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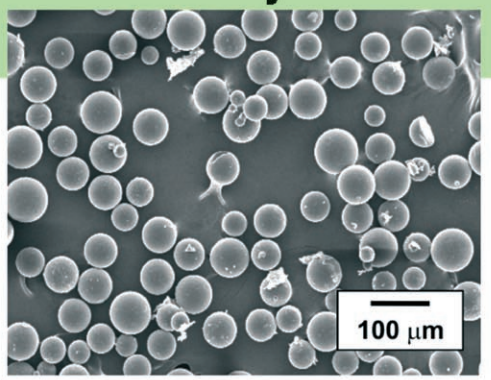
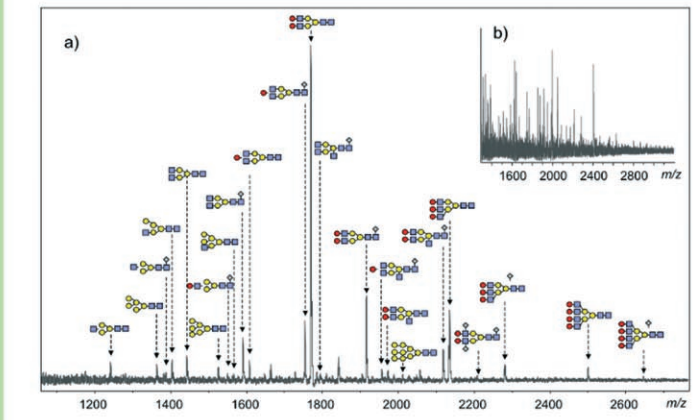
Solid-Phase Glycoblotting and Transoximization for Glycomic and Glycoproteomic Analyses





One-Pot Solid-Phase Glycoblotting and Probing by Transoximization for High-Throughput Glycomics and Glycoproteomics

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Abstract: The development of rapid and efficient methods for high-throughput protein glycomics is of growing importance because the glycoform-focused reverse proteomics/genomics strategy will greatly contribute to the discovery of novel biomarkers closely related to cellular development, differentiation, growth, and aging as well as a variety of diseases such as cancers and viral infection. Recently, we communicated that rapid and efficient purification of carbohydrates can be achieved by employing sugar-specific chemical ligation with aminooxy-functionalized polymers, which we termed "glycoblotting" (see S.-I. Nishimura et al., *Angew. Chem.* **2005**, *117*, 93–98; *Angew. Chem. Int. Ed.* **2005**, *44*, 91–96). The chemoselective blotting of oligosaccharides present in crude biological materials onto synthetic polymers relies on the unique oxime-bond formation between aminooxy group displayed on the supporting materials and aldehyde/ketone group at the reducing terminal of all oligosaccharides, thus enabling highly selective and rapid oligosaccharide purification. Aiming to improve the detection sensitivity of the released oligosaccharides, we introduce here a novel strategy for one-pot solid-phase glycoblotting and probing by transoximization. We found that oligosaccharides captured by the polymer supports via the oxime bond can be released in the presence of excess O-substituted aminooxy derivatives in a weakly acidic condition. The released oligosaccharides could be recovered as newly formed oxime derivatives of the O-substituted aminooxy compound added, thus demonstrating the simultaneous releasing and probing. In addition, we synthesized a novel aminooxy-functionalized monomer, *N*-[2-[2-(2-*tert*-butoxycarbonylaminooxyacetylamino-ethoxy)ethoxy]ethyl]-2-methacrylamide, which allows for the large-scale preparation of a versatile polymer characterized by its high stability, high blotting capacity, and easy use. The one-pot protocol allowed to profile 23 kinds of N-glycan chains of human serum glycoproteins. This concept was further applied for the glycopeptides analysis in a crude mixture followed by galactose oxidase treatment to generate free aldehyde group at the non-reducing terminal of oligosaccharide moiety of glycopeptides. Our technique may be implemented in existing biochemistry and molecular diagnostics laboratories because enriched oligosaccharides and glycopeptides by solid-phase transoximization with high-sensitive labeling reagents are widely applicable in a variety of common analytical methods using two-dimensional HPLC, LC/MS, and capillary electrophoresis as well as modern mass spectrometry.

Keywords: carbohydrates · glycoblotting · glycomics · glycoproteomics · mass spectroscopy

Introduction

Posttranslational protein glycosylation plays an important role in the biological and physical properties of glycoconjugates, which include functions as signals/ligands to control their distribution, antigenicity, metabolic fate, stability, and solubility.[1–3] The structural diversity of oligosaccharide chains of a glycoprotein, namely "glycoform", is also associ-

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ated with various developmental stages and pathological states. Therefore, it is important to investigate the glycan expression profiles (glycomics) and whole structure of glycoproteins (glycoproteomics) in order to understand the functional roles of posttranslational modification by dynamic glycosylation. To date, glycoform analyses have been most commonly carried out by means of chromatographic and electrophoretic techniques in combination with derivatization with some photosensitive reagents,[4] which is a powerful approach for profiling major known oligosaccharides of glycoproteins or proteoglycans.[5] In addition, once oligosaccharide can be purified from other biological materials, a number of derivatization and analytical techniques can be applicable for the qualitative and quantitative analysis. Recent advances in high-performance mass spectrometric technologies such as electrospray ionization mass spectrometry (ESI-MS), matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), and electron capture-induced dissociation using Fourier transform mass spectrometry (ECD-FTMS) have made precise structural characterization of extremely small amount of oligosaccharide materials possible.[6–8] However, we should point out that the difficulty in glycoform analysis greatly depends on the fact that the purification of trace amount of oligosaccharides in the biological samples requires extremely tedious and time-consuming multi-step procedures. This is due to the fact that crude sample mixtures prepared by enzymatic digestion of glycoproteins from, for example, cells, organs, serum, usually contain large amounts of impurities such as peptides, lipids, DNA/RNA and salts.[9] These serious problems in the purification of trace amounts of significant oligosaccharides make it extremely difficult to achieve high-throughput quantitative protein glycomics.

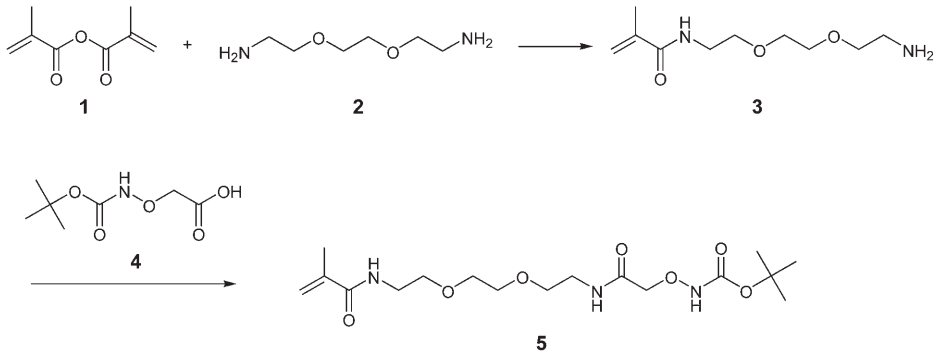
In our previous studies, we demonstrated that aminooxy-functionalized liposomal nanoparticles and water-soluble polymers[10–11] can serve as novel base matrices for selective isolation of oligosaccharides from a crude mixture through a simple procedure, namely "glycoblotting". Oligosaccharides captured by blotting polymers can be released selectively under mild acidic conditions. Chemoselective ligation and cleavage of the oxime bond permitted an efficient "catch-and-release" of the non-labeled reducing oligosaccharides by the liposomal nanoparticles and they were subsequently characterized by MALDI-TOF MS analysis. The glycoblotting method has great potential as a tool for functional protein glycomics study owing to its specific feature of rapid and facile procedure for the chemoselective isolation of oligosaccharides.

