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Site-Specific Glycoprofiling of N-Linked Glycopeptides Using MALDI-TOF MS: Strong Correlation between Signal Strength and Glycoform Quantities

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Site-specific glycoprofiling of N-linked glycopeptides using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is an emerging technique, but its quantitative accuracy lacks documentation. Thus, a systematic study of widely different glycopeptides was performed to determine the relationship between the relative abundances of the individual glycoforms and the MALDI-TOF MS signal strength. Glycopeptides derived from glycoproteins containing neutral glycans (ribonuclease B, IgG, and ovalbumin) were initially profiled and yielded excellent and reproducible quantitation (correlation coefficient $r = 0.9958$, $n = 5$) when evaluated against a normal phase HPLC 2-AB glycan profile. Similarly, precise quantitation was observed for various forms of *N*-glycans (free, permethylated, and fluorescence-labeled) using MS. In addition, three different sialoglycopeptides from fetuin were site-specifically profiled, and good correlation between peak intensities and relative abundances was found with only a minor loss of sialic acids ($r = 0.9664$, $n = 5$). For glycopeptide purification, a range of hydrophilic and graphite materials packed in microcolumn format proved capable of performing desalting without loss of quantitative information, but highlighted the column capacity as a critical parameter. In conclusion, MALDI-TOF MS signal strength of glycopeptides has been found to accurately reflect the relative quantities of glycoforms, providing that certain technical issues are considered, i.e., nonbiased sample handling, matrix choice, and instrumental settings. This enables rapid and sensitive site-specific glycoprofiling of *N*-glycan populations to promote biomarker discovery and elucidation of glycan structure/function relationships.

Aberrant protein N-glycosylation can directly change the function of glycoproteins, and alterations of glycan structures and their relative abundances have been shown to correlate with cancer and other diseases.[1−6] As a result, the focus on glycan characterization has dramatically increased in recent years in order to link glycan structure to function and enable biomarker discovery.

The glycoprofile is a quantitative map of all glycan structures attached to a given protein glycosylation site. Profiling of released *N*-glycans using high-performance liquid chromatography (HPLC) coupled with a fluorescence detector[7] or high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD)[8,9] has been the standard technique for decades. However, low throughput, resolution, and sensitivity limit the applications. Qualitatively, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) represents a rapid and robust method for the structural characterization of glycosylated compounds with high resolution and sensitivity. For MALDI-TOF MS quantitation, accurate glycoprofiling is dependent on equal responses of each glycoform present irrespective of its mass and structure. This has been documented for released *N*-glycans with masses of 1000−10 000 Da when examined on TOF systems, whereas glycans below 1000 Da display a significant reduction in signal strength.[10−13] In addition, it has been found that glycoprofiles of *N*-glycans obtained by MS closely match those obtained by HPLC of fluorescence-labeled compounds.[14−17] Certain technical aspects have been reported to affect the precision of the relative quantitation. The MS settings and

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matrixes should be carefully selected as saturation effects and matrix- and/or MS-induced fragmentation may affect the correlation between sample quantity and response.[18−20] In particular, sialoglycoconjugates are challenging to profile as sialic acids are highly labile in MALDI MS.[21,22] Similarly, for the wide selection of methods available for sample preparation,[23,24] it is important to ensure nonbiased purification and handling. Taken together, it is well-established that the MALDI-TOF MS signal strength of mixtures of released *N*-glycans accurately reflects the amount of analyte on the target when certain conditions are optimized. However, glycan profiling is limited by the loss of site-specificity, which prevents linkage of the glycan structure to its origin (and ultimately to its function) when proteins containing multiple glycosylation sites or glycoprotein mixtures are analyzed. In addition, glycan profiles of purified glycoproteins are easily skewed if contaminants and/or copurified glycoproteins are present.

Site-specific glycoprofiling circumvents these problems. However, in contrast to profiling of released *N*-glycans, the quantitative aspects of glycopeptide profiling using MALDI-TOF MS lack solid documentation and only a few studies have been published on this. In the mid-1990s Sutton et al. showed that populations of glycopeptides having the same peptide moiety ionize equally well, as indicated by good agreement between the MALDI-TOF MS glycoprofiles and reference profiles from other studies (HPAE-PAD, reversed-phase (RP)-HPLC, and fast atom bombardment MS).[25] Sialylated structures were excluded from this conclusion as signals for sialoglycopeptides experienced suppression effects. Recently, various quantitative methods for glycoprofiling were compared in a multi-institutional study.[26] Good quantitation and laboratory-to-laboratory reproducibility of glycopeptide profiling using MALDI and electrospray ionization (ESI) MS were reported, and it was concluded that site-specific profiling of glycopeptides will be a key interfacing technique in proteomics and functional glycomics in the future. As site-specific glycoprofiling using MALDI-TOF MS is an attractive approach, that has become more widely used in recent years[27−31] and is expected to be used increasingly in the future, firm evidence is needed to conclude that glycopeptide signal strength correlates with glycoform quantities.

In this study, we perform a detailed and systematic investigation of the relationship between the MALDI-TOF MS signal strength of glycopeptides from four widely different glycoproteins and the relative abundances of their individual glycoforms. The correlation is evaluated against a normal phase (NP)-HPLC profile of 2-aminobenzamide (2-AB)-derivatized glycans. For comparative purposes, profiling of various glycan derivatives using MALDI-TOF MS is also performed. Furthermore, glycopeptide purification techniques are investigated to identify potential bias using a range of solid-phase extraction materials in microcolumn format.

EXPERIMENTAL SECTION

Glycoproteins. The following glycoproteins were used: bovine ribonuclease B (RNase B) (Sigma, cat. no. R-7884, P61823), recombinant human immunoglobulin G (rhIgG) (gift from Symphogen, Lyngby, Denmark), chicken ovalbumin (Sigma, cat. no. A-5503, P01012), and bovine fetuin (Sigma, cat. no. 2379, P12763). Due to the presence of copurified glycoproteins,[31,32] ovalbumin was isolated using RP-HPLC.

Reduction and Alkylation. The glycoproteins were dissolved in H_2O (ultrahigh quality) (1 nmol/μL) except for rhIgG, which was provided in a concentration of 10 pmol/μL in 5 mM $NaCH_3COO$, pH 5.0. All proteins were reduced and alkylated prior to digestion (except for rhIgG glycan release). 1,4-Dithiothreitol (DTT) was added to glycoprotein samples (40 mM, final concentration), and the mixture was incubated at 56 °C for 30 min before 100 mM iodoacetamide (final concentration) was added, and the samples were incubated for 30 min in the dark. Subsequently, 1 μL of 1 M DTT was added to quench the reaction.

Trypsin, Proteinase K, and *N*-Glycosidase F Digestions. For trypsin digestion, 2 μL of 8 M urea in 0.4 M NH_4HCO_3 was added to 1 nmol of glycoprotein (2 μL), and the mixture was incubated for 30 min at 56 °C before 12 μL of H_2O and 3% (w/w) trypsin (Promega, Madison, WI) were added. The digestion was carried out for a minimum of 8 h at 37 °C. Ovalbumin (1 nmol) was further digested by adding 0.15 U of proteinase K (Sigma) with incubation for another 8 h at 37 °C.

For *N*-glycan release, 2 μL of 1% sodium dodecyl sulfate (SDS) was added to 1 nmol glycoprotein (~2 μL), and the mixture was incubated for 10−20 min at 80 °C before 10 μL of 20 mM Na_2HPO_4, pH 7.2, was added together with 2 U of *N*-glycosidase F (2 μL) (Roche, Mannheim, Germany). The digestion was carried out for 24−48 h at 37 °C. The released glycans were purified using a 10 kDa molecular weight cutoff (MWCO) spin column (Millipore, Billerica, MA). All digestions were monitored for completion using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) (data not shown) and stored at −20 °C until further use.

