CHEMISTRY
A EUROPEAN JOURNAL

DOI: 10.1002/chem.200501267

Direct and Efficient Monitoring of Glycosyltransferase Reactions on Gold Colloidal Nanoparticles by Using Mass Spectrometry

Noriko Nagahori[a] and Shin-Ichiro Nishimura*[a, b]

Abstract: A simple and efficient assay for glycosyltransferase activity on gold colloidal nanoparticles (GCNPs) by using laser desorption/ionization time-of-flight mass spectrometry (LDI-TOF MS) is demonstrated by the enzymatic synthesis of the Lewis X trisaccharide on GCNPs containing GlcNAc residues. GCNPs containing multivalent sugars were well dispersed in aqueous solution and proved to be excellent acceptor substrates for the glycosyltransferase reaction. Direct LDI-TOF MS analysis of these GCNPs provided the ion peaks of the sugar derivatives, chemisorbed through S–Au linkages onto the GCNPs, even in the presence of contaminants such as proteins and salts. Thus, it enabled the rapid and direct detection of the enzymatic reaction on the GCNPs by subjecting a small amount (0.15 μL) of the reaction mixture to MS analysis without purification. Subsequent MS/MS analyses (LDI-LIFT-TOF/TOF method) of the product-carrying GCNPs enabled the structures of the sugar derivatives that had been constructed on the GCNPs by enzymatic glycosylation to be determined. A quantitative inhibition assay for glycosyltransferase by using LDI-TOF MS analysis on the GCNPs was demonstrated by using uridine 5′-diphosphate (UDP) as the inhibitor. This simple assay was then applied to the detection of the enzymatic activity of a crude cell extract of *Escherichia coli*, which produces *Neisseria meningitidis* β-1,4-galactosyltransferase (β-1,4-GalT). In this case, the GCNPs were roughly purified by means of ultrafiltration to remove the buffer and detergents before MS analysis. That the GCNPs are dissolved in solution in the reaction medium but are solid in the purification process is greatly advantageous for the simple and efficient detection of enzymatic activity in crude biological samples. Thus, GCNPs containing a variety of biomolecules may become a versatile and efficient tool for the rapid and direct monitoring of metabolism (metabolomics) in living cells when combined with LDI-TOF MS analysis.

Keywords: carbohydrates · enzymes · glycosylation · mass spectrometry · nanoparticles

Introduction

Glycosyltransferases are important enzymes that catalyze glycoside bond formation to construct a variety of glycoconjugates, such as glycoproteins, glycolipids, and proteoglycans. Glycosylation reactions catalyzed by glycosyltransferases are crucial for the post-translational modifications of proteins and lipids that greatly influence various molecular-recognition processes such as bacterial/viral infections, cell adhesion, immune response, cellular differentiation, development, and regulation, and many other intercellular communication and signal transductions.[1] Glycosyltransferases are indispensable in the chemical and enzymatic synthesis of glycoconjugates.[2] Therefore, facile and rapid methods for monitoring glycosyltransferase reactions are of great significance for the development of automated glycosynthesis as well as novel diagnostic methods. To date, however, there are only a few methods commonly used for analyzing enzymatic sugar-elongation reactions. These methods use synthetic substrates with photosensitive or radioactive probes in combination with high-performance liquid chromatography (HPLC) analysis, and therefore require special labeling of the analyte and extensive sample purification.[3] Metal nanoparticles, including gold colloidal nanoparticles (GCNPs), have been conjugated with various biomolecules[4] and applied in the fields of biochemistry and chemical biology for

[a] Dr. N. Nagahori, Prof. Dr. S.-I. Nishimura
Graduate School of Advanced Life Science
Frontier Research Center for the Post-Genome Science and Technology, Hokkaido University, N21, W11
Sapporo 001-0021 (Japan)
E-mail: shin@glyco.sci.hokudai.ac.jp

[b] Prof. Dr. S.-I. Nishimura
National Institute of Advanced Industrial Science and Technology (AIST)
Sapporo 062-8517 (Japan)
Fax: (+81) 11-706-9043

molecular-recognition assays,[5] fluorescence nanocrystal labeling,[6] surface-enhanced Raman spectrometry,[7] and analyte concentrations for mass spectrometric analysis.[8] These nanoparticles conjugated with biomolecules are soluble in aqueous solution and stable under physiological conditions. Thus, we thought that small GCNPs with a diameter of approximately 10 nm would be a suitable model for general globular proteins. It is likely that GCNPs bearing multivalent sugars will behave like oligosaccharide chains of native glycoproteins. Furthermore, owing to the "glycoside cluster effect"[9] they should display a highly enhanced affinity for enzymes, and are thus expected to be good glycosyl-acceptor substrates.

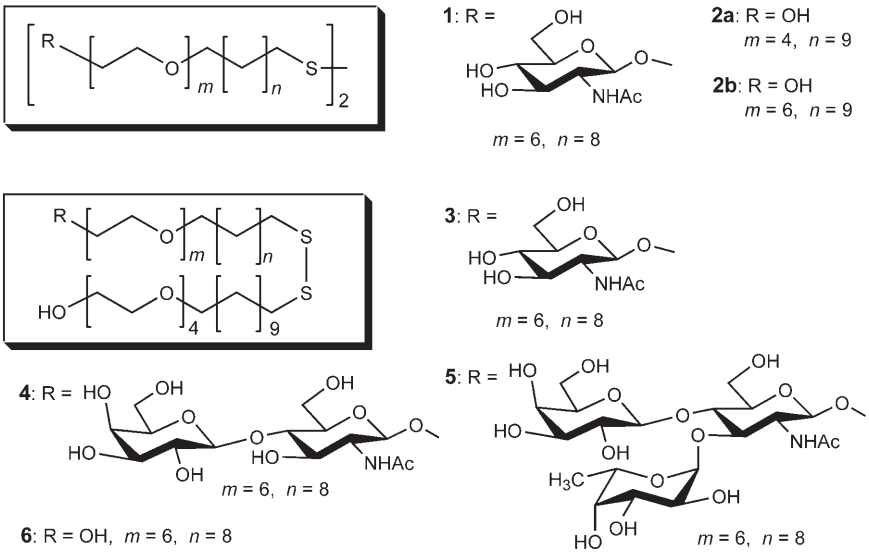
Metal nanoparticles have been used as a matrix for laser desorption/ionization time-of-flight mass spectrometry (LDI-TOF MS) analyses, in which the metal particles serve as a reservoir for photon-energy deposition.[10] In particular, GCNPs (<10 nm) were found to exhibit greater ionization efficiencies than organic matrices owing to the quantum confinement effect.[11] Recently, it was shown that the self-assembled monolayer (SAM) of a thiol compound chemisorbed onto an Au surface could be ionized directly to cleave the Au–S bond by laser irradiation during the matrix-assisted LDI process.[12] Therefore, GCNPs covered with carbohydrates connected through Au–S linkages are expected to be optimal substances for LDI-TOF MS analyses in which the ionization efficiency is highly enhanced. Our attention has been directed toward the use of small GCNPs as a scaffold for immobilizing carbohydrates. It is expected that analyte ionization on the GCNP surface will be highly enhanced by the laser irradiation under LDI-MS conditions. In addition, the surface should become a good scaffold for containing acceptor substrates for glycosyltransferases.

