DOI: 10.1002/chem.200601872

Rapid and Simple Solid-Phase Esterification of Sialic Acid Residues for Quantitative Glycomics by Mass Spectrometry

Yoshiaki Miura, Yasuro Shinohara, Jun-ichi Furukawa, Noriko Nagahori, and Shin-Ichiro Nishimura*[a]

Abstract: A rapid and quantitative method for solid-phase methyl esterification of carboxy groups of various sialylated oligosaccharides has been established. The method employed a triazene derivative, 3-methyl-1-*p*-tolyltriazene, for facile derivatization of oligosaccharides immobilized onto general solid supports such as Affi-Gel Hz and gold colloidal nanoparticles in a multiwell plate. The workflow protocol was optimized for the solid-phase processing of captured sialylated/unsialylated oligosaccharides separated from crude sample mixtures by chemical ligation. From tryptic and/or PNGase F-digest mixtures of glycoproteins, purification by chemoselective immobilization, esterification and recovery were achieved in the same well of the filter plate within three hours when used in conjunction with "glycoblotting technology" (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, High-throughput protein glycomics: Combined use of chemoselective glycoblotting and MALDI-TOF/TOF mass spectrometry: *Angew. Chem.* **2005**, *117*, 93–98; *Angew. Chem. Int. Ed.* **2005**, *44*, 91–96). The recovered materials were directly applicable to subsequent characterization by mass spectrometric techniques such as MALDI-TOF for large-scale glycomics of both neutral and sialylated oligosaccharides. On-bead/on-gold nanoparticle derivatization of glycans containing sialic acids allowed rapid and quantitative glycoform profiling by MALDI-TOF MS with reflector and positive ion mode. In addition to its simplicity and speed, the method eliminates the use of unfavorable halogenated solvents such as chloroform and dichloromethane or volatile solvents such as diethyl ether and hexane, resulting in a practical and green chemical method for automated robotic adaptation.

Keywords: esterification · glycomics · mass spectrometry · oligosaccharides · sialic acid

Introduction

The developments of matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) mass spectrometry (MS) has greatly accelerated the use of MS-based technology in structural analysis of oligosaccharides.[1,2] However, it should be noted that sialic acid-containing acidic oligosaccharides have not yet fully benefited from its usefulness. This is mostly due to the instability of the α-glycoside bond between sialic acid and the adjacent sugar residue under general conditions for ionization/detection processes during mass measurement, giving rise to several inevitable difficulties in the analysis.[3] It has been well documented that various sialylated oligosaccharides, when analysed by mass spectrometry in either positive or negative ion modes, lose sialic acids as a result of the presence of the free carboxy group. In addition, oligosaccharides containing multiple sialic acid residues usually give complicated mass spectra resulting from mixtures of cation adducts when analysed in positive ion mode. It therefore seems likely that structural heterogeneity due to the partial loss of sialic acid residues has often been reduced by the complete removal of terminal sialic acids from the target oligosaccharides on treatment with some acidic solutions.[4]

In view of the importance of glycoforms, including sialic acid residues, in the essential biological roles of glycoconjugates, as well as in potential diagnostic biomarkers of various diseases,[5–7] it is evident that the development of some simple and versatile method for avoiding the risk of desialylation would be very desirable. With the goals of addressing

[a] Dr. Y. Miura, Prof. Dr. Y. Shinohara, Dr. J.-i. Furukawa, Dr. N. Nagahori, Prof. Dr. S.-I. Nishimura
Graduate School of Advanced Life Science
Frontier Research Center for Post-Genome Science and Technology
Hokkaido University, N21, W11, Sapporo 001-0021 (Japan)
Fax: (+81) 11-706-9042
E-mail: shin@glyco.sci.hokudai.ac.jp

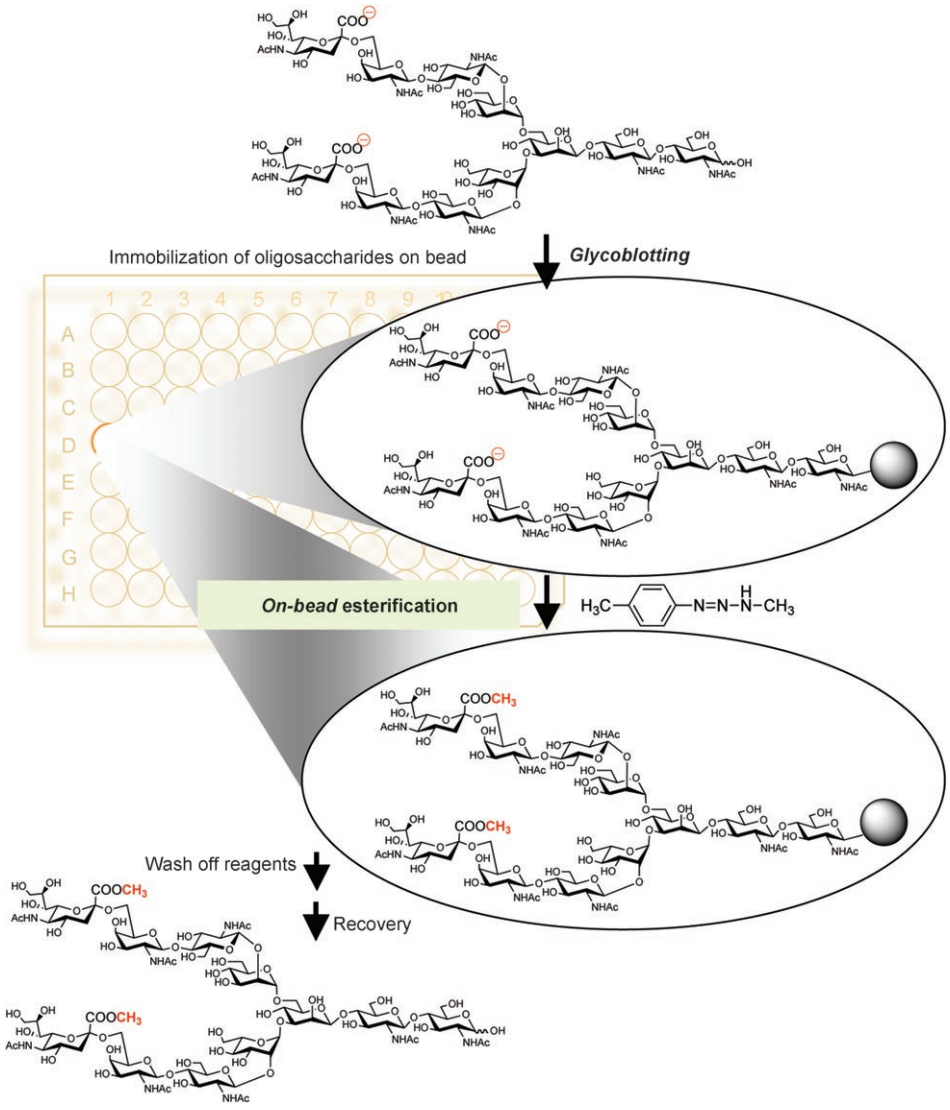
 © 2007 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim WILEY InterScience DISCOVER SOMETHING GREAT