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Glycan Derivatization. Glycans were labeled with both 2-AB and 2-aminobenzoic acid (2-AA) using reductive amination. For 2-AB labeling,[33] 1 nmol of glycan was mixed with 20 μL of 2-AB labeling reagent, which consisted of 0.35 M 2-AB (Sigma-Aldrich, Steinheim, Germany) and 1 M sodium cyanoborohydride in 30% glacial acetic acid in dimethyl sulfoxide (DMSO) (v/v). The mixture was incubated at 65 °C for 2 h before the reaction was stopped by desalting the sample using microcolumns. For 2-AA labeling,[34] 1 nmol of glycan was mixed with 33 μL of 2-AA labeling reagent, which consisted of 3 mg of 2-AA (Sigma-Aldrich) and 2 mg of sodium cyanoborohydride in 100 μL of a solution containing 2% (w/v) boric acid and 4% (w/v) sodium acetate·$3H_2O$ in methanol. The mixture was incubated at 80 °C for 50 min before the reaction was stopped by desalting the sample.

Permethylation was performed essentially as described in Ciucanu and Costello.[35] In brief, 1 nmol of glycan was dried in a clean glass vial and dissolved in 200 μL of solution containing 1 g of NaOH powder in 4 mL of anhydrous DMSO. The sample was then sonicated at room temperature (RT) for 30 min before 100 μL of methyl iodide was added, and the mixture was sonicated at RT for 60 min. The same volumes of NaOH/DMSO and methyl iodide were added, and the mixture was sonicated for another 60 min. A 5-fold volume of chloroform ($CHCl_3$) was then added and washed with water. The organic phase was subsequently dried under argon and redissolved in methanol for C_{18} purification.

Derivatization of sialoglycans by methylation (methyl esterification) was based on a protocol from Powell and Harvey.[22] Briefly, 1 nmol glycan was redissolved in 10 μL of 50 mM $NaHCO_3$, pH 8.4, dried, and redissolved in 2 μL of DMSO. Subsequently, 2 μL of CH_3I was added and the mixture incubated 2 h at RT before the addition of 20 μL of DMSO. Excess methyl iodide was removed by argon flushing.

Sample Preparation. Microcolumns were prepared in GELoader pipet tips (Eppendorf, Hamburg, Germany)[36] by packing various solid-phase extraction materials of different character: (i) hydrophobic, (ii) graphite, and (iii) hydrophilic. (i) Hydrophobic material (Poros R2, 20 μm) (Applied Biosystems, Framingham, MA) for purification of permethylated glycans was packed, equilibrated, loaded, and washed in 5% formic acid (FA), and the sample eluted with 80% acetonitrile (ACN) in water. For glycopeptide purification, the hydrophobic columns were used to fractionate glycopeptides from the total peptide pool. Hence, the flow-through was used as opposed to the retained fraction. (ii) Two sources of graphite were used: HyperCarb (5 μm) (Thermo-Hypersil, Cheshire, U.K.) and commercial LudgerClean EB10 cartridges (Ludger, Oxfordshire, U.K.), which were disassembled and the resin used. These columns were handled as hydrophobic columns, but a C_{18} Empore membrane (3M, St. Paul, MN) was used as a plug for resin retention. (iii) Different hydrophilic and charged materials were used for glycan and glycopeptide purification: ZIC-HILIC (10 μm, 200 Å) (Merck SeQuant, Umeå, Sweden), TSK-gel amide 80 (3 μm, 80 Å) (TOSOH Bioscience, Stuttgart, Germany), PolyHydroxyethyl A (12 μm, 100 Å) (PolyLC, Columbia, MD), PolySulfethyl A (12 μm, 300 Å) (PolyLC), and Titansphere TiO_2 (5 μm) (GL Sciences, Japan). Commercial LudgerClean S cartridges (Ludger) were also used by disassembling the cartridges, slicing the membranes in appropriate sizes, and packing these in GELoader tips. These materials were all handled using hydrophilic interaction liquid chromatography (HILIC) conditions (loading/washing buffer, 80% ACN, 18% H_2O, and 2% FA; elution buffer, 5% FA) as described.[37] In addition, concanavalin A (Con A) sepharose beads (Pharmacia Biotech, Uppsala, Sweden) were used to purify mannose-rich RNase B glycopeptides. In brief, 20 mM Tris-base, 0.5 M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 7.4 was used as binding/washing buffer, and elution was performed with 0.5 M methyl-α-D-mannopyronaside, 20 mM Tris-base, 0.5 M NaCl, pH 7.4.

The cleanup strategies used for the individual experiments are summarized in the following. Glycopeptides of RNase B, rhIgG, and ovalbumin were fractioned from the nonglycosylated peptides by collecting the flow-through of R2 microcolumns and desalted using ZIC-HILIC microcolumns. The multitude of other HILIC materials (and the graphite, TiO_2, and Con A) were in this study used to compare the different sample preparation methods and investigate for bias in the desalting of the fractionated glycopeptides. The fetuin glycopeptides were purified using RP-HPLC and required consequently no further purification. The free glycans and labeled glycans (2-AA or 2-AB) for HPLC and MS were purified using either ZIC-HILIC or LudgerClean S microcolumns, whereas the permethylated glycans were purified using R2 microcolumns. Glycoprotein analyzed on the intact level (RNase B) was sufficiently pure and required no further purification prior to analysis. The entire workflow of the experiments is presented in Figure 1A, where the sample preparations are highlighted in the gray box.

For neutral glycopeptides, 0.5−1 μL of analyte was generally spotted on a MALDI target, 0.5 μL of 2,5-dihydroxybenzoic acid (DHB) (10 mg/mL) in 70% ACN and 0.1% trifluoroacetic acid (TFA) were added, and the mixture was dried. For neutral glycans (free, 2-AB, and permethylated), 0.5−1 μL of analyte was spotted on a MALDI target, 0.5 μL of DHB (10 mg/mL, in 70% ACN and 0.1% TFA) and 0.5 μL of 20 mM NaCl were added, and the mixture was dried. For sialylated glycans and glycopeptides, 2,4,6-trihydroxyacetophenone (THAP) (1 mg/mL) in 50% ACN/50% 20 mM aqueous ammonium citrate was used as a matrix as previously described.[19]

Mass Spectrometry. MALDI-TOF MS glycoprofiling of RNase B, rhIgG, and ovalbumin was performed using a Bruker Ultraflex (Bruker Daltonics, Bremen, Germany). Positive ionization in linear mode was used for quantitative data acquisition. Fetuin glycoprofiles were obtained on a Voyager-DE STR (Applied Biosystems) using linear negative and positive ionization mode. Both instruments were equipped with a pulsed nitrogen laser (337 nm), and as low laser power as possible was used. Delayed extraction times were set to 50−100 ns. Reflector mode was used only to validate the presence of analytes at high resolution. Picomolar amounts (in total 10−60 pmol distributed on the set of glycoforms) were used, as sensitivity was not addressed in this study. The spectra