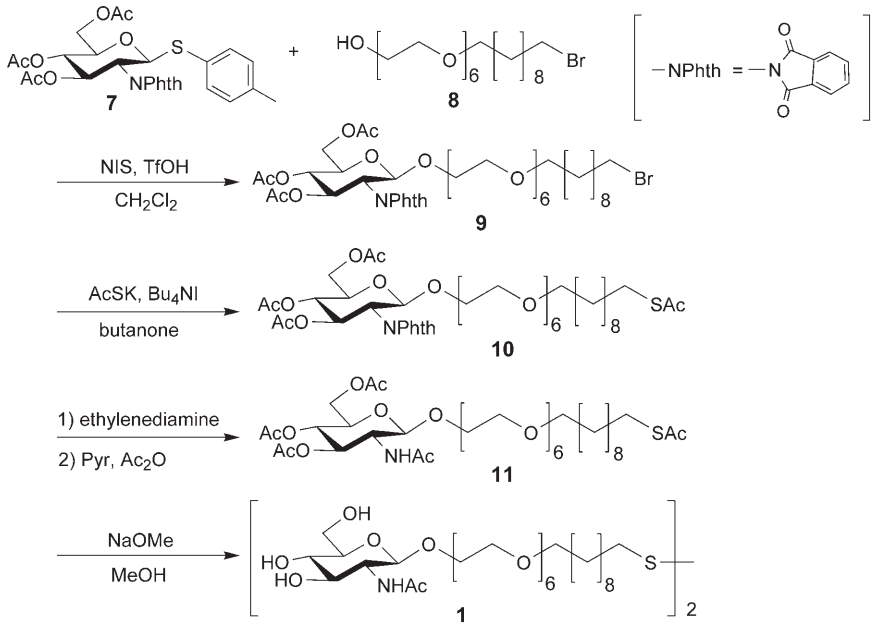
It has also been demonstrated that conjugating small molecules onto a colloidal Au surface makes it possible to sequester and transfer small quantities of analytes with high efficiency.[13] In addition, analytes adsorbed on Au colloids can be easily transferred between instruments and manipulative biological experiments without loss.

In the present paper, we report a novel, simple, and efficient assay for glycosyltransferase activity by demonstrating the direct detection and characterization of Lewis X (Le^x) trisaccharide synthesis on GCNPs, which provide a convenient scaffold that accelerates ionization of the adsorbed molecules.

Results and Discussion

The preparation of the GCNPs involved the stabilization of the metal nanoparticles by providing a monolayer of glycolipid mimetics to generate monodispersed metal nanoparticles. As shown in Scheme 1, stabilizer molecule **1** was synthesized by connecting a GlcNAc residue to a long alkanethiol chain with a hydrophilic oligomeric ethylene glycol linker. Synthetic procedures and conditions are described in the Experimental Section. Reduction of tetrachloroauric acid ($HAuCl_4$) with $NaBH_4$ in methanol was performed in the presence of stabilizer **1** and **2a** under similar conditions to those given in a previous report[14] in which compound **2a** was employed for controlling the sugar density on the GCNPs in biological assays. In this paper, a molar ratio of 1:9 for **1**/**2a** was employed, which seemed to be favorable for the enzymatic glycosylation according to a previous study using glycopolymers as acceptor substrates.[15] Transmission electron microscopy (TEM) of the gold nanoparticles (Figure 1a) showed that the majority were well-separated single particles with diameters ranging from 3.0 to 8.0 nm. LDI-TOF MS analyses of the GCNPs prepared from **1** and **2a** (Figure 1b) clearly indicated the generation of molecular ions due to these stabilizer molecules, and ions at m/z 781 and m/z 1058 were found to correspond to the sodium adducts of homo-disulfide **2a** and hetero-disulfide **3** (composed of **1** and **2a**). The GCNPs were treated with I_2 to cleave the Au–S linkages between the gold surface and the stabilizers prior to the LDI analyses. An MS analysis of this material showed no significant peaks (Figure 1c); this suggests that the laser desorption/ionization shown in Figure 1b had occurred directly on the surface of the GCNPs through the bond rearrangement from Au–S to S–S under laser irradiation. Taking into account the ratio of the two stabilizers employed for synthesizing the GCNPs, it is reasonable that





Scheme 1. Synthetic scheme of compound **1**.

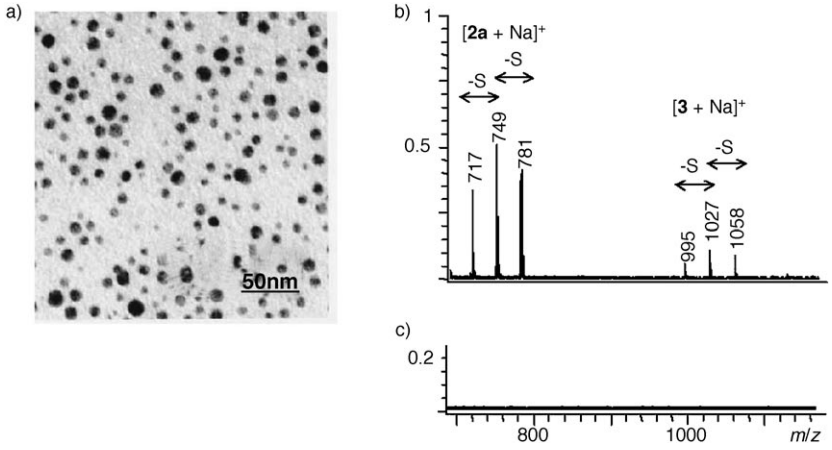


Figure 1. Data for the GCNPs bearing stabilizers **1** and **2**: a) TEM image; LDI-TOF mass spectra of b) the GCNPs (20 pmol) and c) I_2-treated GCNPs (20 pmol).

hetero-disulfide **3** (*m*/*z* 1058) was generated as the major product ion by the bond rearrangement with the counterpart derived from **2a** (*m*/*z* 781) rather than the original homo-disulfide **1**. In addition, peaks for ions in which sulfur had been eliminated at *m*/*z* 1027 and 995 that could be assigned to R-S-R′- and R-R′-type derivatives, respectively, were also produced during laser irradiation. The intensity of these ions can be reduced by controlling the laser power as shown in the following data. Although the mechanism for the formation of the ions containing unusual C−S−C and C−C linkages is not clear,[16] deletion of sulfur atoms from the chemisorbed substances under LDI conditions seems to make the GCNPs a specific catalyst for the development of new reactions. A similar ionization profile was observed in single alkanethiol-type species (*m*/*z*: 680 $[M+Na]^+$; *m*/*z*: 646 $[M-H_2S+Na]^+$) from compound **1** (data not shown).