these issues in measurements of acidic oligosaccharides in mass spectrometry, as well as of enhancing the sensitivity and resolution, several approaches have been employed so far. Although the choice of matrix for MALDI-TOF has a strong influence on the electronic excited state of an individual precursor ion and has often given usable parent ions when sialic acids have been involved,[8] it seems that this approach cannot prevent the significant degree of dissociation of a sialic acid residue from its original structure in the ionization process. While mild ionization methods such as sonic spray ionization (SSI) in combination with negative ion mode measurement may become a potent alternative strategy,[9] chemical modification of target sialic acids in glycoconjugates seems best suited to provide enough stability for subsequent structural characterization by various MS-based approaches. One such approach is to neutralize the negative charge of a sialic acid carboxy group either by methyl esterification[3,10] or by amidation.[11] Although some methods for modification of oligosaccharides incorporating sialic acid moieties have been employed, most such procedures need tedious and time-consuming post-reaction steps to remove large excesses of chemical reagents or to isolate the products, and this in turn prevents rapid, practical and large-scale analysis of picomole levels of total (neutral and acidic) glycome. The use of solid supports should make methods for handling of samples more convenient and improve the ease of system manipulation. It seems likely that the use of "glycoblotting",[12] a promising method in which solid and/or polymer platforms are applied to capture oligosaccharides selectively from crude mixture of biomolecules, should greatly facilitate subsequent manipulation for modification and purification of target derivatives. Recently, Hindsgaul's group has developed an excellent solid-phase derivatization strategy for sugars captured by glycoblotting, known as the "SPOT" method.[12c] We believe that this process has the potential to become a crucial and rate-limiting step for the successful high-throughput analysis of protein glycosylation, one of the most important post-translational modifications.

Here we report a simple and versatile technique for quantitative methyl esterification of sialic acid residues on conventional solid-phase platforms, in which oligosaccharides are adsorbed or covalently attached to the supporting materials and are then subjected to methyl esterification under mild conditions through the use of a simple triazene derivative, 3-methyl-1-*p*-tolyltriazene, in a 96-well filter microplate (Scheme 1).

Results and Discussion

3-Methyl-1-*p*-tolyltriazene (MTT) as an effective methyl esterification reagent for sialic acid residues: 3-Alkyl-1-aryltriazenes[13] and related compounds are known to be excellent alkylating reagents of carboxylic acids in general organ-



Scheme 1. Workflow for "on-bead methyl esterification" of sialic acids for immobilization and recovery of oligosaccharides.

 © 2007 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

ic compounds.[14] While some other common reagents such as diazomethane and its analogues belong to groups of hazardous compounds, alkyl derivatives of triazene are far less toxic, non-explosive and stable unless treated with acids. In spite of their usefulness in organic synthesis, there is no example known to us of alkylation of sugars containing carboxy groups through the use of 3-alkyl-1-aryltriazenes or related compounds. This is due to the poor solubilities of naturally occurring sialyloligosaccharides—which, namely, are extremely hydrophilic macromolecular carboxylic acids bearing multiple hydroxy groups (polyols)—in the general organic volatile solvents, such as chloroform, dichloromethane, ether or hexane, commonly used for alkylation reactions with these reagents. However, the rapid and excellent reaction profiles of this class of alkylating reagents, together with their safe/easy handling characteristics, strongly motivated us to establish a novel and simple protocol for quantitative methyl esterification of sialic acid residues in various glycoconjugates. Our interest first focused on the potential utility and effectiveness of 3-methyl-1-*p*-tolyltriazene (MTT), one of the commercially available alkyl triazenes, in the esterification of sialic acid carboxy groups.

