESI-LTQ-Orbitrap MS/MS detection. Thus, the additional depletion of nonglycosylated peptides in the $IP_{1\%\ TFA}$ PolyHE A-HILIC SPE did not increase the glycopeptide detection. Taken together, the data suggests that including an IPR such as TFA in the mobile phase impacts the enrichment efficiency of charged HILIC resins, such as ZIC-HILIC, to a larger extent than neutral stationary phases. However, it is important to emphasize that irrespective of the stationary phase, the HILIC glycopeptide enrichment benefits from the addition of relatively hydrophobic IPRs by minimizing the hydrophilic overlap of glycopeptides and nonglycosylated peptides.

In addition to documentation of the improved TFA-promoted enrichment efficiency of glycopeptides using HILIC SPE, the reproducibility of the improvement was shown by low sample-to-sample variation when performing multiple technical replicates of ZIC-HILIC SPE enrichments of IgG and the five-glycoprotein mixture (Figures S2 and S3 in the Supporting Information). The close similarity between the spectra originating from the replicas confirms the high reproducibility of the enrichments performed with TFA in the mobile phase. The robustness and wide applicability of the HILIC SPE enrichment using TFA in the mobile phase was shown by performing efficient enrichments from a multitude of samples (i.e., IgG, RNase B, five-glycoprotein mixture, and plasma) spanning a great spectrum of sample complexities (i.e., single glycoprotein to depleted plasma) as well as being capable of being used in different SPE formats (i.e., micro- and somewhat larger mini-columns), with various stationary phases (i.e., ZIC-HILIC and PolyHE A) and being hyphenated to various detection techniques (i.e., MALDI-MS and LC−ESI-MS). Altogether, IP_{TFA} HILIC SPE microcolumns have proven to be a very flexible and robust tool for efficient glycopeptide enrichment that can be used in many contexts and that shows a high reproducibility in terms of low sample-to-sample variability.

CONCLUSIONS

We have documented that the glycopeptide enrichment efficiency can be notably improved by employing TFA as an IPR in the mobile phase when using ZIC-HILIC SPE and other hydrophilic stationary phases. Through the broad range of sample complexities analyzed, we observed that TFA-containing mobile phases enabled us to obtain almost complete glycopep-

tide isolation from single glycoprotein digests using a single SPE enrichment step while offering a marked depletion of nonglycosylated peptides from more complex samples. We believe that this optimized mobile phase will increase the value of the HILIC SPE tool for researchers working with both small-scale glycoprotein characterization and large-scale glycoproteomics. Future work will show whether IPRs having more hydrophobic tails than TFA will increase the hydrophilicity difference between glycopeptides and nonglycosylated peptides even further to enable a close-to-complete separation between the two groups of analytes.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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