Considering that many of functionally important oligosaccharides exist in small quantities and their heterogeneity is usually very high, incorporation of a strategy enabling an efficient derivatization of oligosaccharides will further expand its usefulness in structural and functional glycomic studies. Ideally, it should be a one-pot reaction system from glycoblotting (ligation) to labeling (probing). Recently, Hindsgaul and co-workers reported solid-phase oligosaccharide tagging.[12] They captured oligosaccharides onto aminooxy-functionalized polymer having cleavable linker; subsequently the oxime double bond was reduced and the resulting reactive sp^3 nitrogen atom was selectively labeled with aryl isothiocyanates. Meanwhile, our attention has been focusing on the feasibility of transoximization reaction (oxime-exchange reaction) between small O-substituted hydroxylamine derivatives and oligosaccharides trapped onto polymers through an oxime-bond formation. Transoximization has been implemented recently in a number of cases concerning the development of dynamic combinatorial libraries since the late Nineties,[13–19] but was never applied to the hemiacetal group present at the reducing terminal of oligosaccharide. We thought that transoximization may be perfectly compatible with glycoblotting technology since transoximization may allow simultaneous derivatization upon releasing from the blotting polymer by adding aminooxy functionalized low molecular weight compound(s).

In this study, we also investigated the preferred formula of the base polymer which is ideal for the glycoblotting procedure. We attempted to prepare a high density aminooxy-functionalized polymer, since the blotting efficiency is highly dependent on the concentration of free aminooxy residue as reported in our previous paper.[11] The solid support was designed to be both mechanically and chemically stable, applicable in various formats (e.g., micro-filter plate, packed column), and bulk producible. High chemical stability appeared to be especially important since the polymer should be stable enough against harsh washing with organic solvent and/or detergent to remove non-specifically adsorbed impurities. We prepared a rigid and micron-sized polymer particle by synthesizing a novel aminooxy-functionalized monomer. The proposed one-pot solid-phase glycoblotting and probing by transoximization was applied for high-throughput human serum glycomics. Finally, a novel strategy for high-throughput glycoproteomic analysis that allows for precise structural analysis both of peptide core and oligosaccharide side chains of the chemoselectively enriched glycopeptides will be discussed.

Results and Discussion

Synthesis and characterization of novel polymer particle for glycoblotting: In order to establish a practical method of the glycoblotting for clinical or diagnostic assays, we thought that the base material must be enough stable against harsh washing using organic solvent and/or detergent for removal of adsorbed impurities. Covalently crosslinked solid-phase polymer with a diameter of 10–200 μm was considered to be advantageous in terms of stability and applicability to various separation platforms (e.g., filtration, centrifugation, packed column). We synthesized a vinyl compound with an aminooxy group (Scheme 1). For the subsequent polymerization of compound **5**, the Boc protecting group was removed to give the aminooxy functionalized polymer, which we termed "BlotGlyco". Particle size and shape could be varied by controlling the polymerization conditions such as



Scheme 1. Synthetic route of aminooxy-functionalized monomer.

monomer concentration, kind and amount of dispersion stabilizer, reaction temperature, and stirring speed. Figure 1 shows the SEM image of BlotGlyco, of which particle size

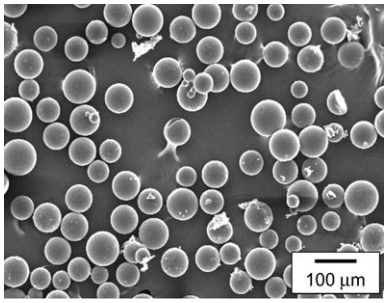


Figure 1. SEM view of polymer particles.

was estimated to be 50–150 μm (average ≈100 μm) in diameter. Particles of such sizes were then employed as they appeared to be suitable for the manipulations with standard centrifugation or filtration.

The theoretical concentration of aminooxy groups in BlotGlyco is as high as ≈3.3 μmol per mg, based on the assumption that the ratio of compound **5** and ethyleneglycol dimethacrylate (EGDMA) units in the polymer is identical to the feed ratio of these molecules. The concentration of active aminooxy groups in the polymer was estimated to be ≈2.7 μmol per mg by quantifying the binding capacity of glucose, thus our attempt to obtain a high-density aminooxy functionalized polymer was proven to be successful. It was also confirmed that the bound glucose via oxime formation was stable enough not to be cleaved during harsh washings by 0.5% sodium dodecyl sulfate (SDS) and/or 50% methanol (data not shown).

The glycoblotting procedure using BlotGlyco was first optimized with *N*-acetyl-D-lactosamine (LacNAc) as a model oligosaccharide. When LacNAc (1 nmol–1 μmol) was incubated with 2 mg of BlotGlyco in 2% acetic acid/acetonitrile at 80°C for 1 h, the amount of active aminooxy groups in the polymer was estimated to be ≈5 μmol (LacNAc was hardly detectable in the flow through fraction, see Figure 2a). This is contrary to the observation that when LacNAc was incubated with Boc protected BlotGlyco, nearly 100% of the added LacNAc was recovered in the flow through fraction (Figure 2b). This result indicates that the chemical ligation of LacNAc onto BlotGlyco is almost quantitative. Note that only five-fold molar excess of aminooxy functionality was found to be sufficient for the quantitative blotting of

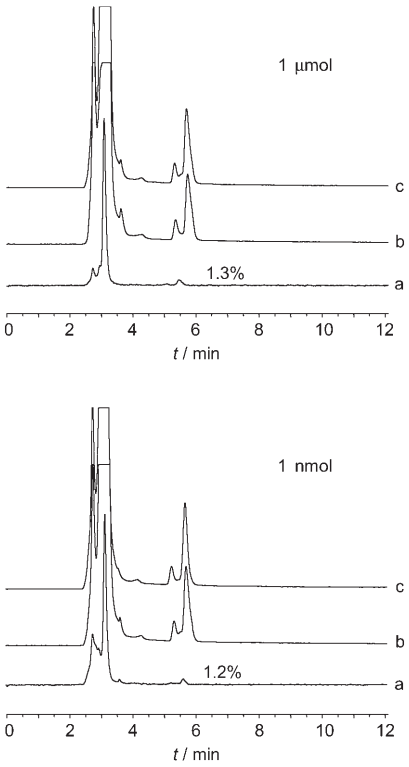


Figure 2. Chromatograms showing the efficiencies at different concentrations of chemoselective ligation of *N*-acetyllactosamine (LacNAc) onto aminooxy functionalized polymer (BlotGlyco). The amount of LacNAc used for blotting is specified on each chromatogram. The unreacted LacNAc in the presence of a) BlotGlyco and b) Boc-protected BlotGlyco were labeled with BODIPY FL hydrazide. They were compared with c) appropriate amount of LacNAc directly labeled with BODIPY FL hydrazide.