After testing several conditions, we found that the combined use of DMSO and acetonitrile provides excellent dissolving capability for common sialyloligosaccharides and allows the desirable quantitative methyl esterification though the employment of MTT in excess. When 2-aminobenzamide-tagged (2AB-tagged) sialyllactose (Neu5Acα2,3-Galβ1,4Glc), serving as a model sialyloligosaccharide, was incubated with MTT (0.1 M) in a DMSO/acetonitrile (1:1) mixture for 1 h at 60 °C, the results suggested that MTT works very well to afford perfect methyl esterification of this trisaccharide, as shown in Figure 1. When an unprotected sialyllactose was analysed by MALDI-TOF in reflector, positive ion mode, three sodiated ions at *m*/*z* 485.5, 776.5, and 798.5—due to $[M-\text{Sia}+\text{Na}]^+$, $[M+\text{Na}]^+$ and $[M-\text{H}+2\text{Na}]^+$—were observed (Figure 1a), together with a protonated ion corresponding to $[M+\text{H}]^+$ at *m*/*z* 754.5. This result clearly indicates that the presence of the one free carboxy group of sialyllactose generated a mixture of four molecular ions, and the ion at *m*/*z* 485.5 was identified as an unfavorable fragment ion caused by in- and/or post-source dissociation of the sialic acid residue from the parent sialyllactose. On the other hand, upon suitable chromatographic purification, MTT-treated sialyllactose gave a simplified monosodiated ion at *m*/*z* 790.5 due to $[M(\text{OMe})+\text{Na}]^+$, without any significant fragmentation, demonstrating that MTT is highly efficient reagent for the methyl esterification of simple oligosaccharides containing sialic acid components under mild solution-phase conditions through the use of a mixture of DMSO and acetonitrile (1:1, v/v).

Solid-phase methyl esterification of sialylated oligosaccharides with MTT: Our attention was next directed toward the applicability of MTT-based esterification for sialylated oligosaccharides enriched on solid supports, as our goal was to establish a practical method for large-scale and quantitative glycomics in a high-throughput, automated manner. We selected a commercially available hydrazide-type resin (Affi-Gel Hz, BioRad) as a tentative platform for glycoblotting, in which carbohydrates captured on solid supports can be recovered reversibly.[12,15] Taking these prerequisites into account, we employed this glycoblotting polymer with a filter plate (MultiScreen Solvinert, Millipore) that was resistant to the organic solvents used in the protocol and showed no leakage of the solvent during all chemical manipulations. Initially, the optimized conditions for the immobilization of porcine fibrinogen oligosaccharides with Affi-Gel Hz were evaluated without MTT-based modification by characterization of 2AB-tagged materials released from the resin as shown in Figure 2a (HPLC) and Figure 3a (MALDI-TOF MS). As anticipated, two major *N*-glycans in porcine fibrinogen,[16] monosialylated A1F (25 min, 30%) and disialylated A2F (35 min, 70%), assigned by HPLC could be detected by MALDI-TOF MS as the ion peaks at *m*/*z* 2122.7 $[M-\text{H}+2\text{Na}]^+$ and *m*/*z* 2435.1 $[M-2\text{H}+3\text{Na}]^+$, respectively. However, MALDI analysis also gave an undesired molecular ion peak at *m*/*z* 1810.0 $[M+\text{Na}]^+$, corresponding to the asialo form of the above *N*-glycans, indicating that a significant degree of dissociation of sialic acid residues had occurred in A1F and/or A2F during the mass measurement.

The benefit of solid-phase MTT-based methyl esterification is evident because both HPLC and MALDI-TOF MS analysis of the 2AB-tagged sugars released after this modification afforded reasonable and satisfactory results after an increase in the concentration of MTT used in the solid-phase manipulation from 0.1 M to 0.5 M (Figures 2b–d and 3b–d). We concluded that on-bead/microplate methyl esterification of immobilized oligosaccharides had proceed-

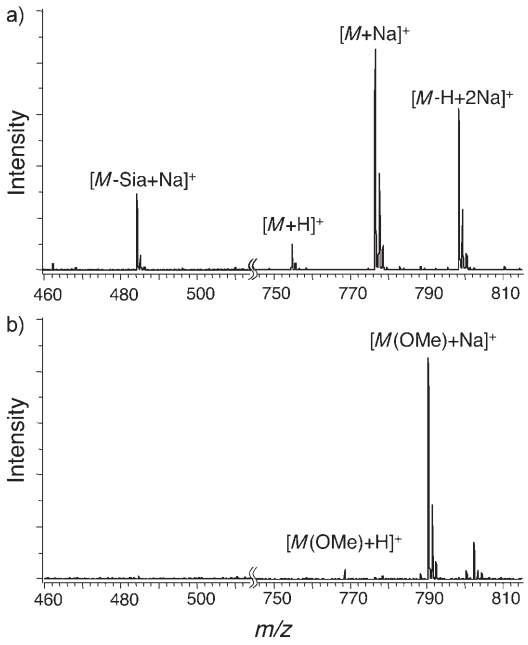


Figure 1. Reflector, positive-ion mode MALDI-TOF mass spectra of 2AB-labeled sialyllactose: a) before, and b) after treatment with MTT in solution phase.