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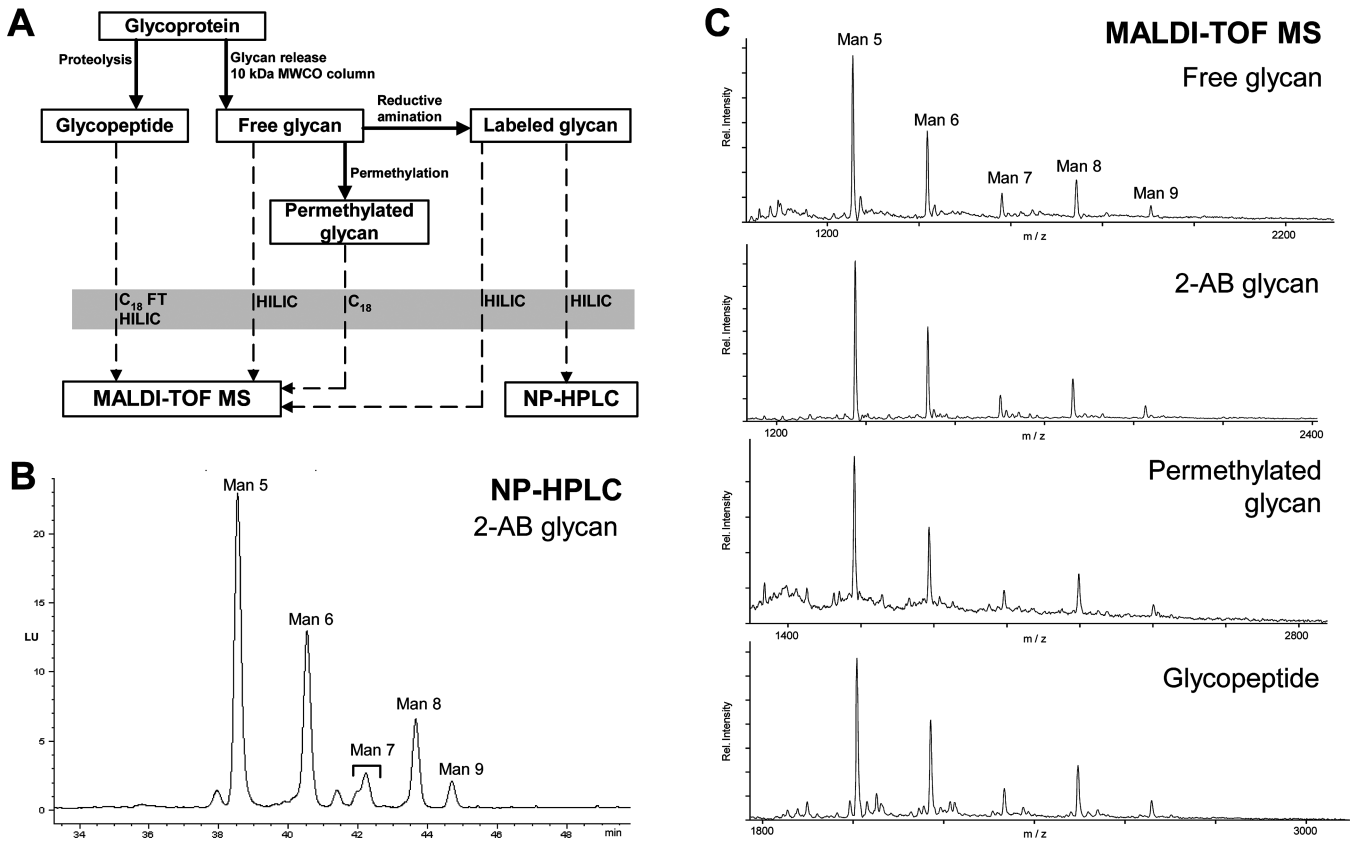

Figure 1. (A) Workflow of the multiple glycoprofiling approaches used in this study. The sample purification techniques for the individual approaches are presented (gray area). Detection was performed using either MALDI-TOF MS or NP-HPLC. Fetuin glycoprofiling deviated from this workflow as glycan release was performed from RP-HPLC-purified glycopeptide fractions. (B) Reference profile obtained using NP-HPLC of 2-AB-labeled RNase B glycans. Fluorescence detection was performed at λ_{ex} 420 nm and λ_{em} 330 nm. (C) MALDI-TOF MS glycan and glycopeptide profiling of RNase B recorded using positive ionization and linear mode. Glycopeptides were detected as $[M + H]^+$ and glycans as $[M + Na]^+$.

were externally calibrated, and good agreement between theoretical and experimental mass values was generally obtained (<0.1% mass accuracy for linear mode and <0.05% for reflector mode). Tandem mass spectrometry (MS/MS) was occasionally used to validate the presence of the expected analytes. An LTQ-XL (Thermo-scientific, Bremen, Germany) was used for ESI MS with direct infusion of the glycoconjugates. A 1:1 mixture of ACN and 0.1% FA in water was used as ESI solvent.

Reversed-Phase and Normal Phase HPLC. RP-HPLC was carried out on an Äkta-Basic system (Amersham Pharmacia Biotech, Uppsala, Sweden) using a Jupiter C_{18} 250 mm × 4.6 mm, 5 μm, 300 Å (Phenomenex, Torrance, CA). The gradient, made of B buffer (0.05% TFA, 90% ACN in water), increased from 5−40% in 30 min, 40−60% in 5 min, 60−90% in 3 min. The A buffer consisted of 0.06% TFA in water.

NP-HPLC (more correctly referred to as HILIC-HPLC under these conditions, but NP is used consistently in this study, as most literature still use this term for this approach) was performed on an Agilent system (1200 series) using a TSK-gel amide 80 (3 μm, 80 Å, 20 mm × 150 mm) (TOSOH, Stuttgart, Germany). Signals were recorded using fluorescence detection (λ_{ex}, 420 nm and λ_{em}, 330 nm). Analytes were collected and confirmed by MALDI-TOF MS. The gradient made of A buffer (50 mM ammonia formate in water, pH 4.4) was dependent on the heterogeneity of the glycan mixture. For RNase B and rhIgG *N*-glycans, the A buffer increased from 20−53% in 40 min and from 53−100% in 5 min. For ovalbumin *N*-glycans, the A buffer increased from 20−60% in 125 min and from 60−100% in 5 min. Due to the heterogeneity of ovalbumin *N*-glycans, not all glycoforms were resolved and their relative quantities were consequently estimated from MS analyses of collected fractions. The B solvent consisted of 100% ACN.

Quantitation. NP-HPLC quantitation was based on relative peak areas of the eluting analytes calculated using ChemStation software (Agilent). Relative quantitation using MALDI-TOF MS detection was based on signal strength as measured by peak height following manual assessment of baselines in the spectra. If present, significant adducts were included in the quantitation. The relationship between signal strength of glycopeptides and glycoform quantities was evaluated by calculating the correlation coefficient (r) for MALDI-TOF MS data and the reference using Pearson product-moment correlation model. The calculations were performed using JMP 8.0 (SAS, Cary, NC) and separated in two: correlation between glycoform quantities as measured by the reference and the signal strength of (a) neutral glycopeptides (N = 110) and (b) sialylated glycopeptides (N = 90).

