Focused Glycoprotemics

DOI: 10.1002/anie.200702919

Reverse Glycoblotting Allows Rapid-Enrichment Glycoproteomics of Biopharmaceuticals and Disease-Related Biomarkers**

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There has been a rapid increase in the number of and demand for approved biopharmaceuticals expressed by using animal-cell cultures over the last few years.[1] Glycan microheterogeneity is an important quality parameter of biopharmaceuticals with regard to drug stability, clinical activity, and immunogenicity.[2,3] Thus, detailed knowledge of protein glycosylation at the proteomics level involving structural information of both the glycan microheterogeneity and the backbone peptide sequence is of growing importance in postgenomic science and clinical research.[4] However, the enrichment and direct determination of individual glycopeptides derived from naturally or engineered glycoproteins in vivo have not been routinely possible to date[5,6] because they typically require tedious and time-consuming separation of the glycosylated peptides of interest from extremely complex mixtures before analysis. Since glycoproteomic approaches may also uncover new clinical biomarkers, there is a substantial need for a more rapid and general method for the enrichment analysis of peptides bearing *N*-glycans of interest.[7,8] Herein we describe such an approach to glycoproteomic analysis that focuses on sialic acid terminated *N*-glycans, the most important human glycoforms. The method, which involves highly selective oxidation of the terminal sialic acid residues of glycopeptides and subsequent enrichment by chemical ligation with a polymer reagent, namely *reverse glycoblotting*, should be of widespread utility for isolating and identifying sialylated glycopeptides present in highly complex peptide mixtures and for glycoproteomics-based discovery research into new classes of disease-related biomarkers.

We devised a method for the selective enrichment of sialylated glycopeptides from a tryptic peptide mixture by using glycoblotting technology[9] in combination with site-specific oxidation of the terminal sialic acid residues (Figure 1 A). With consideration of the fact that periodate oxidation of carbohydrates can be controlled and can afford different types of products according to the conditions employed,[10–12] the optimized conditions used here (1 mM sodium periodate, 0 °C for 15 min) were chosen to assure rapid, selective, and quantitative oxidation of sialic acid residues with terminal linear triols at the C7, C8, and C9 positions into the reactive derivatives with an aldehyde group at the C7 position (Figure 1 B). It should be emphasized that this chemoselective conversion proceeds quantitatively and specifically in all compounds bearing at least one sialic acid residue. The highly reactive aldehyde groups generated on the sialic acid residues can then be enriched quantitatively at 37 °C for 2 h by chemical ligation with Fischer type polymer reagents, such as aminooxy-functionalized polyacrylamide,[9,13] to form stable oxime bonds. We note that hydrazide-carrying polymers can alternatively be used for this purpose. After purification by simple gel filtration to separate nonsialylated glycopeptides and the large excess of peptides, the sialylated glycopeptides captured covalently by Fischer type polymer were treated with 3% trifluoroacetic acid (TFA) aqueous solution at 100 °C for 1 h to digest selectively at the α-glycoside bonds between the sialic acid and adjacent galactose residues. (It should be noted that α-L-fucoside linkages are stable under the above conditions, while α-sialosides of glycopeptides are labile at a range from pH 2 to pH 3 and are selectively hydrolyzed under the conditions optimized in this study.) Finally, recovered asialoglycopeptides were analyzed by MALDI-TOF and TOF/TOF mass spectrometry (MS) to determine both the glycan structure and peptide sequence of each glycopeptide concurrently, essentially as described previously.[14,15] Although lectin-affinity- and hydrophilic-nature-based chromatography have also been employed for the purification of glycopeptides,[5,6] the enrichment efficacy is strongly influenced by the affinity of individual glycopeptides so these methods are not suited for large-scale glycoproteomics with small quantities of glycoproteins, serum, cells, or tissues.