 © 2007 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

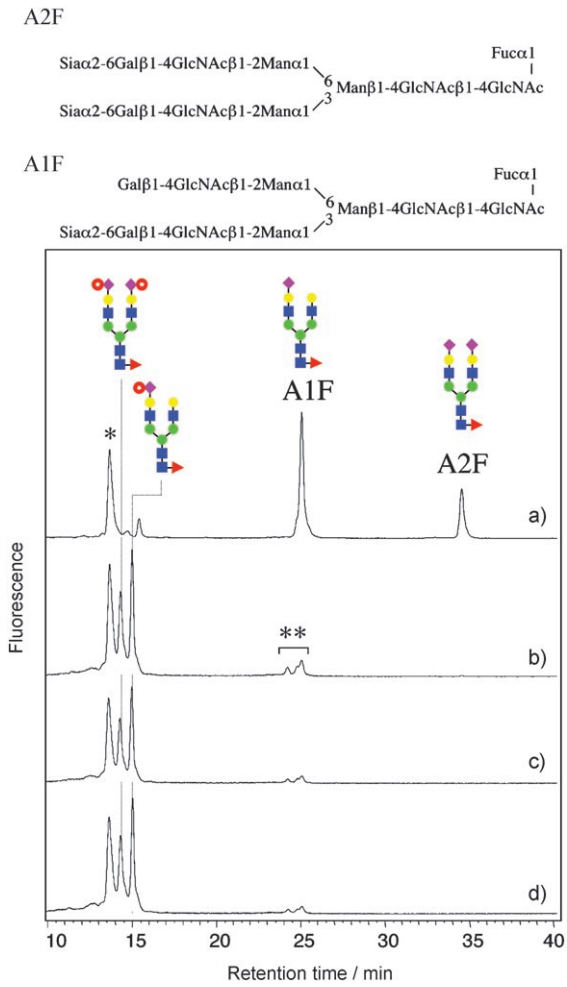


Figure 2. Amine-adsorption HPLC of porcine fibrinogen-derived *N*-glycans labeled with 2AB: a) without MTT, b) 0.1 M MTT, c) 0.2 M MTT, d) 0.5 M MTT. The peaks * and ** represent isomaltoheptaose spiked exogenously as internal standard and incompletely modified sialylated oligosaccharides, respectively. A red ring denotes a methyl ester on a sialic acid residue.

ed smoothly with use of 0.5 M MTT at 60 °C over 2 h and had given close to quantitative derivatization of both A1F and A2F. As shown in Figure 3d, use of MALDI-TOF MS permitted us to generate quite simple, singly charged ion peaks at *m*/*z* 2114.8, due to monomethylated A1F $[M+Na]^+$, and at *m*/*z* 2419.3, due to dimethylated A2F $[M+Na]^+$.

Methyl esterification allowed for quantitative profiling of the mixture of neutral, mono- and disialylated *N*-glycans. As shown in Figure 4a, the ratio of neutral (NA2) and two kinds of mono- (A1) and disialylated (A2) *N*-glycans of the standard mixture used in this study was estimated by HPLC analysis to be 1:2:1. However, when this mixture was directly subjected to MALDI-TOF analysis in positive-ion and reflector mode, the intensities of the ion peaks corresponding to these three oligosaccharides did not reflect the ratio determined by the above HPLC analysis (Figure 4b). The intensity of the ion peak due to A2 observed at *m*/*z* 2410.1 was drastically reduced, while those of the ion peaks at *m*/*z* 2097.4 and 1784.6, due to A1 and NA2, were enhanced, indicating that a significant degree of dissociation of the sialic acid residues from A2 and A1 through in-source and post-source decay had occurred during the MALDI-TOF measurements. On the other hand, on-bead methyl esterification of the mixture by treatment with MTT achieved quantitative profiling of the original glycoforms of NA2 (*m*/*z* 1784.4), A1 (2089.1) and A2 (2393.7), as indicated in Figure 4c. The relative quantities of each precursor ion could be calculated and determined from each monoisotopic peak area. The noticeable good correlation between Figure 4a and 4c for the relative intensities of these *N*-glycan species is shown in Figure 4d. Both HPLC and MALDI-TOF MS analyses gave very similar and reliable quantification results for the mixture composed of neutral (NA), monosialylated (A1) and disialylated (A2) oligosaccharides, demonstrating that the solid-phase methylation of the sialic acid carboxy group provides a means of quantification for the simultaneous analysis of neutral and sialyloligosaccharides.

The versatility of this method was demonstrated by its application for direct monitoring of the enzymatic sialylation of LacNAc residues displayed on gold colloidal nanoparticles (GCNPs). We have recently reported the feasibility of GCNPs as a convenient platform on which to immobilize oligosaccharide derivatives as acceptor substrates for glycosyltransferases.[17] Although on-GCNP MALDI-TOF MS enabled direct ionization and subsequent structural characterization of oligosaccharides constructed on the GCNPs in the presence of β-1,4-galactosyltransferase and α-1,3-fucosyltransferase, glycosylation catalysed by α-2,3- and α-2,6-sialyltransferases could not be detected because of the dissociation of sialic acid residues from the products during in-source and post-source processes as described above. We examined the efficiency of on-GCNP methyl esterification of sialic acid residues by direct MALDI-TOF analysis of the oligosaccharide products synthesized by treatment with rat recombinant α-2,6-sialyltransferase in the presence of CMP-Neu5Ac (Scheme 2). As shown in Figure 5a, direct MALDI-TOF MS of the GCNPs exhibits no detectable ion peak corresponding to a product modified by α-2,6-sialyltransferase, but only the ion peaks due to the starting compound **1** at *m*/*z* 1221.4 $[\mathbf{1}+Na]^+$ and 1237.4 $[\mathbf{1}+K]^+$, when laser irradiation was conducted without any protection for sialic acid residues. However, it was clearly shown that MTT-based methyl esterification on the GCNPs works well, to give a significant ion peak at *m*/*z* 1526.8, due to the sialylated trisaccharide derivative **2** derived from the starting disaccharide derivative **1** (Figure 5b).