RESULTS AND DISCUSSION

Design of Experiment. Four well-characterized glycoproteins containing different populations of *N*-glycans in terms of heterogeneity, size, charge, structure, and type formed the basis of this study and included bovine pancreatic RNase B (P61823), rhIgG

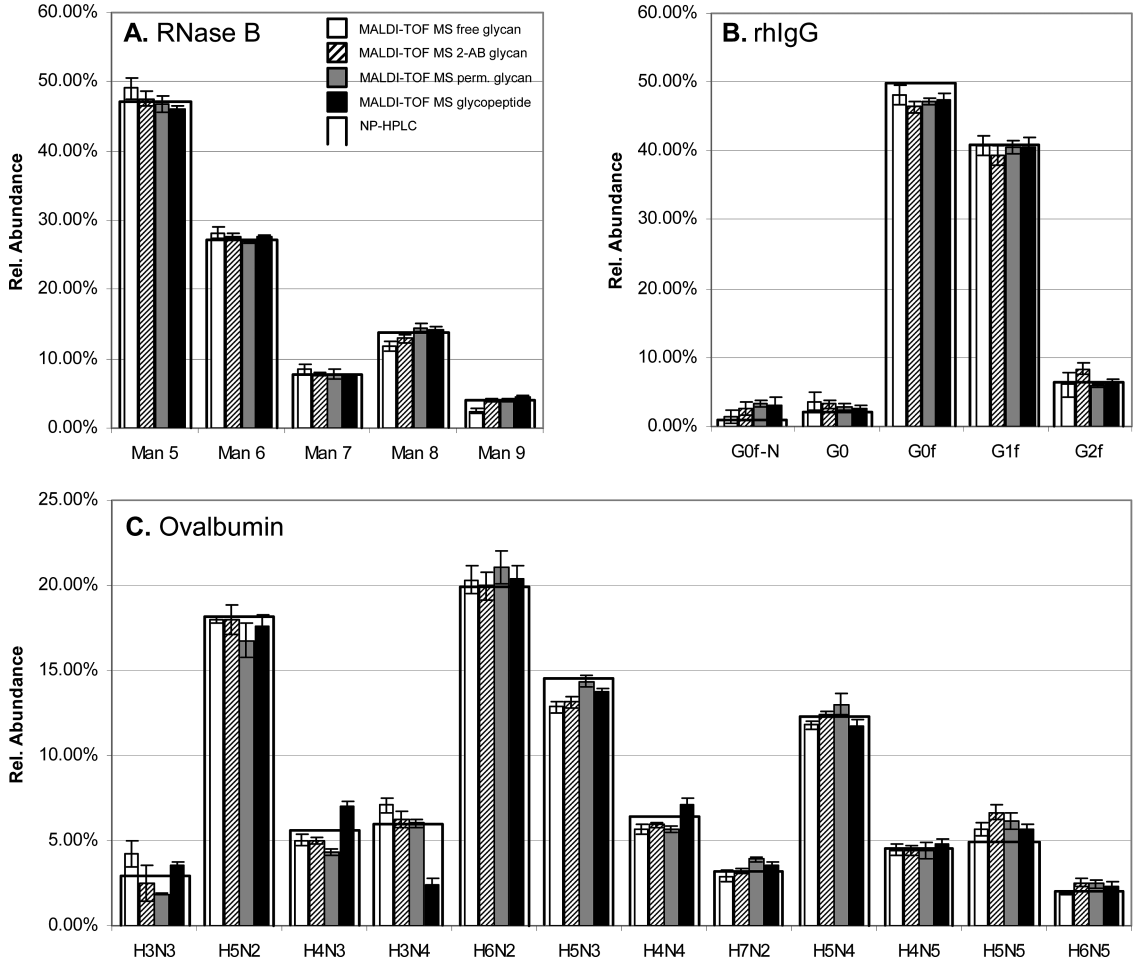

Figure 2. Relative abundances of glycoforms of (A) RNase B, (B) rhIgG, and (C) ovalbumin as measured using the various approaches. Data from five replicates were averaged, and standard deviations are shown. Color coding: MALDI-TOF MS profiling of free glycans (white), 2-AB-labeled glycans (black stripes), permethylated glycans (gray), and glycopeptides (black). These profiles were recorded in positive ionization mode using DHB as matrix and compared to a reference profile (NP-HPLC) (black outline). For simplicity no standard deviations are shown for the reference profile but are listed in Table 1.

derived from CHO cells, chicken ovalbumin (P01012), and bovine fetuin from fetal calf serum (P12763).

Figure 1A illustrates the workflow of the multilevel glycoprofiling setup. The site-specific glycoprofiling was carried out using MALDI-TOF MS quantitation of glycopeptides selectively fractionated from the total peptide pool by serial C_{18} flow-through and HILIC purification. In contrast, loss of site information was a consequence of released glycan profiling, which was performed at three levels using MALDI-TOF MS, i.e., free, permethylated, and fluorescently labeled glycan. Serving as a reference, glycoprofiling of labeled glycans was carried out using NP-HPLC with fluorescence detection. Fetuin glycoprofiling deviated from this workflow, as three glycopeptide-containing fractions were initially purified using RP-HPLC. As illustrated, each approach required a certain type of sample preparation. Examples of RNase B glycoprofiling using MALDI-TOF MS and NP-HPLC are presented in Figure 1, parts B and C, respectively. Throughout the study, the neutral glycopeptides were detected as $[M + H]^+$ and the neutral glycans as $[M + Na]^+$. The NP-HPLC quantities were calculated from the peak area of the eluting analytes, whereas the MS quantitation was based on signal strength as commonly measured by peak height.[10,18,26,38] All MS and HPLC analyses were performed in five replicates ($n = 5$).

Profiling of Glycopeptides Containing Neutral Glycans. Comparative glycoprofiling data of the three glycoproteins containing neutral *N*-glycans of different types, i.e., RNase B (high mannose), rhIgG (complex), and ovalbumin (high mannose/hybrid), are presented in Figure 2A–C. Here, the glycopeptide profiles (MALDI-TOF MS) are compared to the various glycan profiles (MALDI-TOF MS) and ultimately to the reference profile (NP-HPLC).

RNase B is a small glycoprotein containing a single N-glycosylation site (Asn_{34}). In terms of monosaccharide composition, five different glycosylated variants exist with 5–9 mannose residues attached to the chitobiose core, i.e., $Man_{5-9}GlcNAc_2$. However, multiple isomers with different branching patterns are present for the Man 7 and Man 8 glycoforms.[39] The glycopeptide profile matched with high accuracy the reference data for all five glycoforms as shown by the visual comparison in Figure 2A. In addition, very low sample-to-sample variations were observed as reflected by the low standard deviations.

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Table 1. Site-Specific Glycoprofiling of Neutral and Sialoglycans