LacNAc. This high reactivity may be attributable to the high density of aminooxy functionalities on the polymer, as was actually intended.

Probing oligosaccharides by transoximization with aminooxy-containing small compounds: In the previous study, we employed acidic conditions (e.g. 5% trifluoroacetic acid) to cleave the oxime bond and to recover the captured glycans in its underivatized form.[10–11] Aiming to establish more

mild and versatile methods with simultaneous derivatization of the oligosaccharides upon recovery, we investigated the feasibility of transoximization reaction with O-substituted aminooxy compounds (Figure 3). Model oligosaccharides (ribonuclease B glycans containing 1 nmol of high-mannose

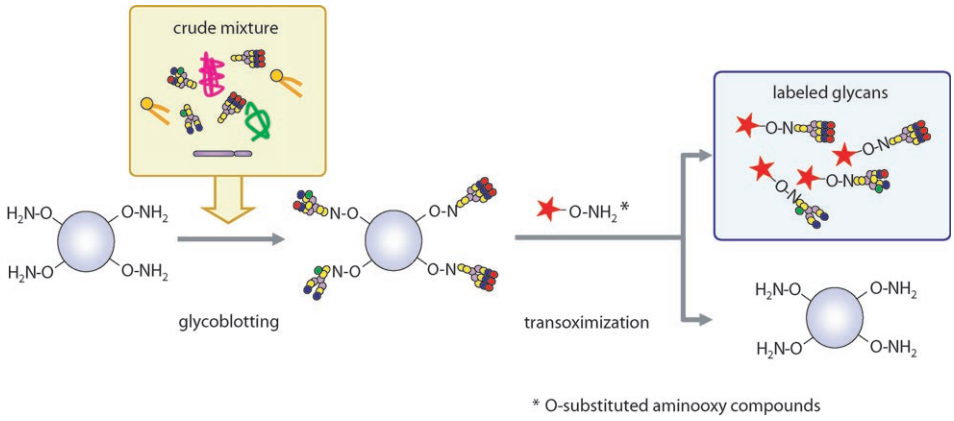


Figure 3. General scheme showing the concept of glycoblotting and on-bead transoximization with O-substituted aminooxy compound(s).

type glycan $Man_5GlcNAc_2$, abbreviated as "M5") prepared from ribonuclease B (RNase B) were blotted onto BlotGlyco, washed successively with 0.5% SDS, 50% methanol and H_2O to remove nonspecifically bound impurities, and the polymer was incubated with either aminooxyacetic acid (aoAA), benzyloxyamine (BOA), *p*-nitrobenzyloxyamine (pNBOA) or dipeptidic aminooxy derivative (aoWR) solution to promote the transoximization. As shown in Figure 4, we found that oligosaccharides covalently bound to the polymer via oxime bond can be released through in the presence of large excess *O*-substituted aminooxy compounds (e.g. aoAA, BOA, pNBOA and aoWR). These results indicate that transoximization of the hemiacetal group present at the reducing terminal of oligosaccharide actually occurred regardless the structure of O-substituted aminooxy derivatives. The proposed mechanism of transoximization and hydrolysis is illustrated in Scheme 2. The pNBOA derivatives of RNase B oligosaccharides were analyzed by normal-phase HPLC and reasonably good separation was achieved as shown in Figure 5. The recovery yield via transoximization by pNBOA was determined to be ≈25% by HPLC. Although the yield was not satisfactory, the relative quantity ratios (e.g. relative intensity on the MALDI-TOF MS analysis, relative peak area on the HPLC chromatography) of the oligosaccharides present in the sample ($Man_5GlcNAc_2$, $Man_6GlcNAc_2$, $Man_7GlcNAc_2$, $Man_8GlcNAc_2$, $Man_9GlcNAc_2$, abbreviated as "M5–M9") were identical to those previously reported,[20] indicating that efficiencies of both blotting and releasing via transoximization are independent of glycan structures. Optimization of the conditions for transoximization is currently in progress in our laboratory aiming to improve the recovery efficiency.

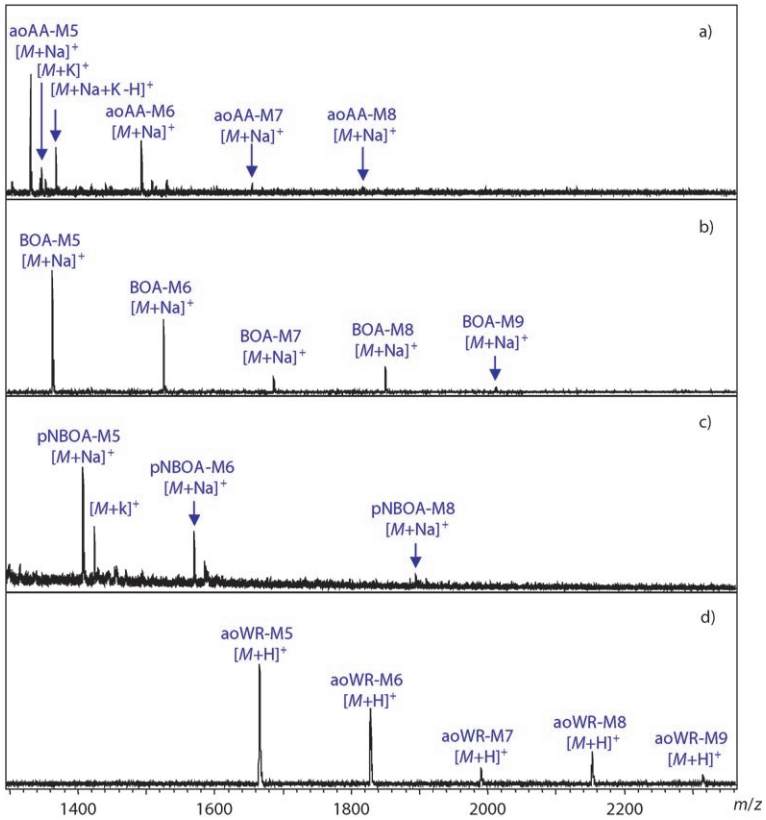
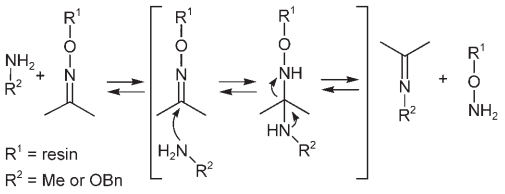


Figure 4. MALDI-TOF spectra showing the simultaneous releasing and probing based on transoximization. Oligosaccharides derived from RNase B were blotted onto BlotGlyco, subsequently recovered by the incubation with a) aminooxy acetic acid (aoAA), b) benzyloxyamine (BOA), c) *p*-nitrobenzyloxyamine (pNBOA), or d) ((aminooxy)acetyl)tryptophanylarginine methyl ester (aoWR).



Scheme 2. Proposed mechanism of transoximization and hydrolysis.

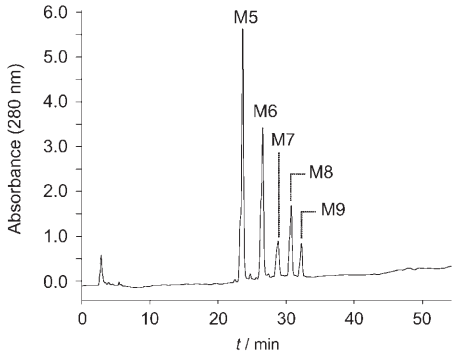


Figure 5. Chromatogram showing the separation of pNBOA derivatized oligosaccharides derived from RNase B by normal-phase chromatography.