Conclusions

We have demonstrated here the versatility of MTT in the solid-phase methyl esterification of general sialic acid(s)-containing oligosaccharides. Unlike other volatile, toxic and hazardous reagents such as diazomethane and iodomethane, MTT is a methylating reagent with great potential, because

 © 2007 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

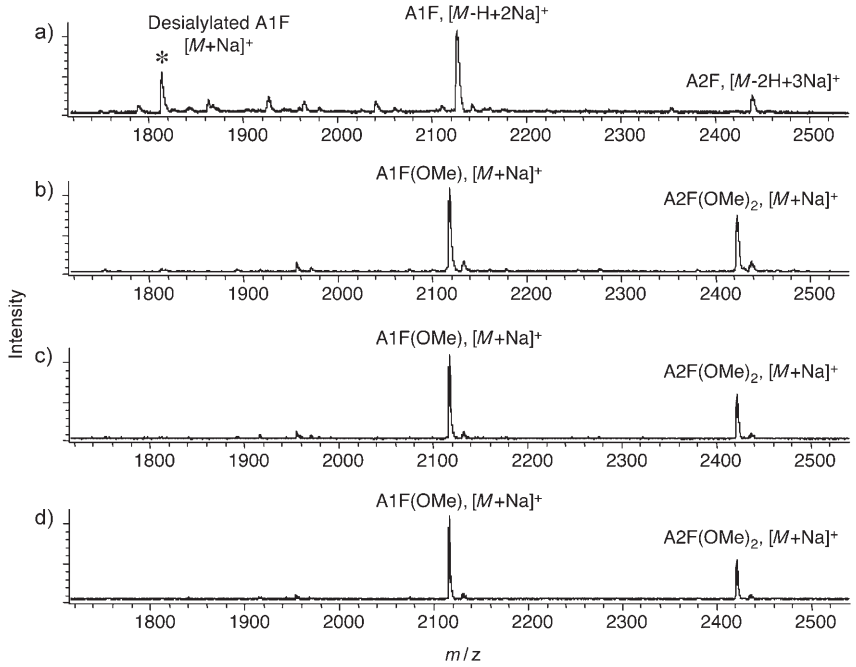


Figure 3. Mass spectra of 2AB-labeled porcine fibrinogen *N*-glycans processed in the absence (a) or presence (b–d) of MTT. Varying concentrations of MTT were used to investigate the effects on the mass profiles of ions: b) 0.1 M MTT, c) 0.2 M MTT, and d) 0.5 M MTT. The ion marked with * corresponds to the neutral glycan produced by complete loss of sialic acid(s). Note that A1F in (a) contains the ion derived from A2F with loss of one sialic acid.

it is a stable solid with high reactivity toward free carboxylic acids. Methyl esterification of sialic acid residues by treatment with MTT is generally complete within 1 h, even though the reaction is conducted on solid surfaces.

Our results clearly demonstrate that the MTT protocol permits high-throughput and quantitative methyl protection of sialic acid residues of general *N*-glycans captured on solid materials. After complete methyl esterification of sialylated oligosaccharides, MALDI-TOF MS provides highly reliable and simple spectra without any detectable loss of sialic acid residues from the original glycoforms. In addition, the mass spectra after methyl esterification become

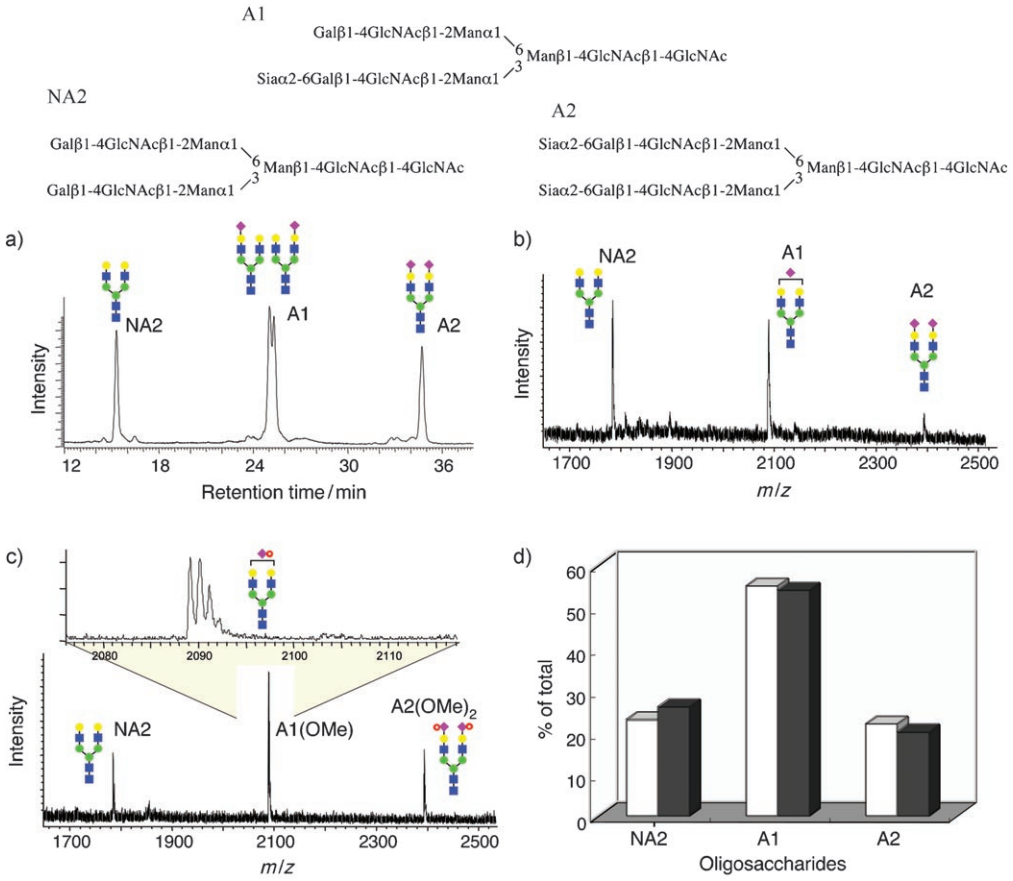
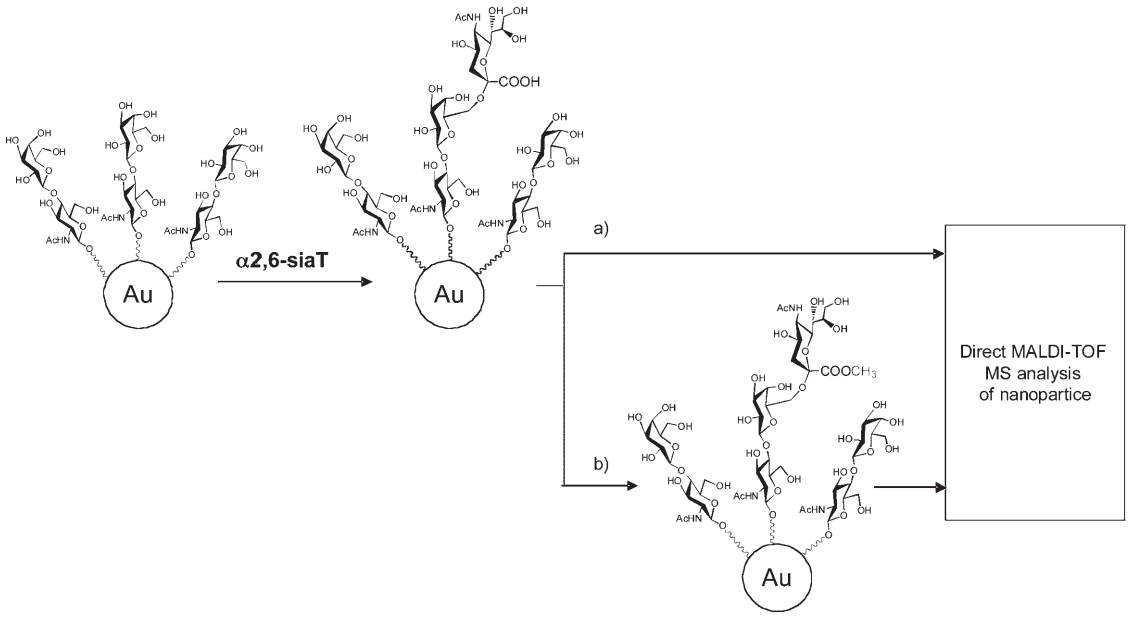