glycoprotein/ glycoforms	monosaccharide composition (glycan type)[a,b]	isomers[c]		MALDI-TOF MS glycopeptide rel abundance/%	NP-HPLC 2-AB glycan rel abundance/% (reference)	MALDI-TOF MS methyl sialoglycan rel abundance/% (reference)
RNase B						
Man 5	$Man_5GlcNAc_2$ (M)	1		46.03 ± 0.57	47.26 ± 0.18	
Man 6	$Man_6GlcNAc_2$ (M)	1		27.66 ± 0.25	27.69 ± 0.19	
Man 7	$Man_7GlcNAc_2$ (M)	3		7.63 ± 0.24	7.58 ± 0.11	
Man 8	$Man_8GlcNAc_2$ (M)	3		14.16 ± 0.45	13.47 ± 0.19	
Man 9	$Man_9GlcNAc_2$ (M)	1		4.52 ± 0.26	4.00 ± 0.17	
rhIgG						
G0-N	$Man_3GlcNAc_3$ (C)	2		3.11 ± 1.23	0.90 ± 0.15	
G0	$Man_3GlcNAc_4$ (C)	1		2.62 ± 0.50	2.34 ± 0.20	
G0f	$Man_3GlcNAc_4Fuc_1$ (C)	1		47.42 ± 0.86	49.97 ± 1.42	
G1f	$Gal_1Man_3GlcNAc_4Fuc_1$ (C)	2		40.55 ± 1.43	40.58 ± 1.19	
G2f	$Gal_2Man_3GlcNAc_4Fuc_1$ (C)	1		6.31 ± 0.55	6.22 ± 0.50	
Ovalbumin						
H3N3	$Man_3GlcNAc_3$ (C/H)	2		3.59 ± 0.19	2.99 ± 0.47	
H5N2	$Man_5GlcNAc_2$ (M)	1		17.57 ± 0.67	18.21 ± 0.86	
H4N3	$Man_4GlcNAc_3$ (H)	1		6.98 ± 0.37	5.56 ± 0.64	
H3N4	$Man_3GlcNAc_4$ (Hb)	2		2.43 ± 0.38	5.84 ± 0.58	
H6N2	$Man_6GlcNAc_2$ (M)	1		20.41 ± 0.77	19.86 ± 0.59	
H5N3	$Man_5GlcNAc_3$ (H)	1		13.76 ± 0.16	14.53 ± 0.35	
H4N4	$Man_4GlcNAc_4$ (Hb)	1		7.10 ± 0.39	6.30 ± 0.21	
H7N2	$Man_7GlcNAc_2$ (M)	1		3.59 ± 0.16	3.08 ± 0.39	
H5N4	$Man_5GlcNAc_4$ (Hb)	1		11.73 ± 0.39	12.15 ± 0.57	
H4N5	$Man_4GlcNAc_5$ (Hb)	1		4.82 ± 0.28	4.61 ± 0.07	
H5N5	$Gal_{0-1}Man_{4-5}GlcNAc_5$ (Hb)	2		5.66 ± 0.32	4.85 ± 0.54	
H6N5	$Gal_1Man_5GlcNAc_5$ (Hb)	1		2.35 ± 0.22	2.02 ± 0.37	
Fetuin						
BS1	$NeuAc_1Gal_2Man_3GlcNAc_4$ (C)	3	Asn_{81}	3.31 ± 0.48		3.84 ± 0.37
			Asn_{138}	3.63 ± 0.38		2.43 ± 0.15
			Asn_{158}	0.00 ± 0.00		0.00 ± 0.00
BS2	$NeuAc_2Gal_2Man_3GlcNAc_4$ (C)	4	Asn_{81}	8.53 ± 0.86		12.05 ± 0.45
			Asn_{138}	24.63 ± 0.62		21.01 ± 0.78
			Asn_{158}	1.38 ± 0.25		6.29 ± 0.64
TS1	$NeuAc_1Gal_3Man_3GlcNAc_5$ (C)	3	Asn_{81}	7.51 ± 0.82		5.12 ± 0.39
			Asn_{138}	3.80 ± 0.30		2.93 ± 0.34
			Asn_{158}	4.60 ± 0.99		0.00 ± 0.00
TS2	$NeuAc_2Gal_3Man_3GlcNAc_5$ (C)	6	Asn_{81}	28.74 ± 0.88		25.55 ± 0.25
			Asn_{138}	14.46 ± 0.84		11.14 ± 0.31
			Asn_{158}	14.30 ± 1.26		7.07 ± 0.76
TS3	$NeuAc_3Gal_3Man_3GlcNAc_5$ (C)	5	Asn_{81}	49.20 ± 0.98		46.62 ± 0.69
			Asn_{138}	47.21 ± 1.42		49.14 ± 0.31
			Asn_{158}	48.59 ± 1.42		46.21 ± 0.59
TS4	$NeuAc_4Gal_3Man_3GlcNAc_5$ (C)	2	Asn_{81}	2.71 ± 0.06		6.82 ± 0.75
			Asn_{138}	6.27 ± 0.80		13.35 ± 0.87
			Asn_{158}	31.13 ± 1.16		40.43 ± 0.95

[a] Key for monosaccharide composition: Man, mannose; GlcNAc, *N*-acetylglucosamine; Gal, galacose; Fuc, fucose; NeuAc, *N*-acetylneuraminic acid. [b] Key for glycan type: M, high mannose; C, complex; H, hybride; b, bisecting GlcNAc. [c] Isomer references: RNase B (ref 39), rhIgG (likely from combinatorial evaluation), ovalbumin (ref 32) fetuin (ref 42).

Similarly, the three glycan profiles matched closely the NP-HPLC reference. All quantitative data for the glycopeptide and reference profiles are listed in Table 1, together with information of the individual glycoform compositions and the number of reported isomeric structures.

IgG is an abundant glycoprotein in human serum and holds an N-glycosylation site (Asn_{297}) in the Fc domain of the heavy chain.[40] rhIgG is of great interest to the pharmaceutical industry as a therapeutic candidate for various diseases, and the detailed characterization of the attached carbohydrate structure is essential. *N*-Glycans typically include biantennary structures with varying amounts of galactose residues (G) with and without core-fucosylation (f). IgG derived from recombinant sources contains, in contrast to human IgG, no significant sialylation. As presented in Figure 2B, high reproducibility and excellent agreement between the glycopeptide profiling and the reference was achieved for the five glycoforms: G0-N, G0, G0f, G1f, and G2f (see Table 1 for further description). Thus, glycoprofiling using MALDI-TOF MS is an ideal technique for rapid monitoring of batch-to-batch glycosylation pattern consistency of recombinant glycoproteins, which is necessary to ensure uniform biological activity.

The single N-glycosylation site of ovalbumin (Asn_{292}) has a heterogeneous population of attached neutral glycans, and these are mostly of the high-mannose (predominantly $Man_5GlcNAc_2$ and $Man_6GlcNAc_2$) and hybrid type.[41] More than 25 distinct, neutral glycans have been reported; however, some of these originate from copurified glycoproteins.[32] Conse-

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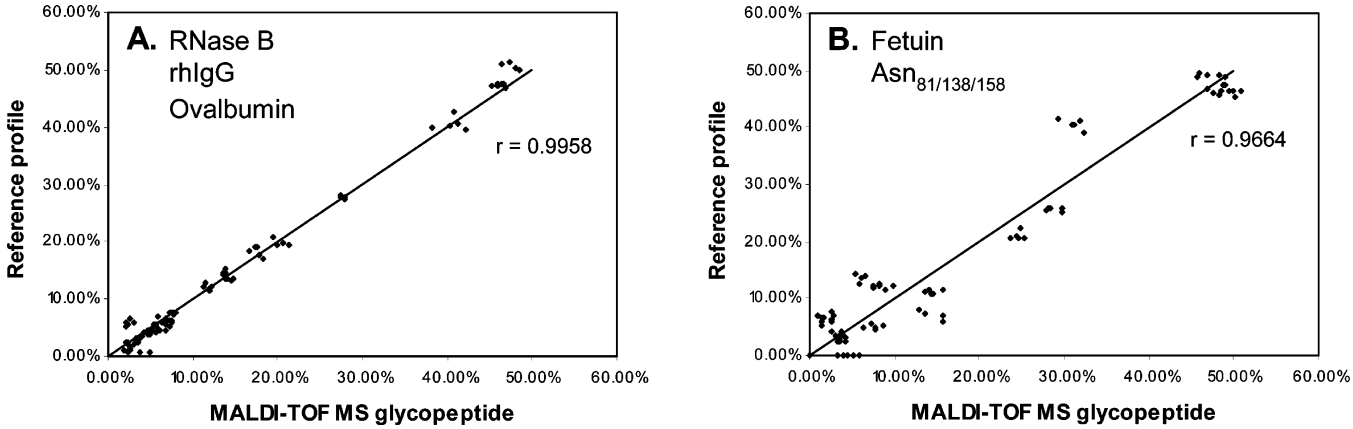

Figure 3. Correlation between MALDI-TOF MS signal strength of glycopeptides and their relative abundances as measured by the reference profiles. The correlation was determined for (A) glycopeptides containing neutral glycans (RNase B, rhIgG, and ovalbumin) and (B) sialoglycopeptides (fetuin, $Asn_{81,138,158}$). A 1:1 line is included to visualize the deviations from perfect correlation. The correlation coefficients (r) are given.

quently, ovalbumin was further purified using RP-HPLC prior to analysis in this study. Consistently, the site-specific glycoprofiling as well as the glycan profiling using MALDI-TOF MS detection showed remarkably accurate and reproducible quantitation when compared to the reference, Figure 2C. As previously reported, Man 5−6 and a number of hybrid structures were the most abundant. More than the 12 glycoforms listed were observed, but these were, due to very low abundance, excluded from the quantitation. The abundance of the glycopeptide containing the complex type glycan $Hex_3HexNAc_4$ (H3N4) deviated markedly from the NP-HPLC glycoprofile. This deviation likely reflects that ovalbumin was not purified completely and a glycan contribution was coming from another source. This was supported by glycoprofiling of nonpurified ovalbumin, which showed high proportions of this particular glycan (data not shown). Although being a minor contribution, this highlighted the importance of performing site-specific glycoprofiling.

In summary, MALDI-TOF MS signal strength of glycopeptides containing neutral glycans appeared to precisely reflect the relative abundances of glycoforms. In addition to the visual comparison (Figure 2), the correlation was statistically supported by a correlation coefficient (r) of 0.9958 ($N = 110$, $p < 0.0001$), Figure 3A.

Profiling of Glycopeptides Containing Sialylated Glycans. Bovine fetuin is a major glycoprotein in fetal calf serum and contains in addition to three O-glycosylation sites also three N-glycosylation sites (Asn_{81}, Asn_{138}, and Asn_{158}). The attached glycans are all of the triantennary (T, major) and biantennary (B, minor) type with a different degree of sialylation (S). Detailed NMR studies have revealed that at least 23 distinct glycoforms exist in the six monosaccharide compositions: BS_1, BS_2, TS_1, TS_2, TS_3, and TS_4.[42] No neutral *N*-glycans are present on fetuin, and it has been reported that the three glycosylation sites are processed differently in terms of glycosylation,[43] stressing the need for site-specific glycoprofiling.

Figure 4A−C presents the Asn_{81}, Asn_{138}, and Asn_{158} glycopeptide and glycan (free and 2-AA-labeled) profiles starting from RP-HPLC-purified glycopeptide fractions. As a result of the many underlying isomeric structures of the sialoglycans, high-quality NP-HPLC data could not be obtained due to unresolved peaks. Instead, a reference profile was generated from methylated sialoglycans as these derivatives have been shown to be stable in MALDI-TOF MS, preventing the loss of the labile sialic acids.[22] This was supported by the lack of BS_1 and TS_1 (or neutral) glycans in the Asn_{158} reference profile, Figure 4C. In addition, the global fetuin glycoprofile, estimated by averaging the three site-specific reference profiles, was found to match published results[42] and increased the confidence of the reference profiles (data not shown). Common for the three glycosylation sites was the high abundance of TS_3, whereas the relative abundances of the other glycans, in particular BS_2, TS_2, and TS_4, as well as the triantennary/biantennary ratio varied greatly among the sites. Good reproducibility and agreement between the glycopeptide profile and the reference was achieved (Table 1), although a minor degree of MS-induced fragmentation of the most sialylated glycans (BS_2 and TS_4) was observed for the glycopeptide and glycan profiles. The less sialylated species were consequently over-represented, in particular TS_1, TS_2, and to some extent TS_3. This indicated pronounced lability of the highly sialylated TS_4 glycoform containing the abnormal α2,6-*N*-acetylneuraminic acid attached to the *N*-acetylglucosamine residue of the antennae.[44] Deviations from the reference profile were in the range of 5−10 percentage points at the highest, and good correlation between the signal strength of the sialoglycopeptides and their relative abundances was statistically observed by a correlation coefficient of 0.9664 ($N = 90$, $p < 0.0001$), Figure 3B.

Glycoprofiling of Released *N*-Glycans. Although not of primary focus in this study, glycoprofiling of various forms of released neutral and sialylated glycans (free, permethylated, and fluorescence-labeled) was also performed. In general, all glycan forms provided accurate and reproducible glycoprofiles (Figures 2 and 4) in agreement with previous reports,[10,11,14−17] but the quality of the spectra varied. Usually, free glycans gave rise to multiple cation adduct signals, whereas 2-AB-labeled glycans

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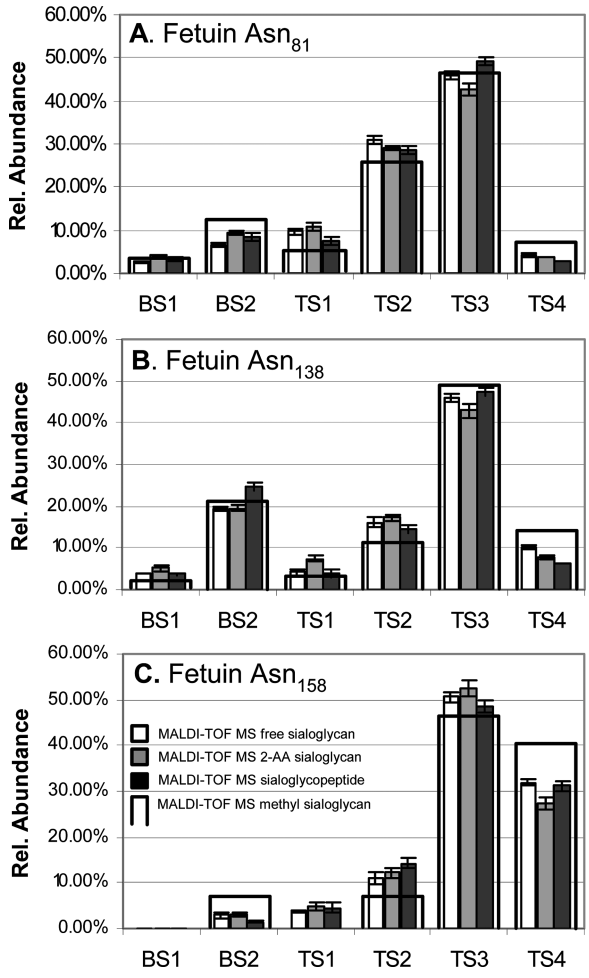

Figure 4. Relative abundances of fetuin glycoforms of (A) Asn_{81}, (B) Asn_{138}, and (C) Asn_{158} as measured using the various approaches. Data from five replicates were averaged, and standard deviations are shown. Color coding: MALDI-TOF MS profiling of free glycans (white), 2-AA-labeled glycans (gray), and glycopeptides (black). These profiles were recorded in negative ionization mode using THAP as matrix. The reference (MALDI-TOF MS profile of methylated sialoglycans, positive ionization mode, DHB) is visualized (black outline). For simplicity no standard deviations are indicated for the reference profile but are listed in Table 1.

mostly provided "clean" spectra and higher signal-to-noise ratios due to high ionization efficiency of the tag. Similar high ionization efficiency was observed for permethylated glycans, which in addition benefited from increased stability, most notably of the sialoglycans. Thus, glycan profiling is preferably performed using derivatized glycans.

The reference profiles were obtained using NP-HPLC and fluorescence detection of released and 2-AB-labeled glycans as this technique represents one of the traditional approaches for highly reproducible N-glycoprofiling. Although it is expected that results obtained using this method reflect the true glycoprofile, the data should not be used uncritically as potential bias may skew the results, e.g., sample handling and differential fluorescence intensities of labeled glycoforms. However, as good agreement between the HPLC and the multiple MS profiles (using different products and ionization techniques) was obtained, we are confident that the presented HPLC glycoprofiles reflect the natural composition of the glycans with reasonable accuracy. For the same reason, the MS detections of the released *N*-glycans are similarly evaluated to be nonbiased. This was expected as glycans in the range of 1000−10 000 Da have been shown to yield quantitative signals using TOF systems.[10]

Technical and Instrumental Considerations. Due to the relatively "hard" ionization of MALDI-TOF MS, in-source (prompt) and postsource fragmentation of the glycan residues, in particular sialic acids, may potentially skew the quantitation. Hence, the choice of matrix and instrumental settings is of immense importance to minimize decay. All MS spectra were recorded in linear mode to avoid the presence of postsource decay fragments, while the character of the carbohydrates defined the ionization polarity. Glycoconjugates containing neutral glycans were recorded in positive ionization mode, where glycopeptides predominantly were detected as $[M + H]^+$ and released glycans as $[M + Na]^+$. In contrast, negative ion mode was used for the sialylated glycans and glycopeptides, which were observed as $[M - H]^-$ species. Improved sensitivity and "cleaner" spectra due to the absence of cation adducts were the reasons for choosing negative ionization. In comparison, it has been reported that no competition for ionization between mono-, di-, and trisialylated species occurs.[19] Since 2-AA contains a carboxylic acid, and thereby readily form $[M - H]^-$ ions, it was chosen for the labeling of sialylated glycans, as these were analyzed in negative ionization mode. In contrast, 2-AB labeling was used for neutral glycans, which were recorded with high sensitivity in positive ionization mode predominantly as the $[M + Na]^+$ species. Due to the typical ionization in different polarity modes, profiling of glycopeptides containing mixtures of neutral and sialylated glycans represents significant challenges and is not addressed here. For spectrum acquisition, low laser power and short delay times (50−100 ns) were used at the cost of resolution, as both parameters have been shown to reduce fragmentation.[45] Recording by means of continuous extraction was also attempted, but the resulting loss of resolution proved too high

Table 2. Overview of Glycopeptides

glycoprotein	proteolytic enzyme	glycopeptide sequence	glycopeptide mass Da (peptide only)[a]	purification from peptide mixture
RNase B	trypsin	^{34}**N**LTKDR^{39}	474.28	R2 flow-through + HILIC[b]
rhIgG	trypsin	^{293}EEQY**N**STYR^{301}	1188.50	R2 flow-through + HILIC[b]
ovalbumin	trypsin + prot. K	^{291}Y**N**LT^{294}	509.25	R2 flow-through + HILIC[b]
fetuin Asn_{81}	trypsin	^{54}RPTGEVYDIEIDTLETTCHVLDPTPLA**N**CSVR^{85}	3670.76	RP-HPLC
Asn_{138}	trypsin	^{127}LCPDCPLLAPL**N**DSR^{141}	1739.83	RP-HPLC
Asn_{158}	trypsin	^{142}VVHAVEVALATFNAES**N**GSYLQLVEISR^{169}	3015.57	RP-HPLC

[a] Monoisotopic masses of sequences with carboxymethylated cysteine residues. [b] Microcolumn format (ref 41).

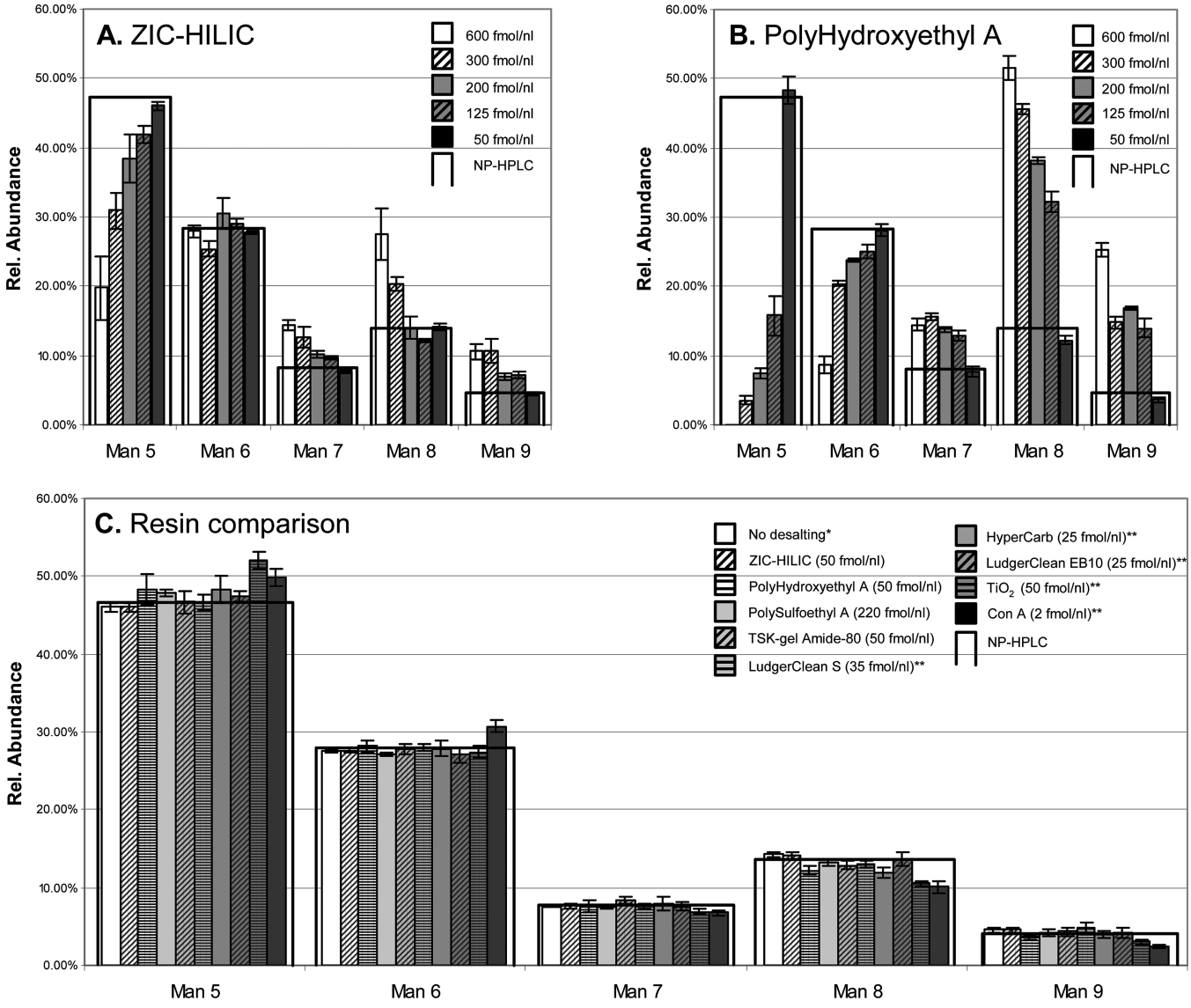

Figure 5. Investigation of potential bias in glycopeptide SPE-purification. RNase B glycopeptides were selectively fractionated from the total peptide pool using C_{18} flow-through and applied to different traditional column materials packed in microcolumn format in various ratios of sample amount/column volume. The relative abundances, as determined using MALDI-TOF MS glycoprofiling, are presented when (A) ZIC-HILIC and (B) PolyHydroxyethyl A were used as column materials (see inset for color coding and sample amount/column volume ratios). (C) A panel of column materials were tested for potential bias (see inset for color coding). The reference profile obtained using NP-HPLC is indicated (black outline). For all experiments $n = 5$, and the standard deviations are shown. Standard deviations for the reference profiles are listed in Table 1. Columns ranging from 3−28 mm in length were used, and these values were converted into volume using a standard curve (Supporting Information Figure S2) to allow the capacity information to be used in other formats. * Samples analyzed without desalting produced spectra of poor quality, but nonetheless provided good quantitation. ** The listed ratios generated nonbiased data, but the binding capacity might be higher as a detailed capacity study was not performed for these materials. Approximate values are given for LudgerClean S and Con A since these were not packed as standard microcolumns.

for data interpretation (data not shown). Generally, 500−1000 shots were summed from various positions of individual spots to minimize matrix heterogeneities. In terms of matrix choice, DHB was readily used for profiling of glycopeptides containing neutral glycans without evident fragmentation. However, a significant loss of sialic acids was observed when DHB was used for sialylated glycopeptides (data not shown), and a "cooler" matrix was required. Hence, THAP was used as it has been shown to be sensitive to sialylated compounds while causing minimal prompt fragmentation in terms of COOH or sialic acid loss.[19] Small amounts of ammonium citrate were added to the THAP matrix to avoid adduct formation thereby increasing the $[M - H]^-$ signal. Both matrixes were too "hot" to allow quantitative data to be recorded using the reflector function (data not shown).

Importance of the Peptide Moiety in Glycopeptide Profiling. Evidently, accurate glycopeptide profiling was obtained irrespective of the nature of the peptide moiety as shown by the variety of peptide lengths and amino acid compositions analyzed, Table 2. Ranging from 4 to 32 amino acid residues, all glycopeptides yielded good quantitative data. In principle, glycoprofiling can also be performed of intact glycoproteins using MALDI-TOF MS as detection, although mostly small glycoproteins are applicable due to limited resolution at high

m/z values. Thus, profiling of intact RNase B was performed and showed good relative quantitation based on the peak intensities, Supporting Information Figure S1, parts A and C, indicating that site-specific glycoprofiling is independent of (poly)peptide length. Electrospray ionization MS is an alternative technique for analyzing glycosylated compounds,[38,46] and this "softer" ionization technique proved useful for precise profiling of RNase B on the intact, glycopeptide, and glycan level (Supporting Information Figure S1A). The sialylated fetuin glycopeptides were also analyzed using ESI MS, and profiles similar to the references were obtained (data not shown). This technique, however, faces the drawbacks of being more time-consuming, less robust, and suffers from charge-dependent ionization efficiencies among the glycoforms (Supporting Information Figure S1B), making deconvolution essential. However, with these obstacles in mind, ESI MS is a well-suited technique for glycoprofiling in particular of labile species, such as sialylated glycans and glycopeptides.

In order to generate glycopeptides of adequate length and separating different glycosylation sites on individual glycopeptides for multiglycosylated proteins, the choice of proteolytic enzyme is important. Trypsin fulfilled these criteria for the glycoproteins used in this study; however, ovalbumin was additionally digested with proteinase K to obtain a shorter glycopeptide. Although not important for the MS quantitation, short glycopeptides (~4−10 amino acid residues) benefit from high hydrophilicity, enabling purification from mixtures of (hydrophobic) peptides with HILIC, graphite, or C_{18} flow-through. Furthermore, short glycopeptides have overall mass values around 1500−4500 Da, where MALDI-TOF MS performs well in terms of mass accuracy, resolution, and sensitivity. Larger glycopeptides are often more difficult to purify based on hydropathity, but size exclusion chromatography has been successfully utilized.[47] In contrast to released glycans, which represent a fairly homogeneous population in terms of hydropathy, size, and structure, the physicochemical properties of glycopeptides vary dramatically depending on the peptide moiety. Consequently, development of universal enrichment strategies for glycopeptides, in particular when starting from complex mixtures, is tremendously challenging.

Sample Preparation of Glycopeptides. Even from pure or semipurified glycoproteins it is not trivial to selectively and quantitatively purify all glycopeptides. Here, different solid-phase extraction materials, mainly graphite or of hydrophilic character, packed in microcolumns (Supporting Information Figure S2) were tested for potential bias in glycopeptide purification, i.e., desalting prior to MS. RNase B glycopeptides were systematically applied to microcolumns in varying sample amount/column volume ratios, and the retained fractions were profiled using MALDI-TOF MS. These analyses were carried out using ZIC-HILIC and PolyHydroxyethyl A (Figure 5, parts A and B). For both materials, ratios of 50 fmol/nL provided accurate glycoprofiles. Increasing the ratio (≥130 fmol/nL) skewed the profile in favor of the highly mannosylated glycopeptides. Hence, exceeding the column capacity resulted in competitive binding among the glycopeptides, and the normally abundant Man 5−6 glycopeptides were outcompeted by the more hydrophilic glycopeptides, Man 7−9. A similar bias was observed for other column materials as well as for released *N*-glycan purifications, where it appeared that high salt concentrations (e.g., introduced for labeling) lowered the binding capacity dramatically (data not shown). Although intuitive, this observation stresses the need for ensuring sufficient column capacity. A range of other column materials were tested for potential bias (Figure 5C): five materials of hydrophilic nature (ZIC-HILIC, PolyHydroxyethyl A, PolySulfethyl A, TSK Amide-80, and LudgerClean S), two types of graphite (Hypersil and LudgerClean EB10), an affinity chromatography lectin (ConA), and TiO_2 (which possesses some HILIC properties). All the HILIC and graphite materials were able to generate nonbiased glycoprofiles, whereas the Con A and TiO_2 purification in our hands deviated slightly from the reference profile. In conclusion, by ensuring sufficient column capacity, a wide variety of materials can be used to desalt glycopeptides without the loss of quantitative information. However, the ability of the individual materials to selectively enrich glycopeptides from peptide mixtures was not addressed here and remains to be investigated. Though all sample handling, in addition to purification, potentially can generate a glycoprofile bias, it appears that the established and commonly used procedures for glycan and glycopeptide manipulation, e.g., protease/glycosidase digestion, fluorescence labeling, and permethylation, all can be employed without introducing a detectable quantitative bias.

CONCLUSION

In recent years, the field of glycoproteomics has gradually gained more popularity as glycoprofiling providing site-specific information is needed to advance the understanding of glycan function. For maximum benefit, methods for rapid, sensitive, and accurate quantitative analyses of glycopeptides are essential to extract valuable data. It is well-known that MALDI-TOF MS features high sensitivity and robustness and can be used in a high-throughput manner. As for its quantitative features, evidence was here presented that confirms the correlation between MALDI-TOF MS signal strength of populations of different glycosylated glycopeptides and their relative abundances. In particular, glycopeptides containing neutral glycans yielded excellent quantitation. The sialoglycopeptides showed a minor loss of sialic acid residues, although good reproducibility and quantitation was still achieved. In terms of sample preparation of glycopeptides, various hydrophilic and graphite materials in microcolumn format all demonstrated quantitative purifications when used as the desalting step prior to MS detection. However, column capacity proved to be a critical parameter and highlighted the importance of ensuring a nonbiased sample handling. In conclusion, this study validated the use of MALDI-TOF MS for accurate site-specific glycoprofiling, and it is anticipated that the technique will be used increasingly in the future for quantitative characterization of N-linked glycoproteins, monitoring of batch-to-batch glycosy-

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lation consistency of recombinant glycoproteins, and for discovery of glycosylated biomarker candidates.

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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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