This protocol was finally applied for the human serum glycomic analysis. Changes in the glycosylation pattern of human serum glycoproteins have been identified as useful markers for detecting various diseases.[21–23] Following the tryptic digestion of human serum, N-glycans were released by peptide:N-glycosidase F (PNGase F). Sialic acids were removed prior to glycoblotting to simplify the spectrum. Figure 6a shows the MALDI-TOF MS spectrum of human serum N-glycans observed as BOA derivatives through one-pot solid-phase glycoblotting and subsequent transoximization. Up to 23 of the precursor ion peaks could be clearly detected and were identified as BOA derivatives of N-glycan chains known to be present in human serum. The observed N-glycan profile was in good agreement with that determined by other techniques both qualitatively and quantitatively.[24] The purification power of this method is evident as a very complicated mass spectrum was obtained when the trypsin and PNGase F treated sample was directly measured by MALDI-TOF MS (Figure 6b). It should also be noted that no attempt to remove blood glucose was made, which would also react with the aminooxy groups on the solid support, though a relatively high concentration (≈5 mM) of glucose is inherently present in the serum. This is explained by the high capacity of the aminooxy functionalities on the newly designed BlotGlyco. Aminooxy groups were estimated to be approximately 100 times in excess compared with blood glucose under the reaction conditions employed in this study (for example, 5 mg polymer particles for 20 μL of human serum). The conventional procedures for human serum glycomic analysis often require tedious and time-consuming purification steps (e.g., gel filtration, ion-exchanging resin and/or other column operations).[9] On the contrary, the described protocol extremely simplifies and accelerates the serum glycomic analysis; the procedures of glycoblotting and derivatization with BOA were simply conducted by means of polymer particles in one-pot manner and are easily automatable.

Application to glycoproteomics: Besides glycomic analysis, we would also like to report on the chemoselective blotting of an enzymatically modified synthetic *O*-GlcNAcylated glycopeptide by using galactosyl transferase (Gal-T) and galactose oxidase (GOD).[10] GOD oxidation quantitatively generates an aldehyde group at the C-6 position of the non-reducing terminal galactose residue, which could be selectively conjugated with aminooxy functionalized compounds. To elucidate the versatility of the present strategy for the GOD-assisted selective blotting and transoximization process of asialo-glycopeptides, a tryptic digest of asialofetuin was tested as a model study. Figure 7a shows the MALDI-TOF MS of BOA-labeled glycopeptides obtained by means of one-pot solid phase glycoblotting and transoximization method as indicated in Figure 8. It was clearly demonstrated that GOD-oxidized glycopeptides were suc-

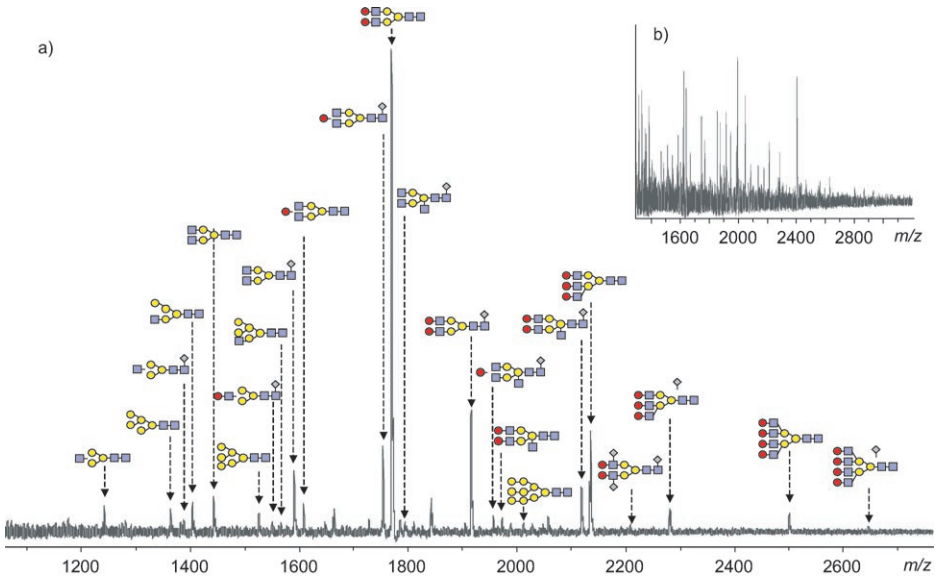


Figure 6. Glycomic analysis of human serum glycoproteins following desialylation. a) MALDI-TOF mass spectrum of benzyloxyamine-labeled *N*-glycans, b) MALDI-TOF mass spectrum of digest mixture without glycoblotting.

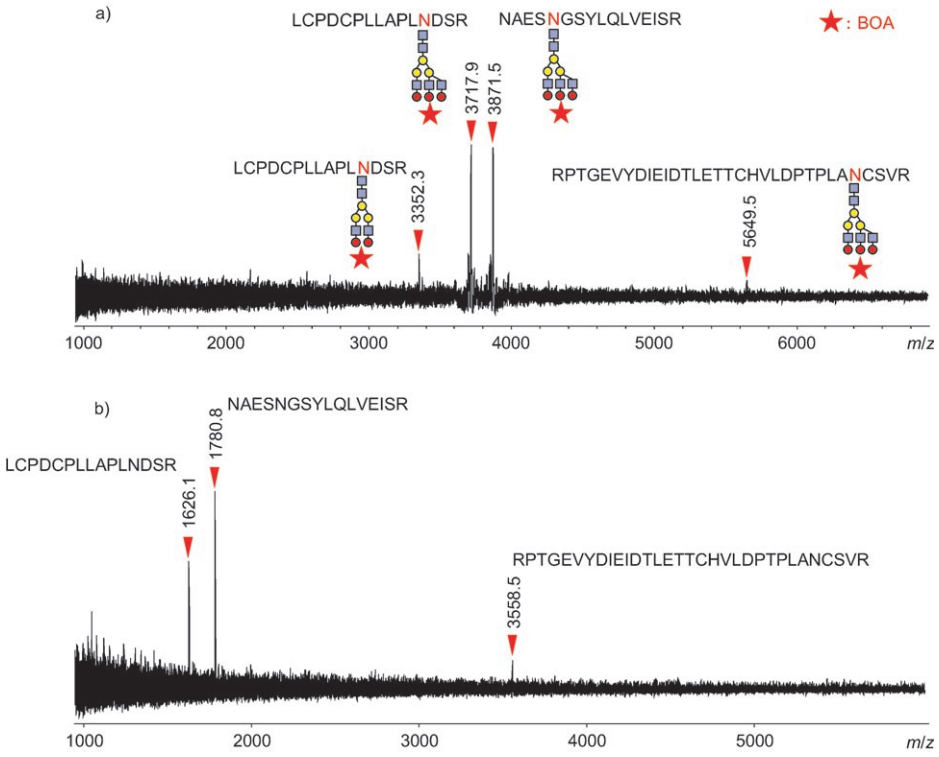


Figure 7. Glycoproteomic analysis of asialofetuin. MALDI-TOF mass spectrum of a) BOA-labeled glycopeptides obtained by one-pot solid phase glycoblotting and transoximization, and b) peptides obtained by glycoblotting and subsequent release by treatment with PNGase F.