Figure 4. Comparison of quantification of neutral and sialylated *N*-glycans by HPLC and by MALDI-TOF MS analysis. a) HPLC chromatograph of untreated *N*-glycans containing neutral (NA2), monosialo (A1) and disialooligosaccharide (A2). b) Mass spectrum of the same oligosaccharide mixture as in a. c) Mass spectrum of the same oligosaccharide mixture as in a), but after treatment with MTT. d) Relative quantities of the oligosaccharide as estimated by HPLC (open bar) and by MALDI-TOF MS (closed bar).

 © 2007 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim



Scheme 2. Workflow for the "on-gold colloidal nanoparticle methyl esterification" of sialic acids for direct monitoring of enzymatic sialylation.

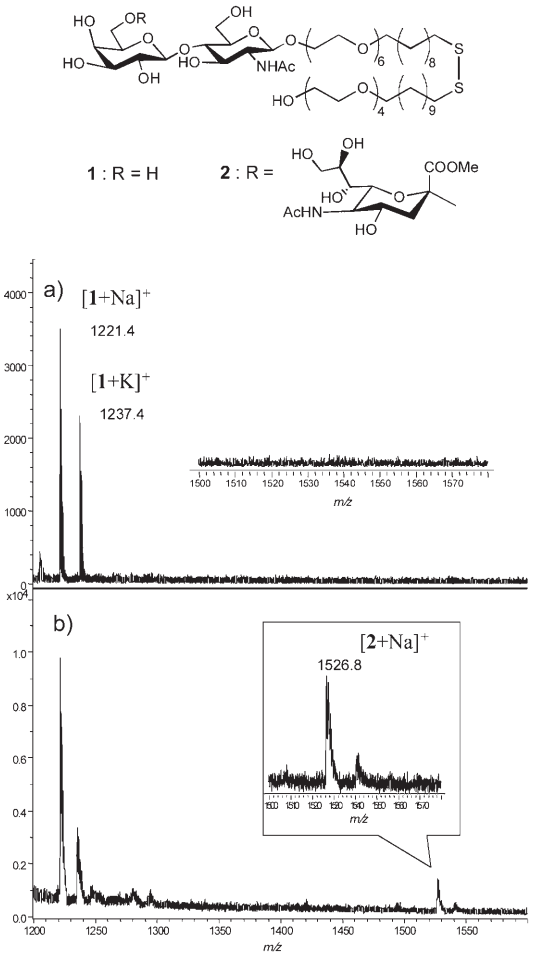


Figure 5. Direct monitoring of recombinant rat α-2,6-sialyltransferase activity with the aid of the GCNP-carrying compound **1**. The modified product, chemisorbed on the GCNP surface, was directly ionized by laser irradiation in the presence of matrix, before (a) and after (b) MTT treatment.

much simpler than those observed without MTT treatment, in which multiply cationized ions are diminished, and hence sensitivity and resolution are enhanced. Although stabilization by amidation with 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) and ammonium chloride also improved structural analysis of sialylated compounds by mass spectrometry,[11] this protocol usually requires incubation of the mixture for 24 h, because of low reactivity. The merit of the present method is evident because rapid and easy esterification by treatment with MTT in DMSO/acetonitrile is suitable for large-scale glycomic approaches based on the concept of glycoblotting, through the use of solid, stable materials.[12] It should also be noted that the combined use of MTT-based methyl esterification and direct mass ionization of designated substrates displayed on GCNP[17,18] should also facilitate quantitative monitoring of activities of various sialyltransferases in living cells as well as in human serum. We are currently investigating the viability of this protocol in large-scale clinical glycomics for the construction of databases of the relationships between glycoforms and various human diseases and the results will be reported as soon as possible. It is our belief that this method should greatly accelerate high-throughput protein glycomics study involving precise structural characterization of terminal sialic acid residues.