When the ionization efficiency of the LDI-TOF MS of **2a** adsorbed on the GCNPs was compared with that of **2a** adsorbed on a gold-coated plate, we observed drastically amplified spectrometric signatures in the results from the GCNPs (Figure 2). GCNPs were spread homogeneously on the TOF MS plate, and they did not cause spot-to-spot variation in signal intensity as often observed in MS analysis using organic matrices, demonstrating that the GCNPs allow highly sensitive LDI analyses of the analyte by single-laser irradiation without any error. LDI analysis by repetitive laser irradiation at the same position on the gold-coated MALDI plate did not show significant ionization (Figure 2a). In the case of the LDI of GCNPs, irradiation performed ten times at the same position still generated the satisfactory ion peak at *m*/*z* 781 signifying the homo-disulfide compound **2a** (Figure 2b). This indicated that the size of the GCNPs is essential for an efficient desorption and ionization process. On an extremely increased metal-nanoparticle surface area, specific laser scattering or diffused reflection can occur, which may greatly accelerate the LDI of chemisorbed small molecules from GCNPs. In addition, it should be noted that the present LDI MS using GCNPs avoided any overlapping with peaks due to matrix observed with common matrix-assisted LDI conditions, as demonstrated in Figure 3b and c.

Next, our attention was focused on the feasibility of GCNP-based LDI-TOF MS for directly monitoring sugar-elongation reactions catalyzed by recombinant glycosyltransferases. Figure 4 shows the results of the sequential enzymatic glycosylation reaction by recombinant human β-1,4-galactosyltransferase (β-1,4-GalT) and recombinant human α-1,3-fucosyltransferase (α-1,3-FucT) on GCNPs bearing **1** as the glycosyl-acceptor substrate. After each defined period of time, a small amount of the reaction mixture (0.5 μL) was

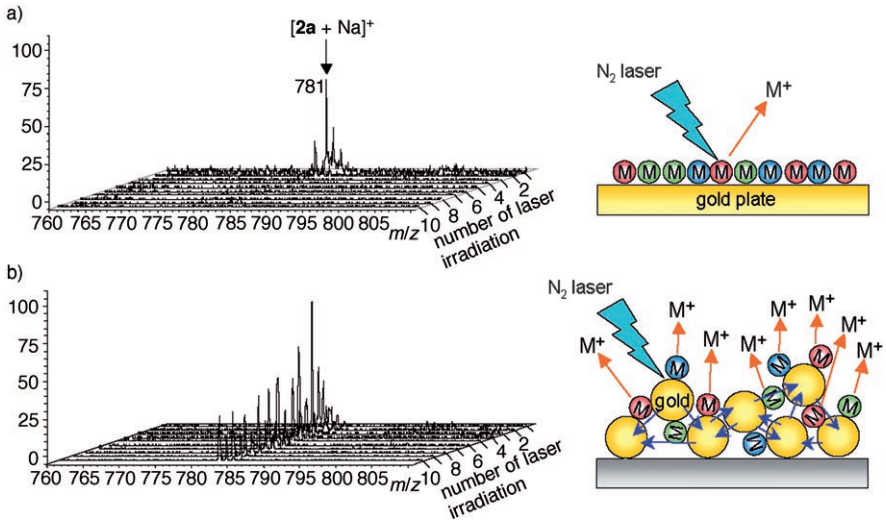


Figure 2. LDI-TOF mass spectra of a) **2a** deposited on the surface of the gold-coated MALDI plate and b) **2a** on the GCNPs.

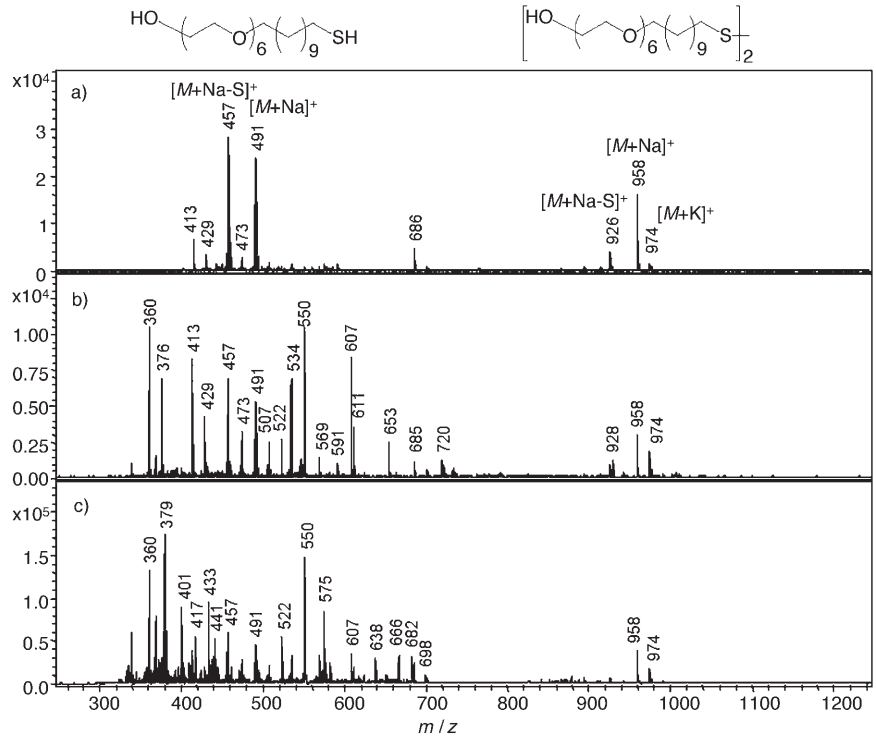


Figure 3. Mass spectra of **2b** on the GCNPs: a) LDI-TOF MS, b) MALDI-TOF MS in the presence of DHB (2,5-dihyroxybenzoic acid), and c) MALDI-TOF MS in the presence of CHCA (α-cyano-4-hydroxycinnamic acid). All experiments were carried out by using 12.5 μg of the GCNPs containing **2b**. 0.5 μL of each matrix solution (10 mg mL^{-1}) was added to the mixture.

subjected to MS analysis directly without any separation step to remove enzymes, excess donor substrates (sugar nucleotides), and impurities in the buffer solution. As the reaction proceeded, new ion peaks at *m*/*z* 1221 and 1367 due to reaction products **4** and **5**, respectively, were generated. It is noteworthy that the enzymes, donor substrates, and impurities were not ionized under the present LDI-TOF MS conditions and they did not affect the selective ionization of chemisorbed oligosaccharide products from the surface of the GCNPs. LDI-TOF/TOF MS analysis of the precursor ion peak at *m*/*z* 1367 produced fragment ion peaks at *m*/*z* 1221, 955, 808, 534, 388, and 372, which are evidence of the production of hetero-disulfide **5**, the appropriate Le^x trisaccharide structure. The formation of a product ion peak at *m*/*z* 855 suggested deglycosylation under LIFT-TOF/TOF[17] fragmentation conditions. In addition, these enzymatic sugar-elongation reactions can easily be monitored as exhibited in Figure 5. It was clearly demonstrated that both the GalT and FucT reactions proceeded smoothly and afforded 80% yields of compounds **4** and **5**, respectively. The precursor ion peaks corresponding to the sugar derivatives were detected as hetero-disulfide compounds similar to the ion peak due to compound **3** generated predominantly from compound **1** (Figure 1). Therefore, it was suggested that the reactions catalyzed by glycosyltransferases could be successfully monitored and evaluated by means of the ratio of peak intensity of product **4** or **5** relative to that of compound **3** as a control.[18] Figure 5c shows the simple inhibition assay using uridine 5′-diphosphate (UDP) as a model inhibitor. Reactions were performed under the same conditions as described above in the presence of UDP (0, 20, 50, 80, 100, 200, 300, 400, and 500 μM). The reaction mixture was incubated for 60 min at 25 °C and the degree of inhibition was directly determined by the intensities of the ion peaks corresponding to **3** and **4** in the LDI-TOF MS analysis performed under the conditions described in the Experimental Section. The IC_{50} (50% inhibitory concentration) was estimated to be 300 μM, which is consistent with previous reports.[19] It should be emphasized that this assay using recombinant glycosyltransferases did not require any purification of the

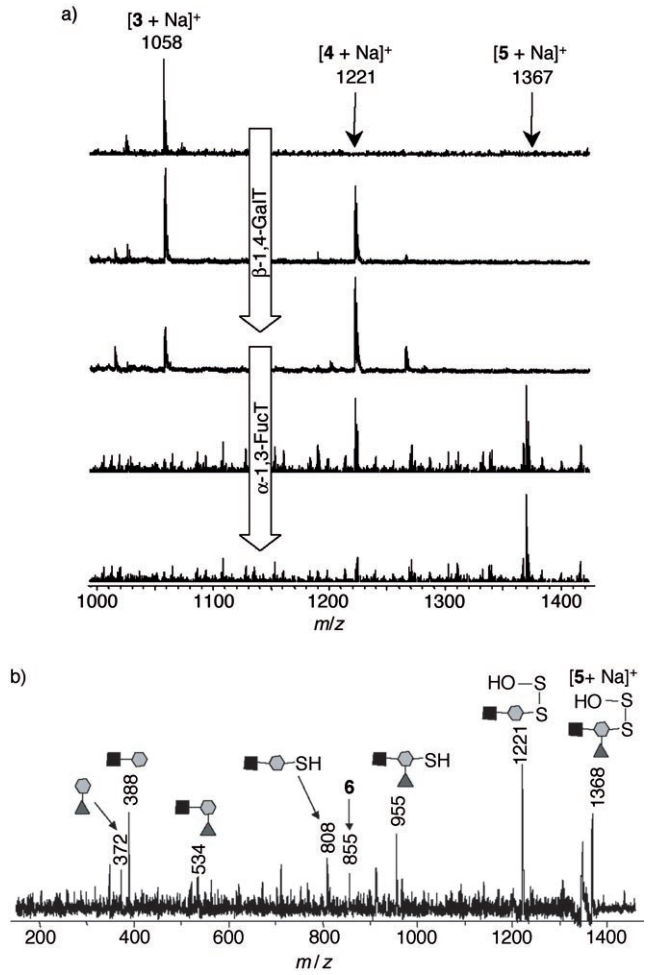


Figure 4. Direct detection of glycosyltransferase reactions by LDI MS using GCNPs with GlcNAc residues. a) Stepwise sugar-elongation reactions on GCNPs by glycosyltransferases. GCNPs carrying compound **3** (*m*/*z* 1058 [**3**+Na]$^+$) were first employed for the treatment with GalT in the presence of UDP-Gal and the reaction mixture was directly subjected to MS analysis. Crude GCNPs carrying product **4** (*m*/*z* 1221 [**4**+Na]$^+$) were subsequently employed for further fucosylation by α-1,3-FucT in the presence of GDP-Fuc and the GCNPs were directly used for LDI-TOF MS analysis without any purification. The precursor ion peak at *m*/*z* 1368 ([**5**+Na]$^+$) was employed for the fragmentation based on the LIFT-TOF/TOF mode according to a previous publication.[17] b) Structural characterization by the LDI-TOF/TOF MS method of Le^X derivative **5** synthesized on the GCNPs by recombinant glycosyltransferases.

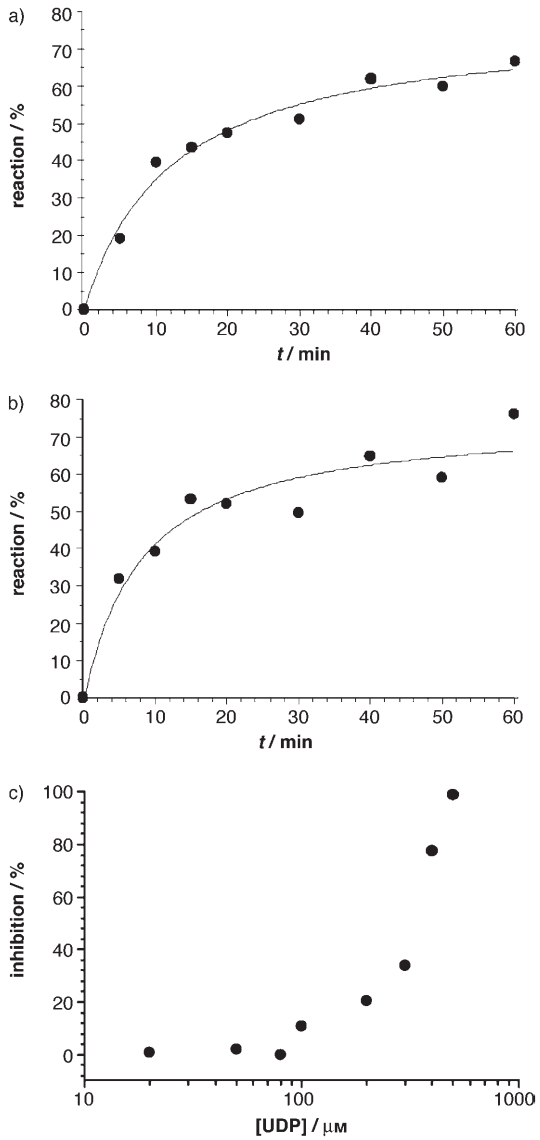


Figure 5. Characterization of glycosyltransferase reactions by means of GCNPs in HEPES buffer (10 mM: pH 7.4, 10 mM $MnCl_2$ and 150 mM NaCl). a) Reaction of glycosylation, monitored by direct LDI-TOF MS on GCNPs carrying **3** (50 μM), by β-1,4-GalT (4 mU) in the presence of UDP-Gal (400 μM). b) Reaction of glycosylation, monitored by direct LDI-TOF MS on GCNPs carrying **4** (50 μM), by α-1,3-FucT (5 mU) in the presence of GDP-Fuc (400 μM). c) Inhibition assay of β-1,4-GalT (4 mU) in the presence of UDP-Gal (400 μM) with UDP (0, 20, 50, 100, 200, 300, 400, and 500 μM).

GCNPs before analysis because the matrix-free LDI-TOF MS analysis allows selective ionization of the oligosaccharide products chemisorbed on the surface of the GCNPs.

Next, GCNPs containing compound **1** (120 μM) and UDP-Gal (50 μM) were directly added to a cell-free extract (final volume 80 μL) of *Escherichia coli*, which produces *Neisseria meningitidis* β-1,4-GalT, and the mixture was incubated for 20 h at 25 °C (Figure 6a). After the GCNPs had been collected from the cell-free extract by means of ultrafiltration to remove the detergent (Triton X-100), they were washed thoroughly with water because we found that some detergent molecules can affect the ionization of the chemisorbed compounds on the GCNPs under the general conditions of the direct LDI process. For this reason, low-molecular-weight impurities such as salts and detergent should be removed by ultrafiltration. However, it should be noted that the GCNPs were still contaminated with significant amounts of other biomacromolecules in the cell extract. The GCNPs were then re-suspended in a small amount of water and subjected to MS analysis. Direct LDI-TOF MS analysis of the GCNPs revealed a precursor ion at *m*/*z* 1221 ([**4**+Na]$^+$, Figure 6b), indicating that the crude extract of *E. coli* contained *N. meningitidis* β-1,4-GalT. This result clearly suggests that the present method does not require tedious purification of the GCNPs because the salts and impurities of the buffer

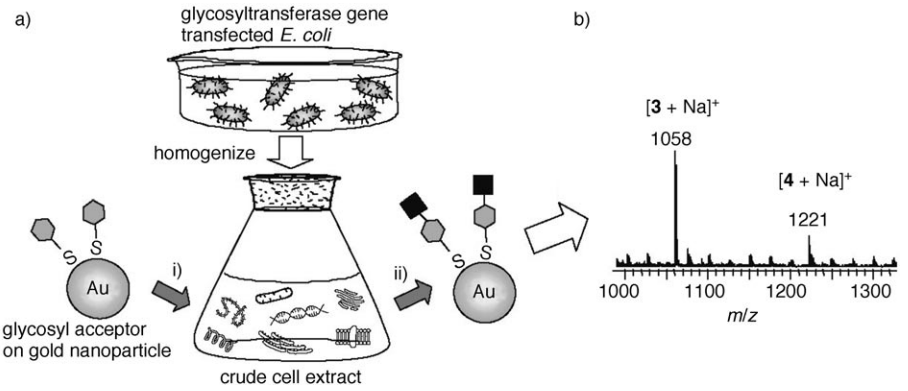


Figure 6. Direct monitoring of bacterial β-1,4-GalT activity in a cell-free extract with GCNPs carrying GlcNAc residues as the acceptor substrate. a) Experimental configuration for the direct detection of *N. meningitidis* β-1,4-GalT expressed in *E. coli*: i) Incubation of the GCNPs with cell-free extract at 25 °C for 20 h (pH 7.4, HEPES buffer containing 0.1 % Triton X-100); ii) Purification of the GCNPs by ultrafiltration using Microcon YM-50 and washed thoroughly with water to remove Triton X-100 before being subjected to the subsequent LDI-TOF MS analysis. b) LDI-TOF mass spectra of **4** at *m*/*z* 1221.6 ([**4**+Na]$^+$) generated from GCNPs collected from crude cell extract.

solution, except detergent, did not affect the ionization of the chemisorbed compounds on the GCNPs. This characteristic will also greatly facilitate high-throughput assays of unknown glycosyltransferases for evaluation of their kinetics and the potency of their inhibitors.

Conclusion

In conclusion, we report the successful LDI-TOF MS analysis of chemisorbed materials on GCNPs by directly monitoring glycosyltransferase reactions using recombinant human β-1,4-GalT, recombinant human α-1,3-FucTVI, and crude cell extracts of *E. coli*, which produces *N. meningitidis* β-1,4-GalT. The LDI-TOF/TOF MS analysis of GCNPs containing a variety of biomolecules is an extremely versatile and efficient method for directly and rapidly monitoring the metabolism (metabolomics) of chemisorbed small-molecule compounds on GCNPs in living cells. This LDI-TOF MS approach using chemisorbed small-molecule compounds on GCNPs displays a high tolerance to the nonspecific ionization of contaminants and no matrix interferences because GCNPs allow the selective desorption/ionization of the substrates/ligands covalently bound onto the surface of the GCNPs through an Au–S linkage in the absence of a conventional organic matrix. The potential applications of the LDI method using GCNPs is broad because general methods for the surface derivatization of GCNPs can easily be tailored for immobilizing target biomolecules.

Experimental Section

General procedure: Unless indicated otherwise, all reagents and solvents were used without further purification. NMR spectra were recorded on an AVANCE 600 instrument (Bruker, Germany). LDI-TOF MS analyses were performed in reflector mode with the Ultraflex instrument (Bruker, Germany). 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. In LDI-TOF/TOF mode, precursor ions were accelerated to 8 kV and selected in a time ion gate. The fragments were further accelerated by 19 kV in the LIFT cell, and their masses were analyzed after the ion-reflector passage. TEM images were obtained with the JEM-100S instrument (JEOL, Japan). Recombinant human β-1,4-galactosyltransferase (β-1,4-GalT) was purchased from Toyobo Co., Ltd. UDP-galactose was obtained from Yamasa Corporation. Recombinant human α-1,3-fucosyltransferase VI (α-1,3-FucT) and GDP-fucose were purchased from Calbiochem. Compounds **2a** and **2b** were prepared as described previously.[20]

Synthesis

Compound 7: Thioglycoside **7** was prepared by using a similar procedure to that described previously:[21] D-Glucosamine hydrochloride (10.0 g, 45.6 mmol) was added to a solution of sodium methoxide (3.0 g, 55.6 mmol) in absolute methanol (60 mL). After stirring the mixture for 30 min at room temperature, phthalic anhydride (6.9 g, 46.6 mmol) was added and the mixture was stirred for a further 20 h. The solvent was removed under reduced pressure and the residue was treated with acetic anhydride (60 mL) and pyridine (50 mL) and stirred for 48 h at room temperature. The reaction mixture was poured into ice/water (200 mL) and stirred for 30 min. Afterwards, the mixture was extracted with $CHCl_3$ and the extract was washed with 3 M H_2SO_4, water, saturated aqueous solution of $NaHCO_3$, and water sequentially, and then concentrated to dryness to give an amorphous solid. After purification by using chromatography on silica gel (hexane/ethyl acetate 1:1), 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranose was obtained (6.08 g, 9.19 mmol, 20.1 %).

1,3,4,6-Tetra-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranose (3.92 g, 5.93 mmol) was dissolved in dry dichloromethane (80 mL), and thiophenol (910 μL, 8.89 mmol) and boron trifluoride–diethyl etherate (980 μL, 7.71 mmol) were added to this solution. The reaction mixture was kept under an argon atmosphere at room temperature for 22 h. The solvent was removed under reduced pressure and the residue was purified by using chromatography on silica gel (hexane/ethyl acetate 1:1) to give compound **7** (3.47 g, 4.79 mmol, 80.7 %). ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 7.88$–7.85 (m, 2H; NPhth); 7.76–7.74 (q, 2H; NPhth); 7.41–7.39 (m, 2H; SPh); 7.31–7.25 (m, 3H; SPh); 5.79 (t, $J = 9.6$ Hz, 1H); 5.71 (d, $J =$ 10.8 Hz, 1H; H-1); 5.13 (t, $J = 9.6$ Hz, 1H); 4.35 (t, $J = 10.2$ Hz, 1H); 4.28 (dd, $J = 12.0$, 5.1 Hz, 1H); 4.21 (dd, $J = 12.6$, 2.4 Hz, 1H); 3.91–3.88 (m, 1H; H-5); 2.10, 2.02, 1.83 ppm (s, 9H; 3×OAc).

Compound 8: NaH (60 % suspended in oil, 212 mg, 5.3 mmol, 1.0 equiv) was added to a solution of hexa(ethylene glycol) (3.0 g, 10.6 mmol) in THF (20 mL) at 0 °C and the mixture was stirred for 1 h. Then, 1,10-dibromodecane (1.6 g, 5.3 mmol, 1 equiv) was added to the mixture. After stirring for 4 h, the reaction mixture was evaporated and the residue was dissolved in chloroform. The solution was washed with water and dried over $MgSO_4$. The residue was concentrated and then purified by using silica gel chromatography (eluent gradient of 0–1 % methanol in chloroform) to give compound **8** (2.2 g, 4.2 mmol, 40 %). ^{1}H NMR ($CDCl_3$, 600 MHz): $\delta = 3.68$–3.57 (m, 24H; -CH_2-O-), 3.44 (t, 2H; -CH_2-O-CH_2-CH_2-CH_2), 3.40 (t, 2H; -CH_2-Br), 1.83 (q, 2H; -CH_2-CH_2-Br), 1.58–1.25 ppm (m, 14H; -CH_2-); MALDI-TOF MS: *m*/*z* calcd for $C_{22}H_{45}BrO_7$: 500.2349; found: 500.815 [*M*+H]$^+$, 522.819 [*M*+Na]$^+$.

Compound 1: *N*-Iodosuccinimide (NIS; 50 mg, 0.222 mmol, 1.2 equiv) was added to a solution of **7** (100 mg, 0.185 mmol, 1 equiv) and **8** (140 mg, 0.277 mmol, 1.5 equiv) in dry dichloromethane (5 mL) at 0 °C. The mixture was stirred for 30 min, then triflic acid (TfOH; 19.6 mL, 0.222 mmol, 1.2 equiv) was added. When the reaction was complete (within 1 h), the solution was neutralized with triethylamine and washed with 5% $Na_2S_2O_3$ and brine, and dried over $MgSO_4$. The residue was concentrated and purified by using silica gel chromatography (eluent gradient of 0–50% EtOAc in hexane) to give **9** (140 mg, 0.152 mmol, 82%). ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 7.85, 7.74 (2×m, 4H; NPh); 5.81 (t, 1H; H-3); 5.43 (d, J = 8.46 Hz, 1H; H-1); 5.17 (t, 1H; H-4); 4.32 (m, 2H; H-2, H-6); 4.18 (d, 1H; H-6); 3.87 (m, 1H; H-5); 3.65–3.32 (m, 28H; -CH_2-O-, -CH_2-Br); 2.12, 2.03, 1.87 (3×s, Ac); 1.86 (m, 2H; -CH_2-CH$_2$-Br); 1.58–1.28 ppm (m, 14H; -CH_2-); MALDI-TOF MS: *m*/*z* calcd for $C_{42}H_{64}BrNO_{16}$: 917.3408; found: 940.076 [*M*+Na]$^+$.

Potassium thioacetate (AcSK; 41 mg, 0.26 mmol) and Bu_4NI were added to the solution of compound **9** (110 mg, 0.12 mol) in butanone (10 mL) and the mixture was stirred for 3 h at 60 °C. The mixture was extracted with EtOAc and washed with water. The organic layer was dried and evaporated. The crude product was purified by using silica gel chromatography (eluent gradient of 0–80% EtOAc in hexane) to give **10** (110 mg, 0.112 mmol, 99%). ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 7.85, 7.74 (NPhta); 5.81 (t, 1H; H-3); 5.42 (d, J = 8.52 Hz, 1H; H-1); 5.17 (t, 1H; H-4); 4.32 (m, 2H; H-2, H-6); 4.18 (d, 1H; H-6); 3.89 (m, 1H; H-5); 3.64–3.31 (m, 26H; -CH_2-O-); 2.85 (t, 2H; -CH_2-SAc); 2.32 (s, 3H; SAc); 2.11, 2.03, 1.86 (3×s, Ac); 1.56 (m, 4H; -CH_2-CH$_2$-O-, -CH_2-CH$_2$-SAc); 1.26 ppm (m, 12H; -CH_2-); MALDI-TOF MS: *m*/*z* calcd for $C_{44}H_{67}NO_{17}S$: 913.4130; found: 952.056 [*M*+K]$^+$.

Ethylenediamine (1 mL, 15 mmol) was added to a solution of compound **10** (100 mg, 0.11 mmol) in ethanol (10 mL), and the mixture was stirred at 80 °C for 18 h. When the reaction was finished, the solvent was evaporated. Methanol was added to the mixture, which was subsequently co-evaporated with methanol three times. The mixture was dissolved in pyridine (Pyr; 4 mL) at 0 °C and acetic anhydride (Ac_2O; 1 mL) was added. The reaction mixture was kept at 23 °C for 21 h. The solvent was evaporated and the crude product was purified by using silica gel chromatography (eluent of chloroform with a methanol gradient from 1 to 2%) to afford **11** (155 mg, 90%). ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 6.52 (d, 2H; N*H*Ac); 5.08 (2×s, 4H; H-3, H-4); 4.78 (d, J = 8.4 Hz, 2H; H-1); 4.25 (dd, 2H; H-6); 4.13 (d, 2H; H-6); 4.13 (m, 2H; H-2); 3.87–3.57 (m, 50H; H-5, -CH_2-O-); 3.43 (t, 2H; -O-CH_2-CH$_2$-CH$_2$-); 2.67 (t, 4H; -CH_2-S-); 2.31 (NHAc); 2.08, 2.00, 1.96 (Ac); 1.65 (m, 4H; -CH_2-); 1.55 (m, 4H; -CH_2-); 1.52–1.27 ppm (m, 24H; -CH_2-); MALDI-TOF MS: *m*/*z* calcd for $C_{72}H_{128}N_2O_{30}S_2$: 1564.799; found: 1587.340 [*M*+Na]$^+$.

NaOMe (200 μL, 28% methanol) was added to a solution of compound **11** (100 mg, 64 μmol) in methanol (10 mL). The reaction mixture was stirred at 23 °C for 19 h, then neutralized by using Dowex 50W-X8 (H^+). The mixture was purified by using silica gel chromatography (eluent of chloroform with a methanol gradient 0–50%) to afford compound **1** (74 mg, 88%). ^{1}H NMR (CD_3OD, 600 MHz): δ = 4.48 (d, J = 8.5 Hz, 2H; H-1), 3.94 (m, 2H), 3.87 (dd, 2H), 3.71–3.63 (m, 48H; -CH_2-O-), 3.57 (m, 4H), 3.47 (t, 4H; -CH_2-O-), 3.43 (t, 2H), 3.30 (m, 2H), 2.68 (t, 4H; -CH_2-S-), 1.99 (s, 6H; NHAc), 1.68 (m, 4H; -CH_2-CH$_2$-S-), 1.56 (m, 4H; -CH$_2$-CH_2-CH$_2$-O-), 1.42–1.29 ppm (m, 24H; -CH_2-); ^{13}C NMR (CD_3OD, 150 MHz): δ = 171.3 (NHAc), 100.2 (C-1), 75.5, 73.8, 69.9, 69.1, 69.0, 68.7, 54.9 (C-2), 37.3 (-CH$_2$-S-), 28.2, 28.1, 28.0, 27.8, 27.7, 26.9, 24.7 ppm; MALDI-TOF MS: *m*/*z* calcd for compound **1**: 1312.736; found: 1335.461 [*M*+Na]$^+$, 1351.361 [*M*+K]$^+$.

Preparation of the GCNPs bearing GlcNAc derivatives 1 and 2a: $NaBH_4$ (70 mg, 1.6 mmol) dissolved in water (5 mL) was added to a solution of **1** (10 mg, 7.6 μmol), **2a** (52 mg, 69 μmol), and $HAuCl_4$ (25.5 mg, 75 μmol) in methanol (35 mL). The clear yellow solution immediately became dark brown. After the solution had been stirred at 23 °C for 12 h, the reaction mixture was subjected to ultrafiltration (Microcon YM50, Millipore) and the collected material was washed with water and methanol and then lyophilized.

MALDI-TOF MS: *m*/*z* calcd for compound **2a**: 758.503; found 781.102 [*M*+Na]$^+$; *m*/*z* calcd for disulfide **3**: 1035.619; found: 1058.297 [*M*+Na]$^+$.

Transmission electron microscopy (TEM): The samples dissolved in deionized water were put on the carbon-grid substrate. The TEM image of the nanoparticles was obtained by using the JEOL JEM-100S instrument, operating at 75 kV.

Enzymatic glycosylation on GCNPs bearing 1: The enzymatic glycosylation of the GCNPs bearing GlcNAc derivative **1** by recombinant human β-1,4-galactosyltransfease (β-1,4-GalT) and α-1,3-fucosyltransferase VI (α-1,3-FucT) was carried out as follows. β-1,4-GalT (4 mU) was added to a solution of GCNPs (50 μM, calculated as the concentration of GlcNAc residues conjugated according to the method reported previously[22]) and UDP-Gal (400 μM) in HEPES buffer (10 mM; pH 7.4, containing 10 mM $MnCl_2$ and 150 mM NaCl), and the mixture was incubated at 25 °C. The reaction was monitored directly by LDI-TOF MS analysis using 0.5 μL of a sample taken at 5, 10, 15, 20, 30, 40, 50 and 60 min. The sample (0.5 μL) was directly placed on the MALDI plate without any purification and was employed for further mass analysis without a matrix. When the GalT reaction was complete, the mixture was subjected to purification by means of ultrafiltration using Microcon YM-50 (Millipore) and the GCNPs were washed with water three times. Next, the GCNPs were dissolved in HEPES buffer solution (50 μL) containing GDP-Fuc (400 μM), then α-1,3-FucT (5 mU) was added. The reaction was monitored directly by LDI-TOF MS analysis as described above.

LDI-TOF MS: *m*/*z* calcd for LacNAc **4**: 1197.726; found: 1221.810 [*M*+Na]$^+$, 1234.539 [*M*+K]$^+$; *m*/*z* calcd for Le^x **5**: 1343.730; found: 1367.609 [*M*+Na]$^+$.

During the reactions, the yield of the product (%) was estimated by using the ratio of the peak intensities from the starting material and the product. For example, the yield of product **4** was calculated as shown in Equation (1):

$$\text{Yield } (\%) = \frac{[\text{compound } \mathbf{4}]}{([\text{compound } \mathbf{4}] + [\text{compound } \mathbf{3}])} \times 100\ (\%) \qquad (1)$$

Inhibition assay for β-1,4-GalT: The inhibitory effect of UDP on the recombinant human β-1,4-GalT reaction was investigated under the same conditions as described above for the galactosylation of GCNPs containing GlcNAc derivative **1**. Reaction mixtures of β-1,4-GalT and UDP-Gal in the presence of UDP (0, 20, 50, 80, 100, 200, 300, 400, and 500 μM) were incubated for 60 min at 25 °C and the degree of inhibition by UDP was determined by the intensities of the ion peaks of **3** and **4** observed in the LDI-TOF MS analysis. The inhibition (%) was estimated by calculating the ratio of the ion peak intensities [Eq. (2)] due to product **4** and hetero-disulfide ion **3**, which is predominantly generated from the starting material (compound **1** attached to the GCNPs):

$$\text{Inhibition } (\%) = 1 - \left(\frac{[\text{product } \mathbf{4}]}{([\text{product } \mathbf{4}] + [\text{compound } \mathbf{3}])} \right) \times 100 \qquad (2)$$

Sugar-elongation reaction by *N. meningitidis* β-1,4-GalT expressed in *E. coli*

Step i (Figure 6a): To a cell-free extract of *E. coli* (8.5 mg mL^{-1} as estimated by Bradford assay[23]), which produces *N. meningitidis* β-1,4-GalT, GCNPs containing **1** and UDP-Gal were added to make a final volume of 80 μL. The final concentrations of the GlcNAc residues chemisorbed on the GCNPs and UDP-Gal were adjusted to be 120 μM and 500 μM in HEPES buffer (pH 7.4 containing 0.1% Triton X-100), respectively. The mixture was incubated at 25 °C for 20 h.

Step ii (Figure 6a): The GCNPs were simply purified by means of ultrafiltration using the Microcon YM-50 instrument (Millipore) and washed with water three times. The residual GCNPs were re-suspended in a small amount of water and used for further LDI-TOF MS analyses. LDI-TOF MS: *m*/*z* calcd for LacNAc **4**: 1197.726; found: 1221.656 [*M*+Na]$^+$.

Comparison of the ionization efficiency of GCNPs and the gold-coated plate: GCNPs bearing **2a** were prepared as described previously. The solution of GCNPs (20 pmol) was applied to the MALDI-TOF MS plate in a spot with a diameter of 2 mm and the plate was dried before use. For the preparation of the monolayer of **2a**, the solution of **2a** (25 pmol) in methanol was placed in a 2 mm-diameter spot on the gold-coated

MALDI-TOF MS plate. After 1 h, the plate was washed with methanol and dried. These materials were subjected to LDI-TOF MS experiments with a nitrogen laser (337 nm) at an intensity of 2 to 50 μJ per pulse.

Acknowledgements

We thank Dr. M. Kurogochi for his help in taking the LIFT-TOF/TOF MS measurements and Dr. T. Hamamoto of Yamasa Corporation for providing samples of the cell-free extract from the *E. coli* expression system of *N. meningitidis* β-1,4-GalT. This work was supported partly by a grant for a "Research Project of the Functional Glycomaterials" and a grant-in-aid (no. 17205015) from the Ministry of Education, Culture, Science, and Technology, Japan.

[1] R. A. Dwek, *Chem. Rev.* **1996**, *96*, 683–720.
[2] S.-I. Nishimura, *Curr. Opin. Chem. Biol.* **2001**, *5*, 325–335.
[3] M. Basu, T. De, K. K. Das, J. W. Kyle, H. C. Chon, R. J. Schaeper, S. Basu, *Methods Enzymol.* **1987**, *138*, 575–607.
[4] E. Katz, I. Willner, *Angew. Chem.* **2004**, *116*, 6166–6235; *Angew. Chem. Int. Ed.* **2004**, *43*, 6042–6108.
[5] J. M. de la Fuente, A. G. Barrientos, T. C. Rojas, J. Rojo, J. Cañada, A. Fernández, S. Penadés, *Angew. Chem.* **2001**, *113*, 2317–2321; *Angew. Chem. Int. Ed.* **2001**, *40*, 2257–2261.
[6] a) J. M. de la Fuente, S. Penadés, *Tetrahedron: Asymmetry* **2005**, *16*, 387–391; b) M. Bruchez, Jr., M. Moronne, P. Gin, S. Weiss, A. P. Alivisatos, *Science* **1998**, *281*, 2013–2016.
[7] S. P. Mulvaney, M. D. Musick, C. D. Keating, M. J. Natan, *Langmuir* **2003**, *19*, 4784–4790.
[8] C. H. Teng, K. C. Ho, Y. S. Lin, Y. C. Chen, *Anal. Chem.* **2004**, *76*, 4337–4342.
[9] Y. C. Lee, R. T. Lee, *Acc. Chem. Res.* **1995**, *28*, 321–327.
[10] K. Tanaka, H. Waki, H. Ido, S. Akita, T. Yoshida, *Rapid Commun. Mass Spectrom.* **1988**, *2*, 151–153.
[11] J. A. McLean, K. A. Stumpo, D. H. Russell, *J. Am. Chem. Soc.* **2005**, *127*, 5304–5305.
[12] S. Mouradian, C. M. Nelson, L. M. Smith, *J. Am. Chem. Soc.* **1996**, *118*, 8639–8645.
[13] J. S. Kirk, P. W. Bohn, *J. Am. Chem. Soc.* **2004**, *126*, 5920–5926.
[14] A. G. Barrientos, J. M. de la Fuente, T. C. Rojas, A. Fernández, S. Penadés, *Chem. Eur. J.* **2003**, *9*, 1909–1921.
[15] a) K. Yamada, E. Fujita, S.-I. Nishimura, *Carbohydr. Res.* **1997**, *305*, 443–461; b) B. T. Houseman, M. Mrksich, *Angew. Chem.* **1999**, *111*, 876–880; *Angew. Chem. Int. Ed.* **1999**, *38*, 782–785.
[16] T. Shibue, T. Nakanishi, T. Matsuda, T. Asahi, T. Osada, *Langmuir* **2002**, *18*, 1528–1534.
[17] a) M. Kurogochi, T. Matsushita, S.-I. Nishimura, *Angew. Chem.* **2004**, *116*, 4163–4167; *Angew. Chem. Int. Ed.* **2004**, *43*, 4071–4075; b) M. Kurogochi, S.-I. Nishimura, *Anal. Chem.* **2004**, *76*, 6097–6101.
[18] The linear correlation between ligand concentration and ion peak intensities in mass spectrometry has been documented, for example, see: D. J. Harvey, *Rapid Commun. Mass Spectrom.* **1993**, *7*, 614–619. In addition, we have checked the reproducibility of the monitoring experiments of glycosyltransferase reactions using the present GCNP-based LDI-TOF MS method as well as the ability to prepare suitable calibration curves.
[19] J. Wu, S. Takayama, C. H. Wong, G. Siuzdak, *Chem. Biol.* **1997**, *4*, 653–657.
[20] C. Pale-Grosdemange, E. S. Simon, K. L. Prime, G. M. Whitesides, *J. Am. Chem. Soc.* **1991**, *113*, 12–20.
[21] G. Balavoine, S. Berteina, A. Gref, J. Fischer, A. Lubineau, *J. Carbohydr. Chem.* **1995**, *14*, 1217–1236.
[22] M. J. Hostetler, J. E. Wingate, C.-J. Zhong, J. E. Harris, R. W. Vachet, M. R. Clark, J. D. Londono, S. J. Green, J. J. Stokes, G. D. Wignall, G. L. Glish, M. D. Porter, N. D. Evans, R. W. Murray, *Langmuir* **1998**, *14*, 17–30. The concentration of the GCNPs, as described in this study, was the amount of the compound adsorbed on the surface of the GCNPs calculated from the dried weight and the diameter of the GCNP estimated from the TEM image.
[23] M. M. Bradford, *Anal. Biochem.* **1976**, *72*, 248–254.

Received: October 12, 2005
Revised: February 10, 2006
Published online: June 14, 2006