To illustrate the new method, three glycoproteins, human fetal-cord serum α-fetoprotein (AFP), bovine pancreas fibrinogen, and recombinant human erythropoietin (rHuEPO), expressed in Chinese hamster ovary (CHO) cells were subjected to protease digestion and subsequent

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[**] This work was partly supported by funding from the Japan Science and Technology Agency (JST) for "Development of system and technology for advanced measurement and analysis (SENTAN)" and for "Innovation COE Program for Future Drug Discovery and Medical Care". We are grateful to Dr. Y. Shinohara, Dr. Y. Miura, and Dr. H. Nakagawa for valuable suggestions and discussions on mouse serum *N*-glycan profiling.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

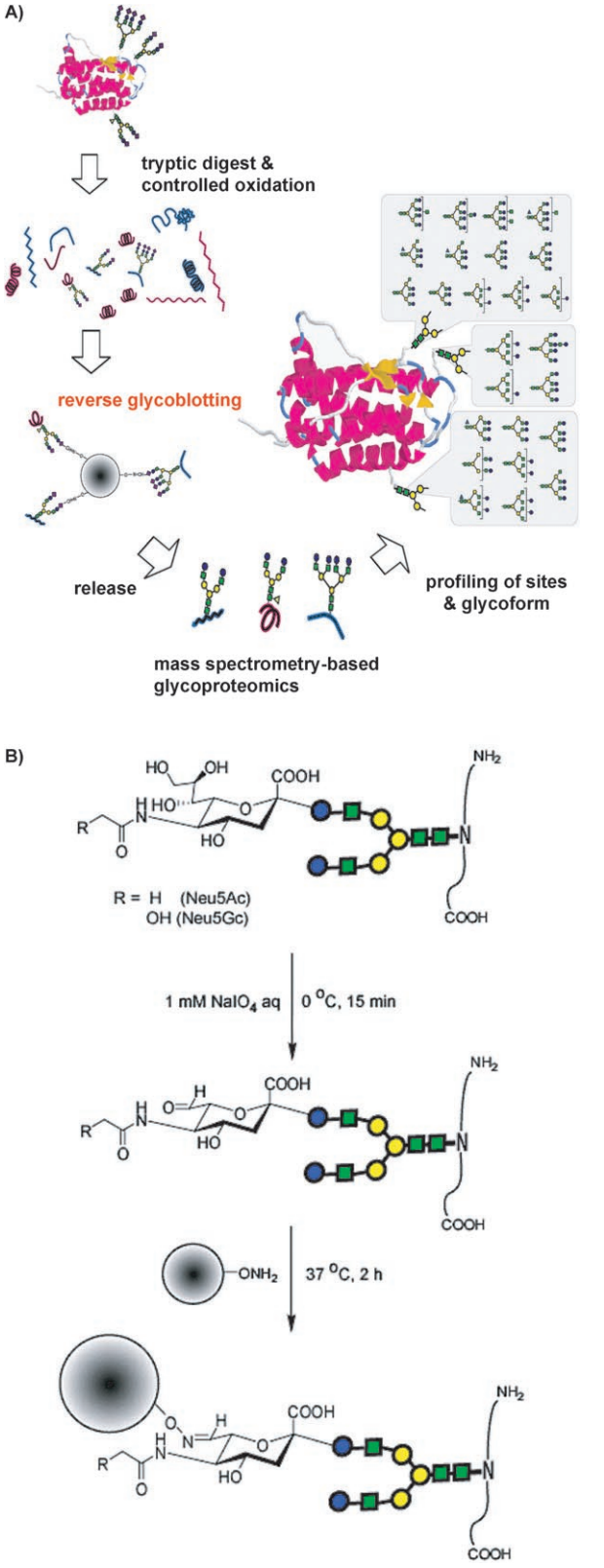

Figure 1. General concept for reverse-glycoblotting-based glycoproteomics. A) Schematic representation showing the protocol of the glycoproteomic approach for profiling glycan microheterogeneity and sites of glycosylation. B) Reagents and conditions for the selective oxidation and reverse glycoblotting (oxime bond formation) of sialic acid residues. Neu5Ac: *N*-acetylneuraminic acid; Neu5Gc: *N*-glycolylneuraminic acid; green squares: *N*-acetyl-D-glucosamine (GlcNAc); yellow circles: D-mannose (Man); blue circles: D-galactose (Gal); triangles: L-fucose (Fuc).