Experimental Section

Materials: Peptide *N*-glycosidase F (PNGase F) was purchased from Roche, Indianapolis. 3-Methyl-1-*p*-tolyltriazene (MTT), sialyllactose and dithiothreitol (DTT) were from Sigma–Aldrich. MTT was recrystallized from *n*-hexane before use. RapiGest SF was from Millipore. Bovine fibrinogen was purchased from Seikagaku Corp., Tokyo. Affi-Gel Hz was obtained from BioRad. Gold colloidal nanoparticles (GCNPs) were pre-

 © 2007 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

pared by the previously reported method.[17] Disialylated biantennary oligosaccharide, abbreviated as A2 glycan and used as a model for major complex-type *N*-linked-type glycans capped with sialic acids, was prepared from a sialylated glycopeptide purified from egg yolks as described previously.[19] The A2 glycan was partially hydrolysed by treatment with aqueous acetic acid (0.5 M) at 80 °C for 20 min to give a suitable mixture of neutral (NA2) and mono- (A1) and disialylated (A2) *N*-glycans. 2,5-Dihydroxybenzoic acid sodium salt (DHB) was from Wako Pure Chemicals, Tokyo.

Efficiency of MTT-based methyl esterification of 2AB-glycans in solution: To evaluate the efficiency of MTT-based methyl esterification of sialic acid carboxy groups, some model oligosaccharides labelled with 2-aminobenzamide (2AB) were prepared. Fluorescence-tagging of oligosaccharides with 2AB and removal of excess reagents were carried out by the general procedure reported previously.[20,21] Briefly, the freeze-dried oligosaccharide materials were incubated with the 2AB reaction mixture (20 μL). After the reaction was complete, purification of labeled oligosaccharides was performed on a spin column with a pipette tip containing silica gel beads. 2AB-tagged glycans were eluted in acetonitrile/water (1:1, 3×20 μL). The resulting materials were then passed through DOWEX-50 (H^+) and freeze-dried with use of a centrifugal concentrator. The purity of the 2AB-labelled oligosaccharides used in this study was characterized by amine-adsorption HPLC on a Microsorb MV amino (Varian), column, based on the number of negative charges and size of oligosaccharides as described previously.[22]

MTT solution (0.1 M, 20 μL) in DMSO/acetonitrile (1:1) was added to an aliquot of the above 2AB-labelled glycans (1 nmol scale), and the mixture was incubated at 60 °C for 60 min. After the reaction was complete, acetonitrile (200 μL) was added to the solution and the crude oligosaccharide derivatives were subjected to the general procedure for the purification on a silica gel spin column employed in the above 2AB tagging procedure.

Enzymatic release and immobilization of bovine fibrinogen *N*-glycans: Crude porcine fibrinogen (20 mg mL^{-1} in 50 mM ammonium bicarbonate, 500 μL) was mixed with aqueous RapiGest SF solution (0.2%, 500 μL) and the mixture was incubated at 37 °C for 10 min. DTT in water (1 M, 10 μL) was added to the solution and incubation was carried out at 60 °C for 30 min. Iodoacetamide (1 M, 21 μL) was then added to the solution and the mixture was incubated for an hour in the dark at room temperature. PNGase F (5 units) was directly added to the solution and the mixture was incubated for 12 h at 37 °C.

An aliquot of the digest mix (typically 5–10 μL) was directly subjected to immobilization on Affi-Gel Hz beads as follows. The suspension of Affi-Gel Hz beads (50 μL) was transferred to a well (MultiScreen Solvinert, Millipore Corporation, Bedford) and the beads were washed with acetic acid (1 M, 300 μL)). After washing with acetonitrile (300 μL), the digest mixture containing oligosaccharides (5 to 10 μL) was applied to the beads and made up to 20 μL with water. Acetic acid in acetonitrile (2%, 200 μL) was added to the well and the plate was incubated at 80 °C for 30 min. After the incubation, the beads were dried down and washed successively with guanidine hydrochloride (6 M, 3×300 μL) in ammonium bicarbonate (50 mM), HCl (10 mM), acetonitrile in water (50%) and acetonitrile.

On-bead esterification of sialylated oligosaccharides by MTT: MTT solution (0.1 M to 0.5 M, 100 μL) in DMSO/acetonitrile (1:1) was added to the above plate containing oligosaccharide-immobilized Affi-Gel beads, and the mixture was incubated at 60 °C for 2 h. The solution was filtered off under vacuum, and the beads were washed with acetonitrile (2×300 μL), ammonium bicarbonate (10 mM), and water. The modified oligosaccharides on the Affi-Gel beads were released by treatment with TFA in water (0.1%, 100 μL) at 60 °C for 20 min. The solid material (Affi-Gel beads) was filtered off and the solution was freeze-dried with use of a centrifugal evaporator. The residual material was subjected to the 2AB-labeling step by the same procedure as described in the preparation of model 2AB-tagged glycans.