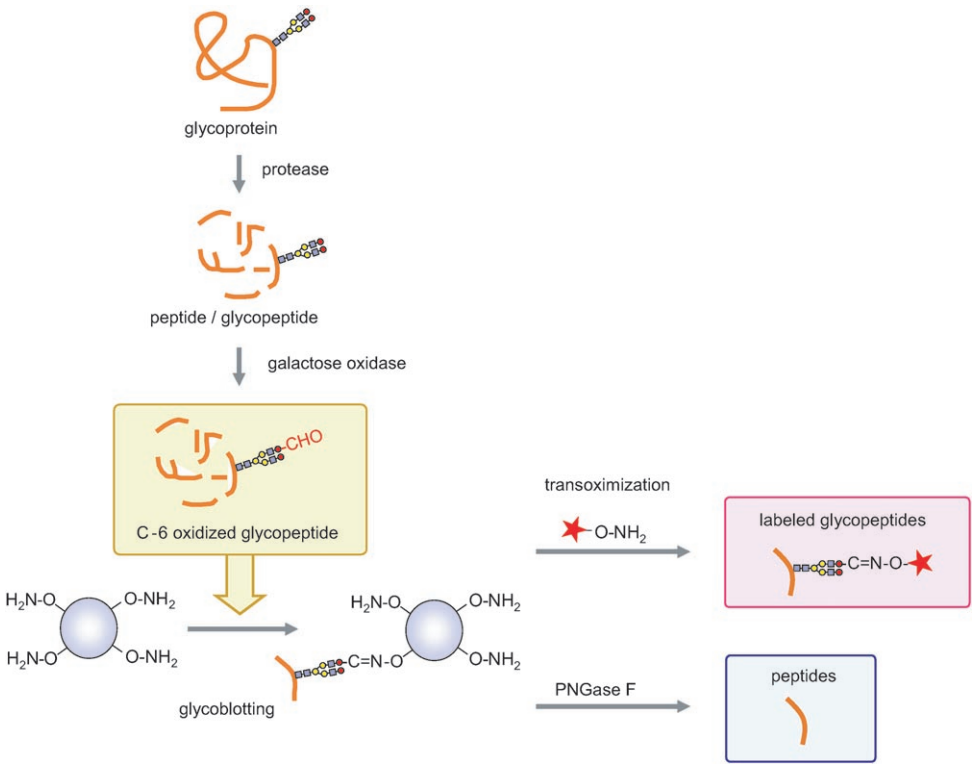


Figure 8. General scheme showing the concept that allows the selective enrichment of glycopeptides whose antennae terminates in galactose and subsequent structural analysis of both peptide core and oligosaccharide side chains.

cessfully captured by polymer particle and were recovered as BOA derivatives. MALDI-TOF MS analysis performed without any further purification revealed that four dominant ion peaks were observed; three peaks at *m*/*z* 3352.3, 3717.9 and 5649.5 were assigned to the precursor ions of $[M+H]^+$ of BOA-labeled derivatives corresponding to the expected tryptic glycopeptides of asialofetuin: [LCPDCPLLAPLNDSR + $(Gal)_2(GlcNAc)_2$ + $(Man)_3$-$(GlcNAc)_2$] (*m*/*z* calcd 3352.4), LCPDCPLLAPLNDSR + $(Gal)_3(GlcNAc)_3$ + $(Man)_3$-$(GlcNAc)_2$] (*m*/*z* calcd 3717.6), and [RPTGEVYDIEIDTLETTCHVLDPTPLANCSVR + $(Gal)_3(GlcNAc)_3$ + $(Man)_3$-$(GlcNAc)_2$] (*m*/*z* calcd 5648.5), respectively. The other signal appeared at *m*/*z* 3871.5 could not be assigned as any known tryptic glycopeptide of asialofetuin, although MALDI-TOF/TOF MS[25] analysis suggested that this ion peak can be assigned as $[M+H]^+$ of BOA-labeled glycopeptide due to [NAESNGSYLQLVEISR + $(Gal)_3(GlcNAc)_3$ + $(Man)_3$-$(GlcNAc)_2$] (*m*/*z* calcd 3871.6); its peptide sequence is consisted with a part of known tryptic glycopeptide (VVHAVEVALATFNAESNGSYLQLVEISR). The occurrence of such an irregular cleavage by trypsin may be explained by the contamination of other protease since the trypsin was not treated with *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK). When the glycopeptides-blotted polymer was treated with PNGase F, three dominant ion peaks due to peptides were observed as shown in Figure 7b. All *m*/*z* values were identical to the deglycosylated forms of above described glycopeptides. The precursor ion peaks at *m*/*z* 1626.1, 3558.5, and 1780.8 were corresponding to $[M+H]^+$ of

LCPDCPLLAPLDDSR (*m*/*z* calcd 1627.8), RPTGEVYDIEIDTLETTCHVLDPTPLADCSVR (*m*/*z* calcd 3558.7), and NAESDGSY LQLVEISR (*m*/*z* calcd 1780.9), respectively. (Conversion of the linker asparagines into the aspartic acids was observed as a mass shift of one mass unit, when the oligosaccharides were released from the glycopeptides by treating with PNGase F.) The peptide sequence of the latter unpredicted tryptic peptide was confirmed by MALDI-TOF/TOF analysis.

Selective manipulation using PNGase F and transoximization of glycopeptides captured through GOD-assisted glycoblotting greatly facilitated systematic proteomics and glycoproteomics analyses. To the best of our knowledge, although a few promising techniques have already been available for the identification of glycosylation sites of peptide moieties,[26–28] the present method is the only strategy which allows for a precise structural analysis both of the peptide core and oligosaccharide side chains of the chemoselectively enriched glycopeptides without excluding glycomic information. In addition, it was revealed that combined use of glycoblotting and matrix dependent selective fragmentation method (MDSF)[25] by MALDI-TOF/TOFMS greatly accelerates high-throughput glycoproteomics. Note that, not only oligosaccharides with a terminal galactose but also terminal sialic acid can also be trapped by this method. The feasibility of this strategy was preliminarily demonstrated by trapping the tryptic digest of fetuin in which the diols at C-8 and C-9 positions of sialic acids were selectively converted to aldehyde groups by treating with 1 mM $NaIO_4$ solution at 0 °C for 30 min.[10] Though the present method can thus far not be used for the analysis of glycopeptides displaying glycans whose antenna do not terminate either galactose or sialic acid, it may become a powerful alternative to lectin-based affinity enrichment for a focused glycoproteomics.

Conclusion

We described a novel concept for a high-throughput glycomics and glycoproteomics based on one-pot solid-phase chemical manipulations including glycoblotting and subsequent transoximization. Using this method for a human serum glycomic analysis, it was demonstrated that 23 kinds of N-glycans can be detected by MALDI-TOF MS as derivatives of the O-substituted aminooxy compound (BOA) with appropriate relative quantitative ratios. The applicability of transoximization targeted towards the hemiacetal group present at the reducing terminal of oligosaccharide was proven for the first time. Furthermore, we demonstrated that this method allowed simultaneous identification of both glycan structure and peptide sequence with the information of the glycosylation sites. These results demonstrate that the combination of glycoblotting and transoximization has a great potential for high-throughput glycomic and glycoproteomics analysis by allowing both rapid, efficient purification and derivatization of oligosaccharide and glycopeptide. We have recently established a novel solid-phase methyl esterification of sialic acid residues.[29] The combined use of the methyl esterification with one-pot solid-phase glycoblotting and probing by transoximization will be described elsewhere. The power of transoximization assisted simultaneous releasing and probing also lies in its versatility; that is, glycan can be recovered as an arbitrary derivative in accordance with the intended uses by using appropriately designed aminooxy-functionalized small compounds. For practical applications, however, the transoximization efficiency must be improved.

Experimental Section

General methods and materials: All commercially available starting materials and solvents were reagent grade and used as received. Methacrylic anhydride (MAH), ethylenedioxy-bisethylamine (EDBEA), ethyleneglycol dimethacrylate (EGDMA), α,α′-azobisisobutylonitrile (AIBN), aminooxy acetic acid (aoAA), benzyloxyamine hydrochloride (BOA) and *p*-nitrobenzyloxyamine (pNBOA) were purchased from Sigma-Aldrich. 4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid, hydrazide (BODIPY FL hydrazide) was obtained from Invitrogen (Carlsbad, CA). *tert*-Butyloxycarbonyl-aminooxyacetic acid (Boc-aoAA) was purchased from NovaBiochem. Polyvinyl alcohol (M_w 2000) was purchased from Wako Pure Chemistries. Dipeptidic aminooxy derivative (aoWR) was prepared according to the method described in our previous paper.[30] All other chemicals were ultra pure grade and purchased from Wako Pure Chemistries. Asialofetuin, ribonuclease B (RNase B), human serum, trypsin and other proteins were purchased from Sigma-Aldrich. Peptide:N-glycosidase F (PNGase F) was purchased from Roche Applied Science. ^{1}H NMR spectra were measured at 600 MHz on a Bruker DPX-600 spectrometer using $CDCl_3$ as the solvent. All chemical reactions were performed in anhydrous solvents under nitrogen atmosphere in the dark unless noted otherwise. TLC was performed on Merck precoated plates (20×20 cm; layer thickness, 0.25 mm; silica gel 60F254); spots were visualized by spraying a solution of 90:5:5 methanol–*p*-anisaldehyde–concentrated sulfuric acid and heating at 180 °C for about 30 s, and by UV light (256 or 365 nm) when applicable. Flash column chromatography was performed on silica Gel 60 (spherical type, particle size 40–50 μm; Wako Pure Chemical Co. Ltd.) with the solvent systems specified, and the ratio of solvent systems was given in v/v. Organic extracts were dried over anhydrous $MgSO_4$, and solutions were concentrated under diminished pressure below 50 °C.

Synthesis of aminooxy-functionalized monomer 5

***N*-[2-[2-(aminoethoxy)ethoxy]ethyl]-2-methacrylamide (3)**: EDBEA (25 g, 0.169 mol) was dissolved in chloroform (200 mL) in a round-bottomed flask and cooled by ice bath. MAH (5.0 g, 0.032 mol) was added to the flask, followed by incubation under nitrogen atmosphere for 16 h with stirring and the reaction was monitored by TLC. After the reaction was completed, solvent was removed by evaporation and the residue was subjected to the silica gel chromatography with chloroform/methanol 9:1 to give syrupy compound **3** (9.0 g, 65 %). ^{1}H NMR (600 MHz, $CDCl_3$): δ = 1.35–1.45 (m, 2H), 1.97 (s, 3H), 2.85–2.9 (m, 2H), 3.4–3.9 (m, 10H), 5.32 (s, 1H), 5.7 (s, 1H), and 6.39 (s, 1H); MALDI-TOFMS: *m*/*z*: calcd for $C_{10}H_{21}N_2O_3$: 217.2853; found: 217.25 $[M+H]^+$, 239.18 $[M+Na]^+$.

***N*-[2-[2-(2-*tert*-Butoxycarbonylaminooxyacetylamino-ethoxy)ethoxy]ethyl]-2-methacrylamide (5)**: EDC (10 g, 0.052 mol) and Boc-aoAA (5.0 g, 0.026 mol) were added to the solution of **3** (5.6 g, 0.026 mol) in chloroform (200 mL), and the mixture was stirred under nitrogen atmosphere for 16 h. After the reaction was completed on TLC test, solvent was removed by evaporation and the residue was purified by using silica gel chromatography with chloroform/methanol 9:1 to afford pure compound **5** (5.6 g, 55 %). ^{1}H NMR (600 MHz, $CDCl_3$): δ = 1.48 (s, 9H), 1.97 (s, 3H), 3.4–3.55 (m, 2H), 3.55–3.7 (m, 10H), 4.34 (s, 2H), 5.33 (s, 1H), 5.71 (s, 1H), 6.43 (s, 1H), 7.75–8.1 (m, 2H); MALDI-TOFMS: *m*/*z*: calcd for

$C_{17}H_{32}N_3O_7$: 390.4519; found: 390.03 $[M+H]^+$, 412.11 $[M+Na]^+$, 428.13 $[M+K]^+$; $n=1.453$ (25 °C); $\rho=1.09\ g\,cm^{-3}$ (25 °C).

Dispersion polymerization: Polymerization of compound **5** was performed according to the common procedure for the dispersion polymerization reactions.[31] PVA (50 mg) was dissolved in distilled water (30 mL) in a 50 mL three-necked flask. Compound **5** (1.0 g) and EGDMA (5 mol% vs monomer) were put into the above PVA solution. The dispersion mixture was kept at 60 °C and stirred under nitrogen atmosphere. AIBN (0.3 g) was added into the mixture to initiate the polymerization reaction and stirred for 16 h. The polymer particles were collected by centrifugation and harshly washed with methanol and distilled water to remove unreacted materials and PVA. The polymer particles were suspended in a trifluoroacetic acid/methanol 1:3 solution at 40 °C and the mixture was stirred for 16 h to remove the Boc group. The resulted polymer particles were washed with methanol and distilled water, and stored at 4 °C before use. The yield of polymerization was 23% (0.23 g).

SEM observation of polymer particle: The morphological features of the polymer particles were observed by scanning electron microscope (SEM). The suspension of particles in water was desiccated by using vacuum drying, and the residue was coated with Pt/Pd (300 Å in thickness) with an E-102 Hitachi Ion Sputter. SEM pictures were taken using S-2250N Hitachi Scanning Electron Microscope at 15–25 kV/90–120 mA.

Estimation of the concentration of active aminooxy groups in the polymer: The polymer particle (20 mg) was incubated with theoretically equivalent amount of glucose (100 mM, 680 μL in 10 mM acetate buffer, pH 4.0) at 60 °C for 24 h in a disposable column (Mobicol polypropylene column, *Φ*6.8 mm, MoBiTec, Germany). After the reaction, the unreacted glucose was recovered by filtration, and was quantified by HPLC following derivatization with pNBOA.[32] HPLC analysis of pNBOA-labeled oligosaccharide was performed using a Hitachi LaChrom Elite HPLC system operated by D-7000 control unit equipped with L-7100 pump unit, L-7405 UV detector, L-7485 fluorescent detector, column oven and autosampler. The column used was silica gel column containing amino groups (Varian Microsorb-MV 100-5 amino, 250×4.6 mm *Φ*). The separation was performed at 30 °C using a linear gradient of 2% acetic acid in acetonitrile (solvent A) and 5% acetic acid in water containing 3% triethylamine (solvent B). Initial isocratic elution with 20% solvent B was performed for 10 min followed by a linear increase to 90% solvent B for 80 min. Then the column was washed with 90% B for 15 min. The detection wavelength was performed at 280 nm. Quantification was performed by absolute calibration method upon injecting a series of known concentrations of the pNBOA-labeled glucose onto HPLC.

Preparation of crude glycopeptides and oligosaccharides mixtures from model glycoproteins: Asialofetuin or RNase B (5 mg) was dissolved in 0.1 M Tris-HCl buffer (pH 8.0) including trypsin (50 μg), and was incubated for 16 h at 37 °C. Then, the reaction mixture was boiled for 5 min to terminate the reaction. For the glycopeptide-blotting for glycoproteomics, this material was directly used without further treatment. For the glycomic analysis, the mixture was further incubated with PNGase F (5 unit) for 20 h at 37 °C. The concentrations of oligosaccharides in each digested sample were determined by HPLC following fluorescent derivatization with BODIPY FL hydrazide: an aliquot (1 μL) was suspended in distilled water (20 μL) and added with 20 mM BODIPY FL hydrazide (1 μL) and acetonitrile (180 μL) containing 2% acetic acid followed by incubation at 80 °C for 1 h. The HPLC conditions were the same as those for pNBOA-labeled carbohydrate except that the detection was performed with fluorescence detection (Ex: 505, Em: 510 nm).

Estimation of the glycoblotting efficiency of the polymer particles: Quantitative analysis of glycoblotting efficiency of the novel polymer particles was performed using LacNAc as a model reducing disaccharide. Briefly, aqueous LacNAc solution (20 μL, either 50, 5, 0.5 or 0.05 mM) and acetonitrile (180 μL) containing 2% acetic acid were added to 2 mg (dry weight) of the polymer particles in a disposable column (*Φ*6.8 mm, MoBiTec, Germany). Boc-protected polymer was also used as negative control that does not expose free aminooxy functionalities. The glycoblotting reactions were performed in a disposable column by directly incubating the column at 80 °C for 1 h. Then, the column was washed with distilled water (200 μL) three times to recover the unreacted LacNAc. The recovered fractions were evaporated in vacuo, and were derivatized with BODIPY FL hydrazide for the HPLC analysis as described above.

Transoximization procedure: Recovery of the polymer-surface blotted glycans by transoximization reaction was evaluated using four aminooxy-containing compounds, aoAA, BOA, pNBOA and aoWR. Oligosaccharides derived from RNase B, of which concentration of M5 is ≈1 nmol, were ligated onto polymer particle as described, followed by washing successively with 0.5% SDS aqueous solution, 50% methanol, and distilled water. Then, 100 μL of either aoAA, BOA, pNBOA or aoWR solution (100 mM, pH was adjusted to 4.0 with acetic acid) was added to the polymer and incubated at 80 °C until the solvent dried out (≈2 h). Recovery of the labeled oligosaccharides was performed by adding 50% acetonitrile/water three times and collecting the eluent. Following evaporation, excess reagent was removed using simple solid-phase extraction.[33] Briefly, a disposable column packed with ≈20 mg of silica gel (Iatrobeads, 6RS-8060, Iatron Laboratories, Tokyo, Japan) was first preconditioned successively with 1 M acetic acid and acetonitrile. The labeled oligosaccharide sample was dissolved with water (50 μL) and then diluted with acetonitrile (950 μL). The solution was applied to the silica gel followed by washing successively with acetonitrile and acetonitrile/water 96:4, three times each, and eluted with acetonitrile/water 50:50. Each sample was subjected to MALDI-TOF analysis using 2,5-dihydroxy benzoic acid (DHB) as matrix for the derivatives of aoAA, BOA and aoWR, and 4-hydroxyazobenzene-2-carboxylic acid (HABA) for pNBOA derivative.[34] The transoximization efficiency was evaluated by quantifying the recovered pNBOA-M5 by HPLC. The HPLC conditions were the same as those for BODIPY labeled oligosaccharides except that the detection was performed at 280 nm by UV detector.

Human serum glycomic analysis: Human serum (100 μL) was added to 100 mM ammonium bicarbonate (4 μL, pH ≈7.8), H_2O (21 μL) and trypsin (5 μL, 400 U), followed by incubation at 37 °C for 1 h. Following the heat denatured at 80 °C for 15 min, PNGase F (2 U) was added and incubated at 37 °C for 24 h. Sialic acid was removed by adjusting the digested sample solution to pH 2.0 with 0.1 M HCl and was heated at 90 °C for 1 h. Thus prepared deglycosylated serum sample was lyophilized, dissolved in water (400 μL), and one-tenth was directly used for the blotting onto the polymer as described. Following the serial washing with 0.5% SDS, 50% methanol and distilled water, the polymer was incubated with BOA (100 μL, 100 mM, pH was adjusted to 4.0 with acetic acid) at 80 °C for ≈2 h. The recovered BOA labeled glycans were recovered by washing the polymer with 50% acetonitrile/water three times and the collected solution was evaporated in vacuo. The recovered sample was dissolved in DHB solution and was directly subjected to MALDI-TOF analysis.

General procedure for glycoblotting and transoximization of glycopeptides on the polymer particle: A glycopeptide mixture prepared by tryptic digestion of asialofetuin described above was treated with galactose oxidase (GOD) to elaborate the aldehyde groups at C-6 position of terminal D-galactose residues as follows: To a solution of crude glycopeptides in phosphate buffer (200 μL, pH 6.0 including copper sulfate) were added GOD (20 units) and trace amount of catalase. The mixture was incubated at room temperature for 16 h in the dark. The generation of the aldehyde group was preliminarily confirmed by using the Purpald reagent (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole), which react with free aldehyde to give purple color.[35] Then, the mixture was suspended with the polymer particles (20 mg) in pH 4.0 (0.1 M acetate buffer) and the final volume was adjusted by suited saline to be 400 μL. The reaction mixture was incubated at 60 °C for 16 h under vigorous stirring. Then, the polymer particles were washed with 0.5% SDS, 50% methanol, and distilled water to remove non-specifically bound impurities. After the washing, the polymer particle was treated with 100 mM BOA for 1 h at 90 °C. Alternatively, the polymer particles were treated PNGase F (1 unit, Tris/HCl buffer, pH 8.0) at 37 °C for 12 h to release core peptide chains as deglycosylated forms of captured glycopeptides. Both BOA-labeled glycopeptides and their deglycosylated peptides were directly subjected to proteomics study by MALDI-TOFMS without any other purification.

MALDI-TOF mass spectrometry: All measurements were performed using an Ultraflex TOF/TOF mass spectrometer equipped with a reflector, and controlled by the FlexControl 2.1 software package (Bruker Dal-

tonics GmbsH, Bremen, Germany) according to the general protocols reported in the previous papers.[10,25] In MALDI TOF-MS reflector mode, ions generated by a pulsed UV laser beam (nitrogen laser, $\lambda = 337$ nm, 5 Hz) were accelerated to a kinetic energy of 23.5 kV. Metastable ions generated by laser-induced decomposition of the selected precursor ions were analyzed without any additional collision gas. In MALDI-TOF/TOF mode, precursor ions were accelerated to 8 kV and selected in a timed ion gate. The fragments were further accelerated by 19 kV in the LIFT cell (LIFT means "lifting" the potential energy for the second acceleration of ion source), and their masses were analyzed after the ion reflector passage. Masses were automatically annotated by using FlexAnalysis 2.0 software package. External calibration of MALDI mass spectra was carried out using singly charged monoisotopic peaks of a mixture of human angiotensin II (m/z 1046.542), bombesin (m/z 1619.823), ACTH (18–39) (m/z 2465.199), and somatostatin 28 (m/z 3147.472). The mixture of these peptides was measured on the central spot of a 3×3 square by using external calibration. To achieve mass accuracy better than 60 ppm, internal calibration was carried out by doping the matrix solution with a mixture of the calibration peptides. Calibration of these mass spectra was performed automatically by utilizing a customized macro command of the FlexControl 2.1 software package. The macro command was used for the calibration of the monoisotopic singly charged peaks of the above-mentioned peptides. TOF/TOF spectra were annotated with the BioTools 2.1 software package.

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[1] R. A. Dwek, *Chem. Rev.* **1996**, *96*, 683–720.
[2] A. Corthay, J. Backlund, J. Broddefalk, E. Michaelsson, T. J. Goldschmidt, J. Kihlberg, R. Holmdahl, *Eur. J. Immunol.* **1998**, *28*, 2580–2590.
[3] N. Mitra, S. Sinha, T. N. Ramya, A. Surolia, *Trends Biochem. Sci.* **2006**, *31*, 156–163.
[4] S. Hase, *J. Chromatogr.* **1996**, *720*, 173–182.
[5] M. J. Davies, E. F. Hounsell, *J. Chromatogr.* **1996**, *720*, 227–233.
[6] A. Dell, H. R. Morris, *Science* **2001**, *291*, 2351–2356.
[7] F. Kjeldsen, K. F. Haselmann, B. A. Budnik, E. S. Sorensen, R. A. Zubarev, *Anal. Chem.* **2003**, *75*, 2355–2361.
[8] K. Hakansson, M. J. Chalmers, J. P. Quinn, M. A. McFarlan, C. L. Hendrickson, A. G. Marshall, *Anal. Chem.* **2003**, *75*, 3256–3262.
[9] C. Campbell, K. J. Yarema, *Adv. Genome Biol.* **2005**, 6, 236–242.
[10] S.-I. Nishimura, K. Niikura, M. Kurogochi, T. Matsushita, M. Fumoto, H. Hinou, R. Kamitani, H. Nakagawa, K. Deguchi, N. Miura, K. Monde, H. Kondo, *Angew. Chem.* **2005**, *117*, 93–98; *Angew. Chem. Int. Ed.* **2005**, *44*, 91–96.
[11] K. Niikura, R. Kamitani, M. Kurogochi, R. Uematsu, Y. Shinohara, H. Nakagawa, K. Deguchi, K. Monde. H. Kondo, S.-I. Nishimura, *Chem. Eur. J.* **2005**, *11*, 3825–3835.
[12] A. Lohse, R. Martins, M. R. Jorgensen, O. Hindsgaul, *Angew. Chem.* **2006**, *118*, 4273–4278; *Angew. Chem. Int. Ed.* **2006**, *45*, 4167–4172.
[13] W. P. Jencks, *J. Am. Chem. Soc.* **1959**, *81*, 475–481.
[14] L. Amaral, W. A. Sandstrom, E. H. Cordes, *J. Am. Chem. Soc.* **1966**, *88*, 2225–2233.
[15] V. A. Polyakov, M. I. Nelen, N. Nazarpack-Kandlousy, A. D. Ryabov, A. V. Eliseev, *J. Phys. Org. Chem.* **1999**, *12*, 357–363.
[16] G. R. L. Cousins, S.-A. Poulsen, J. K. M. Sanders, *Chem. Commun.* **1999**, 1575–1576.
[17] V. Goral, M. I. Nelen, A. V. Eliseev, J.-M. Lehn, *Proc. Nat. Acad. Sci. USA* **2001**, *98*, 1347–1352.
[18] *Functional Synthetic Receptors* (Eds: T. Schrader, A. D. Hamilton, Wiley, New York, **2005**, pp. 299–332.
[19] Y. Takaoka, H. Tsutsumi, N. Kasagi, E. Nakata, I. Hamachi, *J. Am. Chem. Soc.* **2006**, *128*, 3273–3280.
[20] C.-J. Liang, K. Yamashita, A. Kobata, *J. Biochem.* **1980**, *88*, 51–58.
[21] N. Callewaert, H. V. Vlierberghe, A. V. Hecke, W. Laroy, J. Delanghe, R. Contreras, *Nat. Med.* **2004**, *10*, 429–434.
[22] M. Farooq, N. Takahashi, H. Arrol, M. Drayson, R. Jefferis, *Glycoconjugate J.* **1997**, *14*, 489–492.
[23] T. M. Block, M. A. Comunale, M. Lowman, L. F. Steel, P. R. Romano, C. Fimmel, B. C. Tennent, W. T. London, A. A. Evans, B. S. Blumberg, R. A. Dwek, T. S. Matts, A. S. Mehta, *Proc. Nat. Acad. Sci. USA* **2005**, *102*, 779–784.
[24] H. Nakagawa, Y. Kawamura, K. Kato, I, Shimada, Y. Arata, N. Takahashi, *Anal. Biochem.* **1995**, *226*, 130–138.
[25] M. Kurogochi, S.-I. Nishimura, *Anal. Chem.* **2004**, *76*, 6097–6101.
[26] H. Zhang, X.-j. Li, D. B. Martin, R. Aebersold, *Nat. Biotech.* **2003**, *21*, 660–666.
[27] T. Liu, W.-J. Qian, M. A. Gritsenko, D. G. Gamp II, M. E. Monroe, R. J. Moore, R. D. Smith, *J. Proteome Res.* **2005**, *4*, 2070–2080.
[28] H. Kaji, H. Saito, Y. Yamauchi, T. Shinkawa, M. Taoka, J. Hirabayashi, K. Kasai, N. Takahashi, T. Isobe, *Nat. Biotech.* **2003**, *21*, 667–672.
[29] Y. Miura, Y. Shinohara, J.-i. Furukawa, N. Nagahori, S.-I. Nishimura, unpublished result.
[30] R. Uematsu, J. Furukawa, H. Nakagawa, Y. Shinohara, K. Deguchi, K. Monde, S.-I. Nishimura, *Mol. Cell. Proteomics* **2005**, *4*, 1977–1989.
[31] H. Kawaguchi, *Prog. Polym. Sci.* **2000**, *25*, 1171–1210.
[32] M. Pauly, W. S. York, R. Guillen, P. Albersheim, A. G. Darvill, *Carbohydr. Res.* **1996**, *282*, 1–12.
[33] Y. Miura, H. H. Freeze, *Glycobiology* **1998**, *8*, 813–819.
[34] K. R. Nielsen, M. W. Pennington, *Lett. Pept. Sci.* **1996**, *2*, 301–305.
[35] M. S. Quesenberry, Y. C. Lee, *Anal. Biochem.* **1996**, *234*, 50–55.

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