treatment for selective enrichment as described above. Glycopeptides enriched by the above protocol were analyzed by MALDI-TOF/TOF MS and identified. As indicated by a typical procedure that identified glycoforms of AFP, the merit of the present strategy is evident. From the MALDI-TOF mass spectra of the tryptic digest measured before (Figure 2A) and after (Figure 2B) enrichment by the reverse-glycoblotting technique, an important feature of the method is immediately apparent because two precursor ions observed at *m*/*z* 2601.40 and 2747.45 (Figure 2B) due to the major glycosylated peptides of AFP can not be detected in the mass spectrum of all the tryptic peptides (the entire tryptic digest without enrichment by reverse glycoblotting) at all (Figure 2A). This result also indicates that glycosylation drastically suppresses the ionization efficacy of the parent (naked) peptides. Therefore, it seems likely that selective enrichment of glycosylated peptides from the whole tryptic peptide mixture, or complete removal of the nonglycosylated peptides, is crucial for a practical glycoproteomic approach on the basis of mass spectrometry. TOF/TOF MS analysis of the two precursor ions mentioned above by using the matrix-dependent selective-fragmentation (MDSF) method[14] revealed that the peptide sequence of the enriched two glycopeptides is ^{232}V*N*FTEIQK^{239}, in which the N233 (asparagine) residue has two different glycoforms, (Galβ1,4GlcNAcβ1,6Man)$_2$α1,3&α1,6Manβ1,4GlcNAcβ1,4GlcNAc and (Galβ1,4GlcNAcβ1,6Man)$_2$α1,3&α1,6Manβ1,4GlcNAcβ1,4-(Fucα1,6)GlcNAc, respectively (Figure 2C and Supporting Information). Interestingly, this microheterogeneity in the fucosylation of human AFP seems to be a more sensitive diagnostic biomarker for the status of a liver disease such as human hepatocellular carcinoma (HCC) in liver cirrhosis (LC) patients than the total serum AFP concentration.[16] Therefore, glycoproteomic analysis of known protein biomarkers should reveal novel structural features that lead to much more specific clinical signals for use in predicting the precise disease status than those obtained only by common proteome-based characterization.

In a similar manner, glycoforms of bovine pancreas fibrinogen and rHuEPO were analyzed and identified (Table 1). Structural characterization of glycopeptides enriched from bovine pancreas fibrinogen yields two and three different glycoforms of ^{73}QVE*N*K^{77} and ^{368}VGE*N*R^{372}, respectively (Supporting Information). Three ion peaks observed at *m*/*z* 2060.9, 1831.3, and 2034.8 were identified as peptides bearing a biantennary glycan chain with an alternative terminal Man or GlcNAc residue, thereby demonstrating that monosialylated glycans can also be enriched efficiently by the reverse-glycoblotting technique. We demonstrated that rHuEPO exhibits 8, 4, and 12 different glycoforms of ^{43}LLEAKEAE*N*I^{52}, ^{63}NE*N*I^{66}, and ^{108}LV*N*SSQPW^{115}, which indicates that this method potentially makes it possible to identify the quality of biopharmaceuticals in terms of the asialo-*N*-glycan microheterogeneity at the individual glycosylation sites (Figure 2D; see also Supporting Information). These results clearly indicate that three glycosylation sites of rHuEPO have highly heterogeneous glycoforms composed of bi-, tri-, and tetraantennary *N*-glycans in addition to the microheterogeneity due to the

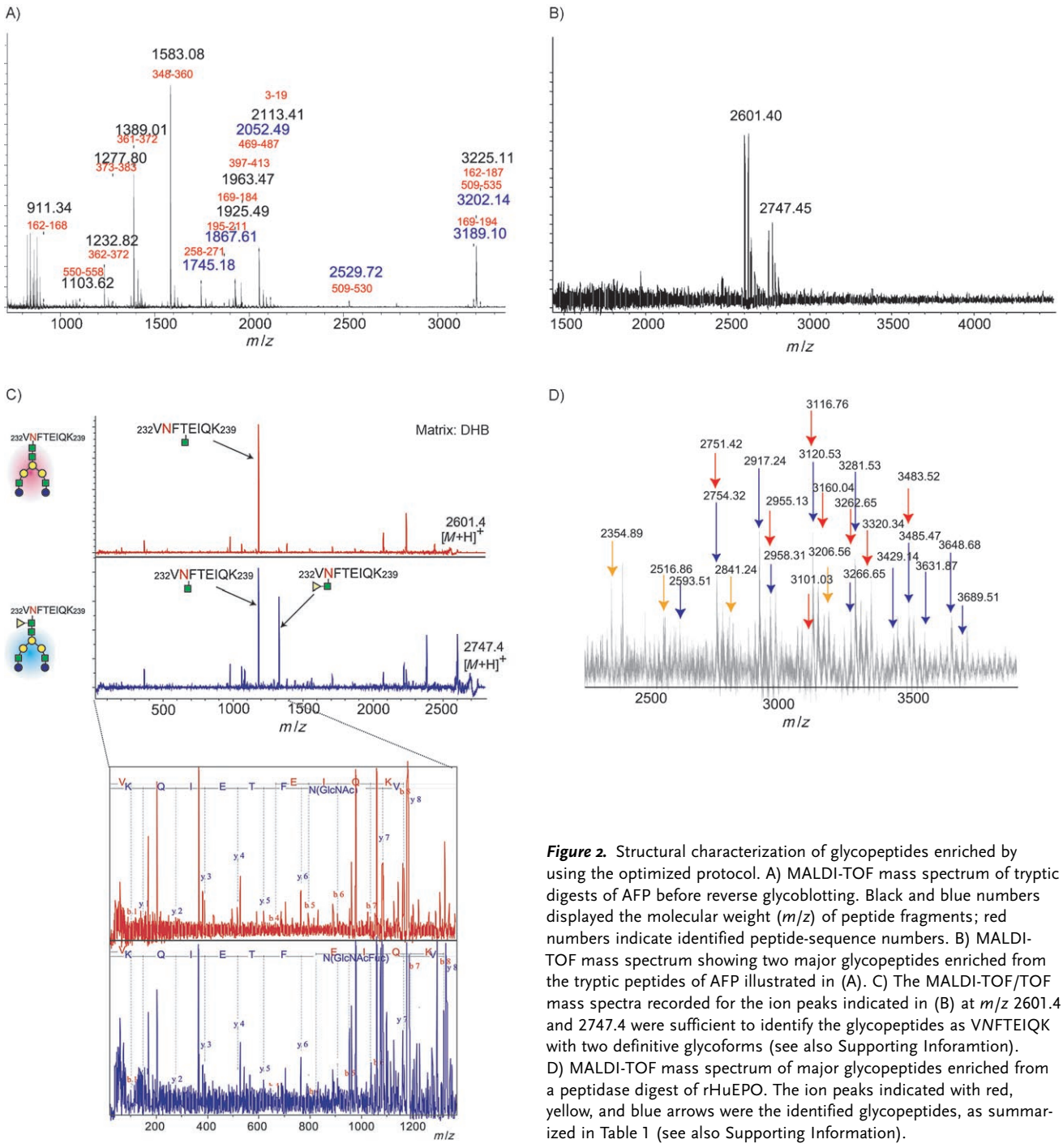

Figure 2. Structural characterization of glycopeptides enriched by using the optimized protocol. A) MALDI-TOF mass spectrum of tryptic digests of AFP before reverse glycoblotting. Black and blue numbers displayed the molecular weight (*m*/*z*) of peptide fragments; red numbers indicate identified peptide-sequence numbers. B) MALDI-TOF mass spectrum showing two major glycopeptides enriched from the tryptic peptides of AFP illustrated in (A). C) The MALDI-TOF/TOF mass spectra recorded for the ion peaks indicated in (B) at *m*/*z* 2601.4 and 2747.4 were sufficient to identify the glycopeptides as VNFTEIQK with two definitive glycoforms (see also Supporting Inforamtion). D) MALDI-TOF mass spectrum of major glycopeptides enriched from a peptidase digest of rHuEPO. The ion peaks indicated with red, yellow, and blue arrows were the identified glycopeptides, as summarized in Table 1 (see also Supporting Information).

fucosylation at the chitobiose core region.[17] It has been reported that neutralizing antierythropoietin antibodies and pure red-cell aplasia can develop in patients with the anemia of chronic renal failure during treatment with rHuEPO produced by using CHO cells.[2] Glycoforms of rHuEPO also seem to have great influence on potential biological functions as tissue-protective agents.[18] Therefore, we think that reverse-glycoblotting-based analysis will become a nice tool for the rapid and highly efficient characterization of widespread recombinant glycoprotein drugs. When galactose oxidase is employed for the selective oxidation, asialoglycopeptides can be enriched from tryptic peptide mixtures through a coupling reaction with the aldehyde generated at the C6 position of the terminal galactose residue.[9] When it is considered that mammalian complex and hybrid-type *N*-glycans usually involve a terminal sialic acid residue or at least a galactose residue (desialylated forms of abundant *N*-glycans), the reverse-glycoblotting approach should greatly facilitate quantitative enrichment analysis of major glycopeptides of interest. However, we note that this method still needs to be improved because the terminal sialic acid residues must eventually be deleted from the enriched glycopeptides

Table 1: Glycoproteomic analysis of AFP, fibrinogen, and rHuEPO.

Protein	Peptide sequence[a] peptide mol. weight (calcd)	Glycoform glycan mol. weight (calcd)	Glycopeptide found mass (calcd mass $[M+H]^+$)
α-fetoprotein	$_{232}$VNFTEIQK$_{239}$ 977.52	1640.59	2747.45 (2747.16)
		1786.57	2601.40 (2601.10)
fibrinogen	$_{73}$QVENK$_{77}$ 616.32	1640.59	2222.80 (2239.91)
		1478.55	2060.90 (2077.87)
	$_{368}$VGENR$_{372}$ 573.29	1640.59	2196.80 (2196.87)
		1478.55	2034.78 (2034.83)
		1275.48	1831.30 (1831.81)
huEPO	$_{63}$LLEAKEAENI$_{52}$ 1128.60	2151.68	3483.52 (3482.30)
		2005.65	3320.34 (3319.26)
		2046.63	3116.76 (3117.24)
		2370.71	2751.42 (2751.18)
		1640.59	3262.65 (3262.27)
		1843.61	3160.04 (3158.22)
		1989.64	2955.13 (2954.2)
		2208.67	3101.03 (3100.23)
	$_{63}$NENI$_{66}$ 488.22	2046.63	3206.56 (3205.98)
		2735.77	2841.24 (2840.92)
		1884.59	25.16.86 (2516.84)
		2370.71	2354.89 (2354.80)
	$_{108}$LVNSSQPW$_{115}$ 929.46	2573.73	3689.51 (3688.20)
		2735.77	3429.14 (3428.19)
		2776.75	2958.31 (2958.08)
		2719.76	3648.68 (3647.22)
		2208.67	3281.53 (3282.16)
		2354.70	2917.24 (2917.10)
		2370.71	3631.87 (3631.21)
		2516.74	3266.65 (3266.15)
		2005.65	2754.32 (2755.06)
		1843.61	3485.47 (3485.18)
		2046.63	3120.53 (3120.12)
		1681.57	2593.51 (2593.02)

[a] The red N indicates the asparagine residue that is the site of glycosylation.

in the present protocol. This feature will be addressed by integrating procedures for 1) quantitative methyl esterification of sialic acid residues,[19] 2) *trans*-oximization (iminization) based tagging,[20,21] and 3) highly sensitive and stable isotope labeling.[15,22–25] In the case of glycopeptides bearing high-mannose-type *N*-glycan chains, we may use conventional lectin (Con A) affinity based enrichment, although this approach is suited for relatively high abundance glycoproteins, as exemplified in mouse-skin glycoproteomics.[15]

Our interest was next directed toward the feasibility of this method in the glycoform-focused reverse-proteomics/genomics approach[15] toward the discovery of new disease-related biomarkers. To this end, we chose to test all of the serum glycoproteins of diabetes-model (C57BL/KsJ *db*/*db*) and control (age-matched *db*/+) mice. In this case, we preliminarily applied our optimized protocol for general glycomic analysis to profile asialo-*N*-glycans derived from sialylated glycopeptides of interest. The modified protocol includes the following sequential steps: 1) Peptidase digests of glycoproteins prepared from mouse total serum (5 μL) are subjected to treatment with protein *N*-glycanase F (PNGase F, 100 mU) at 37 °C for 3 h to release whole *N*-glycans from the core peptides. 2) The mixtures of all of the *N*-glycans and peptides are allowed to react with 1 mM sodium periodate at 0 °C for 15 min and subjected to reverse glycoblotting with a Fischer type polymer reagent (1 μg μL^{-1}) at 37 °C for 2 h. Notably, the optimized conditions employed for selective enrichment of the aldehyde groups of the oxidized sialic acid residues (37 °C for 2 h) does not cause a reaction with any hemiacetal groups of common oligosaccharides. As described in our previous reports,[9,19–21] glycoblotting for general sugar hemiacetals (free glycans) needs to be conducted at a much higher temperature (80 °C) than that for aldehydes or reactive carbonyl groups.[13] To remove the large excess of peptides and asialo-*N*-glycans, the polymer is purified by simple gel filtration with a Sephadex G-50 column. 3) Isolated polymer is treated with 3 % TFA at 100 °C for 1 h to release the target oligosaccharides as asialo-*N*-glycans. Finally, the purified asialo-*N*-glycans are analyzed and identified by MALDI-TOF MS. It was demonstrated that the peak intensity in MALDI-TOF MS can be directly used for semiquantitative profiling of asialo-*N*-glycans because common neutral oligosaccharides exhibit quite similar ionization efficacy under positive-ion mode.[14] We expected that reverse-glycoblotting-based (sialic acid targeted) total-serum glycomics of control and diabetic mice would provide significant differences in their glycoforms to be focused for further reverse-proteomic/genomic analysis. As expected, we identified nine major precursor-ion peaks due to enriched glycoforms as asialo-*N*-glycans that were detectable in both samples and we normalized their abundance to the total peak-height intensity. We found a significant increase in the level of core α-1,6-fucosylation observed at *m*/*z* 1809.66 and 2174.80 in case of the diabetic mice (Figure 3 A). To identify the glycoproteins bearing the above-mentioned glycoforms (*N*-glycans containing both sialic acid(s) and core α-1,6-fucose residues), we next carried out focused glycoproteomics by means of the reverse-glycoblotting-based enrichment protocol for total-serum glycoproteins (Figure 3 B). Although the results shown in the MALDI-TOF mass spectra confirmed that the glycopeptides enriched from the diabetic mice can be readily detected as a more complex mixture than those of the control mice, we subjected the enriched glycopeptides obtained from the total serum to offline HPLC–MALDI-TOF MS (Supporting Information) and identified the abundant glycopeptide ions at *m*/*z* 3160 and 3306 (Figure 3 C) as a definitive signature of the presence or absence of core α-1,6-fucosylation. Further precise structural characterization by TOF/TOF MS and

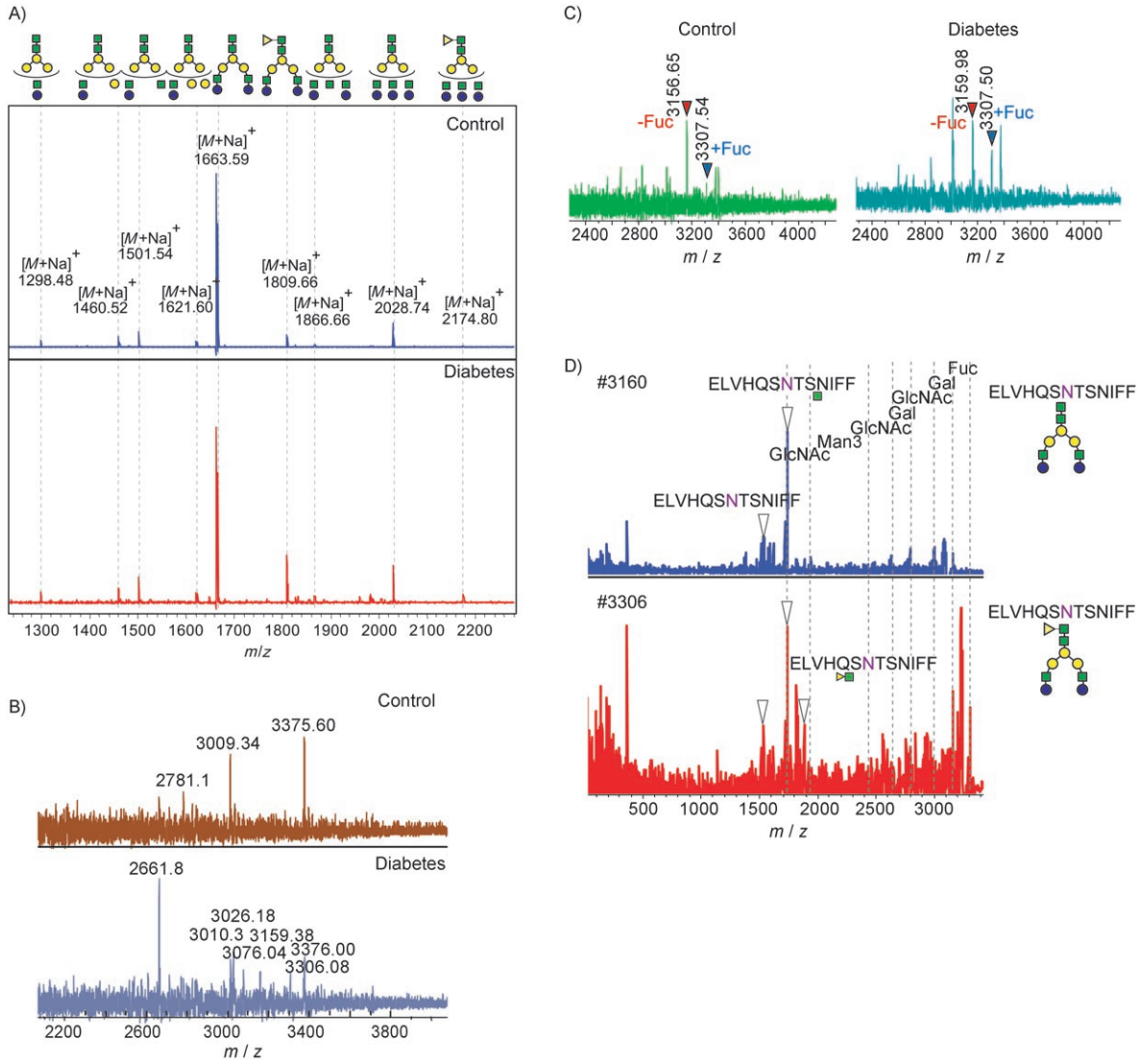

Figure 3. A rapid and efficient strategy for the determination of mouse diabetes biomarkers based on the combined glycomic and glycoproteomic approaches by using the reverse-glycoblotting technique. A) MALDI-TOF mass spectra showing major asialo-*N*-glycans enriched from the total serum of control and diabetic mice by using the modified reverse-glycoblotting technique for focused (sialic acid targeted) glycomics. B) MALDI-TOF mass spectra showing whole glycopeptides enriched from the peptidase-digest mixtures of control and diabetic mice. C) MALDI-TOF mass spectra showing identified glycopeptides recorded after fractionation (see Supporting Information). The blue and red arrows indicate significant differences in the glycan microheterogeneity due to the presence (*m*/*z* 3307) or absence (*m*/*z* 3160) of a fucose residue. D) Identification of a new glycoprotein biomarker for mouse diabetes was carried out by MALDI-TOF/TOF mass analysis of the precursor ions suggested in (C) (see also Supporting Information). Our results revealed that an ELVHQS*N*TSNIFF peptide arising from serine proteinase inhibitor 1 displays two definitive glycoforms.

Mascot search analysis (Figure 3D and Supporting Information) revealed that the ELVHQS*N*TSNIFF peptides carrying the identified glycoforms arise from the abundant glycoprotein serine proteinase inhibitor 1 (Q8VC20_MOUSE). These results clearly suggest that the enhanced core α-1,6-fucosylation observed in both glycomic (Figure 3A) and glycoproteomic analyses (Figure 3C) of the diabetic mice greatly depends on the glycan microheterogeneity of serum serine proteinase inhibitor 1. This result clearly means that this type of distinct glycoform alteration in the specified glycoprotein becomes a potential candidate for new diagnostic biomarkers. However, we could not identify the glycoprotein(s) with a triantennary *N*-glycan chain and a core α-1,6-fucose residue because the glycopeptide(s) seem to arise from lower abundance glycoprotein(s), as seen in the results of the glycan profiling (Figure 3A). Given that the number of enriched glycopeptides from the mouse total serum seems to be more than several thousand, even when the reverse-glycoblotting technique is used, fractionations either before or after this enrichment protocol should greatly facilitate identification of low-abundance sialylated glycoproteins.[12,26] We also identified other glycopeptides arising from serine proteinase inhibitor 1 or its isoform (NP-033270_MOUSE) and from pregnancy zone protein (Q6PEM2_MOUSE) as having non-fucosylated bi- and triantennary *N*-glycans, respectively (Supporting Information). We are currently investigating the use of a stable isotope tag[15a,27] to achieve quantitative glycoproteomics in reverse glycoblotting and the result will be reported shortly.

In summary, we have described a method for enriching tryptic glycopeptides with sialic acid residue(s) and for subsequently identifying glycoforms (glycan microheterogeneity) of both asialo-*N*-glycans and *N*-glycosylation sites. This protocol appears to be well suited to rapid and high-throughput sialylglycopeptide enrichment from complex tryptic peptide mixtures before MS analyses. It should be emphasized that the reverse-glycoblotting technique greatly facilitates glycoproteomic analysis of a wide range of naturally occurring and engineered glycoproteins involved in biopharmaceuticals expressed by various animal-cell cultures. Here, we demonstrate for the first time that the glycoform-focused proteomic approach by using reverse glycoblotting is feasible for discovery research into disease-related biomarkers with total serum. With its additional utility for describing quantitative changes in the *N*-glycosylation state of glycoproteins of interest through the use of on-bead manipulations, such as methyl esterification of sialic acids[19] and modification with appropriate stable isotope tags,[15,20,21] the method could enhance the potential of reverse-glycoblotting-based glycoproteomics and expand its scope in the near future.

Experimental Section

Selective and quantitative oxidation of glycoproteins: Fibrinogen (50 μg), AFP (50 μg), rHuEPO (30 μg), or mouse total serum (5 μL) were treated with 10 mM dithiothreitol (DTT; 100 μL) in 100 mM NH_4HCO_3 at 60 °C for 1 h. The mixture was then added to and incubated with 50 mM iodoacetamide (100 μL) in 100 mM NH_4HCO_3 at 37 °C for 30 min. After precipitation by addition of acetone (1.8 mL), the alkylated glycoproteins were subjected to digestion with trypsin (for AFP) or a mixture of trypsin and α-chymotrypsin (for fibrinogen, rHuEPO, and mouse total serum) in 50 mM NH_4HCO_3 (the peptidase concentration was adjusted to be approximately 5 mg mL^{-1} and employed with a ratio of 1:50 against the glycoproteins) at 37 °C for 16 h. After evaporation of the solution, the residual peptidase digests were mixed with 3 mM $NaIO_4$ (50 μL) and allowed to react at 0 °C for 15 min. The reaction was quenched by addition of an equivalent amount of 15 mM $Na_2S_2O_5$ (10 μL) to prevent additional oxidation and the mixture was used directly for the next glycoblotting.

Enrichment of oxidized sialylglycopeptides by reverse glycoblotting: Fischer type polymer (40 μL; final concentration of the polymer was adjusted to be 1 μg μL^{-1}) was added to the solution of oxidized glycopeptides (60 μL) and the mixture was incubated at 37 °C for 2 h. The polymer solution was subjected to purification by Sephadex G-50 column chromatography (0.6 × 10 cm, 3 mL) with water as the eluent. The isolated polymer fraction (approximately 1 mL) was mixed and treated with 10% trifluoroacetic acid (TFA) solution (300 μL) at 100 °C for 1 h to cleave the glycoside bond between the sialic acid and galactose residues. Neutral asialoglycopeptides were then isolated by purification by Sephadex G-50 column chromatography (0.6 × 10 cm, 3 mL). To remove trace amounts of TFA, the crude products were subjected to coevaporation with 50 mM NH_4HCO_3 by using a centrifugal evaporation system because a few picomoles of glycopeptide usually give satisfactory MALDI-TOF mass spectra in the absence of TFA.

Received: July 1, 2007
Published online: October 17, 2007

Keywords: glycoblotting · glycoproteins · proteomics · reverse genomics · sialic acids

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