Modification of oligosaccharides with MTT on gold colloidal nanoparticles: GCNPs displaying LacNAc (Galβ1,4GlcNAcβ1→) residues (**1**) were prepared by the previously reported method.[17] Sialylation of chemisorbed **1** on GCNP was performed with the use of recombinant rat α-2,6-sialyltransferase (10 mU, Calbiochem.) in cacodylate buffer (50 mM, pH 6.0, 0.5% Trion CF-54) containing CMP-Neu5Ac (800 μM, Yamasa, Japan) at 37 °C for 24 h. The GCNP was simply washed with deionized water and purified by ultrafiltration on YM-50 (Millipore). The enzymatically sialylated GCNP was dissolved in deionized water (5 μL) and mixed with HCl (50 mM, 1 μL) and acetonitrile (200 μL), and the mixture was then centrifuged at 15000 rpm for 5 min. The precipitated GCNP was collected and dried in vacuo. MTT stock solution (0.2 M in DMSO/acetonitrile 1:1, 5 μL) was added to an aliquot of the residual GCNP and the mixture was placed on a heat block at 60 °C for 1.5 h. The reaction mixture was mixed with deionized water (10 μL) and centrifuged at 15000 rpm for 5 min to remove excess reagent and solvents. The precipitated GCNP was collected and directly employed for further MALDI-TOF MS measurement without any purification under conditions similar to those described in the preceding paper.[17]

Mass spectrometry: All MS measurements were performed with an Ultraflex TOF/TOF mass spectrometer fitted with a reflector and controlled through a FlexControl 2.2 software package (Bruker Daltonics) with use of a mixture of DHB and DHB sodium salt (9:1, 10 mg mL^{-1} each) in 30% acetonitrile as a matrix. 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.[8]

Acknowledgement

This work was supported partly by a grant for "Development of Systems and Technology for Advanced Measurement and Analysis (SENTAN)" from the Japan Science and Technology Agency (JST) and a grant-in-aid (no. 17205015) from the Ministry of Education, Culture, Science, and Technology, Japan.

[1] J. Zaia, *Mass Spectrom. Rev.* **2004**, *23*, 161–227.
[2] A. Dell, H. R. Morris, *Science* **2001**, *291*, 2351–2356.
[3] A. K. Powell, D. J. Harvey, *Rapid Commun. Mass Spectrom.* **1996**, *10*, 1027–1032.
[4] M. Hato, H. Nakagawa, M. Kurogochi, T. O. Akama, J. D. Marth, M. N. Fukuda, S.-I. Nishimura, *Mol. Cell. Proteomics* **2006**, *5*, 2146–2157.
[5] P. R. Crocker, A. Varki, *Trends Immunol.* **2001**, *22*, 337–342.
[6] J. W. Dennis, M. Granovsky, C. E. Warren, *Biochim. Biophys. Acta* **1999**, *1473*, 21–37.
[7] I. Brockhausen, *Biochim. Biophys. Acta* **1999**, *1473*, 67–95.
[8] M. Kurogochi, S.-I. Nishimura, *Anal. Chem.* **2004**, *76*, 6097–6101.
[9] Y. Takegawa, K. Deguchi, S. Ito, S. Yoshioka, H. Nakagawa, S.-I. Nishimura, *Anal. Chem.* **2005**, *77*, 2097–2106.
[10] P. Kang, Y. Mechref, I. Klouckova, M. V. Novotny, *Rapid Commun. Mass Spectrom.* **2005**, *19*, 3421–3428.
[11] S. Sekiya, Y. Wada, K. Tanaka, *Anal. Chem.* **2005**, *77*, 4962–4968.
[12] a) 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; b) K. Niikura, R. Kamitani, M. Kurogochi, R. Uematsu, Y. Shinohara, H. Nakagawa, K. Deguchi, K. Monde K, H. Kondo, S.-I. Nishimura, *Chem. Eur. J.* **2005**, *11*, 3825–3835; c) A. Lohse, R. Martins, M. R. Jorgensen, O. Hindsgaul, *Angew. Chem.* **2006**, *118*, 4273–4278; *Angew. Chem. Int. Ed.* **2006**, *45*, 4167–4172; d) H. Shimaoka, H. Kuramoto, J-i. Furukawa, Y. Miura, M. Kurogochi, Y. Kita, H. Hinou, Y. Shinohara, S.-I. Nishimura, *Chem. Eur. J.* **2007**, *13*, 1664–1673.
[13] E. H. White, H. Scherrer, *Tetrahedron Lett.* **1961**, *2*, 758–762.
[14] J. Rademann, J. Smerdka, G. Jung, P. Grosche, D. Schmid, *Angew. Chem.* **2001**, *113*, 390–393; *Angew. Chem. Int. Ed.* **2001**, *40*, 381–385.

 © 2007 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

[15] K. Larsen, M. B. Thygesen, F. Guillaumie, W. G. Willats, K. J. Jensen, *Carbohydr. Res.* **2006**, *341*, 1209–1234.
[16] M. L. Da Silva, T. Tamura, T. McBroom, K. G. Rice, *Arch. Biochem. Biophys.* **1994**, *312*, 151–157.
[17] N. Nagahori, S.-I. Nishimura, *Chem. Eur. J.* **2006**, *12*, 6478–6485.
[18] J. M. de La Fuente, A. G. Barrientos, T. C. Rojas, J. Rojo, J. Canada, A. Fernandez, S. Penades, *Angew. Chem.* **2001**, *113*, 2317–2321; *Angew. Chem. Int. Ed.* **2001**, *40*, 2257–2261.
[19] A. Seko, M. Koketsu, M. Nishizono, Y. Enoki, H. R. Ibrahim, L. R. Juneja, M. Kim, T. Yamamoto, *Biochim. Biophys. Acta* **1997**, *1335*, 23–32.
[20] Y. Miura, Y. Ding, A. Manzi, O. Hindsgaul, H. H. Freeze, *Glycobiology* **1999**, *9*, 1053–1060.
[21] J. C. Bigge, T. P. Patel, J. A. Bruce, P. N. Goulding, S. M. Charles, R. B. Parekh, *Anal. Biochem.* **1995**, *230*, 229–238.
[22] Y. Miura, H. H. Freeze, *Glycobiology* **1998**, *8*, 813–819.

Received: December 23, 2006
Published online: March 20, 2007

 © 2007 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim