Glycosyltransferase Microarray Displayed on the Glycolipid LB Membrane

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Abstract: β(1→4) Galactosyltransferase expressed as a fusion protein with maltose binding protein (MBP-GalT) was displayed specifically on a Langmuir–Blodgett (LB) membrane prepared by photopolymerization of maltotriose-carrying glycolipid (**1**) with 1,2-bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphocholine (**2**). The catalytic activity of MBP-GalT on the LB film was directly monitored by the surface plasmon resonance (SPR) method using a GlcNAc-carrying water-soluble polymer (**3**) as an acceptor substrate. Highly sensitive sigmoidal-type signals were obtained upon the addition of the acceptor substrate in the presence of the donor substrate, UDP-galactose (UDP-Gal), while the binding of **3** was not detected in the absence of UDP-Gal. The intensities of the signals were dependent on the amount of immobilized MBP-GalT on the LB film, which was estimated from the images obtained by atomic force microscope (AFM).

Keywords: carbohydrate synthesis; cluster effect; enzyme catalysis; glycolipid membrane; glycosyltransferase; immobilization; maltose-binding protein; surface plasmon resonance

Introduction

Real-time monitoring of enzymatic glycosylating processes is desirable not only for establishing efficient automated synthetic methods for various carbohydrates[1] but also for understanding the catalytic mechanism of glycosyltransferases.[2,3] In addition, a high throughput assay is also required for screening novel inhibitors or modulators of glycosyltransferases among a large number of combinatorial libraries in the pharmaceutical fields. Although a few practical methods for continual monitoring of glycosyltransferase activity in aqueous solutions, such as a spectrophotometric assay using a pyruvate kinase-based reaction[4] and fluorescence resonance energy transfer between donor and acceptor substrates[5] have been reported, these methods are not suited for the direct monitoring of the sugar elongation reactions by glycosyltransferases immobilized on the polymer supports.[6] From the viewpoint of automated synthesis on some solid phase materials, such spectrophotometric methods as well as NMR and MALDI-TOF mass spectroscopy[7] cannot be used as facile and efficient tools for direct and real-time monitoring of the progress of glycosylation reactions.

In the course of our research on the enzyme-assisted synthesis of glycoconjugate,[6,8] we have employed some glycosyltransferases expressed as fusion proteins with maltose-binding protein (MBP) in order to immobilize these enzymes on the surface of polymer supports having maltotriose branches. Since MBP-combined enzymes exhibited highly specific and strong affinity with maltooligosaccharide-carrying materials, it was suggested that glycosyltransferases can be simply immobilized on the basis of "specific sugar-protein interaction" without any reagent to cross-link enzymes with supporting materials.[9] These findings prompted us to apply this efficient immobilization process for preparing a highly-oriented glycosyltransferase microarray on a biosensor chip used in an SPR technique. Here we are pleased to communicate a practical and versatile method for the real-time monitoring of sugar elongation reactions catalyzed by a galactosyltransferase microarray displayed on the surface of glycolipid LB thin films.

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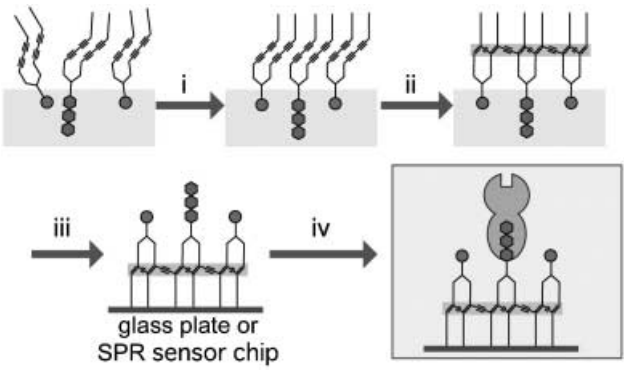
Results and Discussion

Although some self-assembled monolayers of alkanethiolates on gold have been employed as a platform for immobilizing carbohydrates,[10] we have designed and synthesized a novel type of photopolymerizable glycolipids that will widely be applied for the preparation of carbohydrate-based microarrays.[11] Scheme 1 indicates a basic concept for the construction of a glycosyltransferase microarray displayed on the LB membrane. Firstly, the monolayer prepared from a photopolymerizable glyceroglycolipid **1** with a matrix lipid **2** (Figure 1) was polymerized by UV irradiation at 254 nm to produce the polydiacetylene thin film (steps i and ii) according to the previous reports.[12] Then, the glycopolydiacetylene membrane was transferred onto a glass plate or a SPR sensor chip (step iii). Finally, the glass plate and sensor chip were immersed in MBP-GalT solution to immobilize a fusion protein through the specific interaction between MBP and maltotriose residue.



Scheme 1. Procedure to construct a glycosyltransferase biosensor chip: (i) formation of a monolayer, (ii) photopolymerization by UV irradiation, (iii) transfer of the polymerized membrane onto a solid surface, (iv) immobilization of MBP-GalT through maltotriose-MBP interaction.

An advantage of the use of the polydiacetylene-type glycolipid LB membrane is that polymerized films prevent a collapse of the monolayer by flip-flop of the monomeric glycolipids[13] when they are exposed into the air during the transfer of the LB membrane to a plate or sensor chip. In addition, polymerization would provide a stable platform for MBP-GalT (a guest fusion protein) on the sensor chip and avoid the dissociation of the glycolipid-MBP-GalT complex from the film. Here, the diacetylene-containing maltotriose-carrying glycolipid **1** was designed and synthesized for the immobilization of MBP-GalT *via* specific maltotriose-MBP interaction (Scheme 2). Synthetic details are given in the Experimental Section. Diacetylene-containing phosphatidylcholine **2** was selected as the matrix lipid because phosphatidylcholine is an abundant lipid in

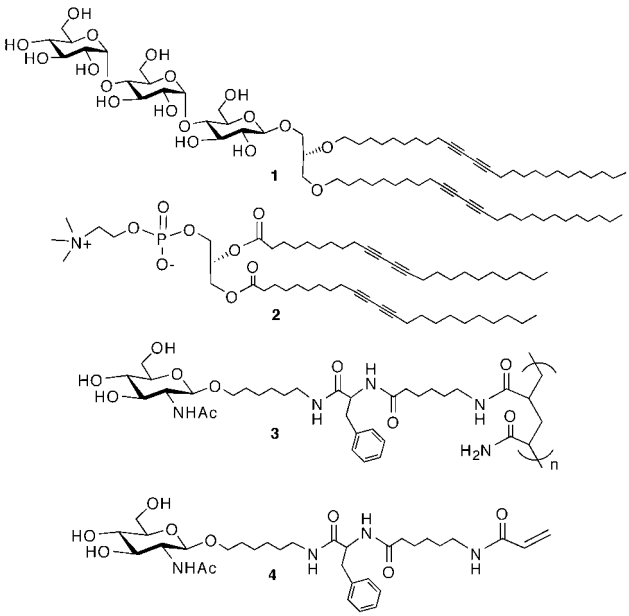
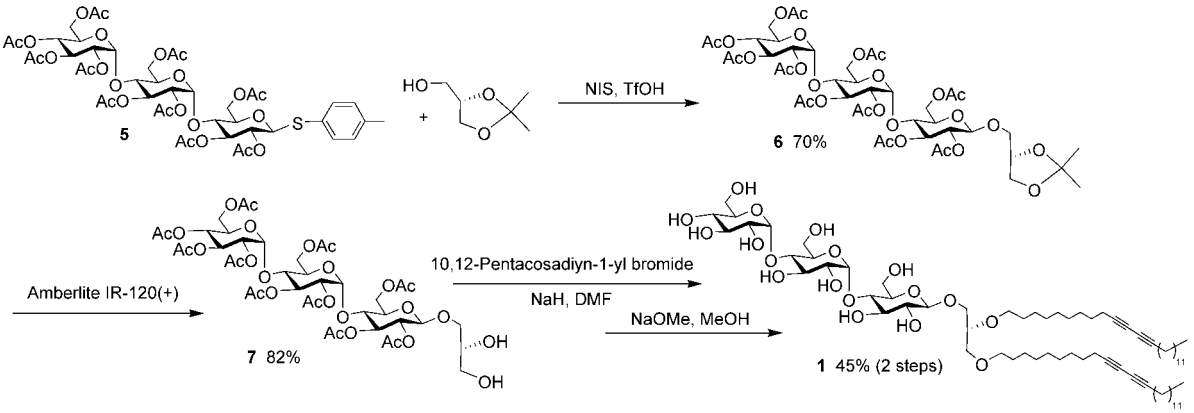


Figure 1. Chemical structures of photopolymerizable lipids for the preparation of LB films and acceptor substrates for the sugar elongation reaction by MBP-GalT.



Scheme 2. Synthesis of photopolymerizable glycolipid **1**.

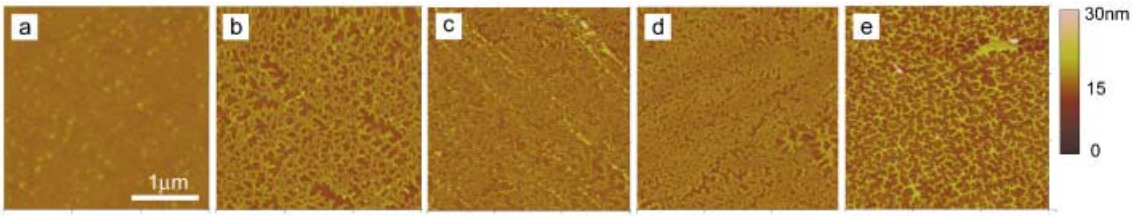


Figure 2. AFM images of MBP-GalT immobilized on the glycolipid LB films. The density of glycolipid **1** in the matrix lipid **2** was (**a**) 0%, (**b**) 5%, (**c**) 10%, (**d**), 20% and (**e**) 100%.

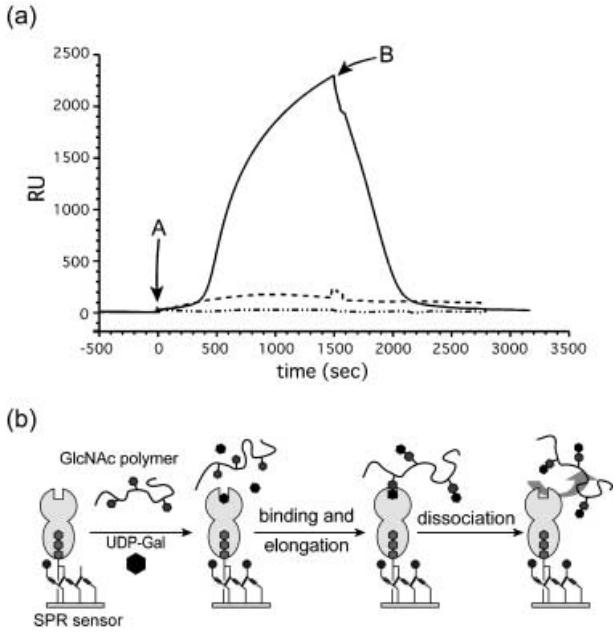


Figure 3. (**a**) SPR sensorgram upon adding of glycosyl donor and/or acceptor substrates. **A**, injection of the substrate mixture. **B**, washing the flow cell with buffer solution. The mixture of acceptor **3** (42 μM based on GlcNAc) and UDP-Gal (0.5 mM) was injected (solid line), or acceptor **3** alone (- - -), or acceptor **4** (42 μM) with UDP-Gal (- · · · · -). (**b**) The plausible process of galactosylation on the SPR sensor chip, suggested from the sensorgram.

biomembranes and often suppresses the non-specific adsorption of proteins.[14] It was confirmed by π-A isotherms that the mixture of **1/2** and also **1** alone formed stable monolayers at the air-water interface (see supporting information).

The morphology of MBP-GalT bound on the surface of LB films was observed by using atomic force microscope (AFM). Figure 2 shows the AFM images of MBP-GalT adsorbed on the glycopolydiacetylene films prepared from monolayers with various lipid compositions of **1** and **2**. The AFM images exhibited the formation of the specific protein arrays such as networks and dendrites, depending on the carbohydrate density in the LB film. It was also suggested from the section views that MBP-GalT formed a single layer (monolayer) on the glycolipid LB membrane in all cases (see supporting information). When MBP-GalT was incubated with the film composed of the matrix lipid **2** alone as a control experiment (photo **a**), only a small amount of protein was adsorbed on the film, indicating that MBP-GalT was bound to the glycolipid LB membrane by the specific carbohydrate-protein interaction between maltotriose residue and MBP-GalT. It seems that there might be a suited sugar density or morphology to immobilize protein efficiently because LB films containing 5% (photo **b**), 10% (photo **c**), or 20% (photo **d**) of **1** were covered by much larger amounts of protein molecules than the film prepared with 100% glycolipid **1** (photo **e**) [data shown partly in Figure **4** (**b**)].

Immobilized MBP-GalT on gold sensor chips (ratio of **1**:**2** = 1:4) were employed for further activity evaluation study by SPR (Biacore-X, Biacore AB) with the addition of the solutions containing glycosyl donor (UDP-Gal) and/or acceptor substrates (compound **3** or **4**). Figure 3 (solid line) shows a typical SPR sensorgram demonstrating galactose transfer reaction from UDP-Gal to acceptor polymer **3** catalyzed by MBP-GalT on a sensor chip. In this experiment, the glycosyl acceptor **3** was added at the point arrowed by (**A**) and the flow cell was rinsed with buffer solution at the time arrowed by (**B**). When the polymer **3** was injected in the presence of 0.5 mM UDP-Gal, a large sigmoidal response (2300 RU) was recorded (solid line). The value of 2300 RU seems to be reasonable when it is assumed that a single layer of the polymer covers the sensor surface. On the other hand, no response was monitored when the same amount of acceptor **3** was injected in the absence of UDP-Gal (dashed line). The large sigmoidal response of SPR found in the presence of both UDP-Gal and polymeric acceptor **3** suggests that activation of GalT by the predominant binding with UDP-Gal may be an essential step for successful galactosylating process to the acceptor substrate as illustrated in Figure 3b. Recently, X-ray crystallographic analysis of the complex of GalT with UDP-Gal also showed a significant conformational change and this crucial step may be necessary for creating a properly arranged acceptor-binding pocket.[3]

When a monomeric GlcNAc derivative **4** was injected with UDP-Gal, negligibly low affinity was detected (dash-dot line). These results correspond with our

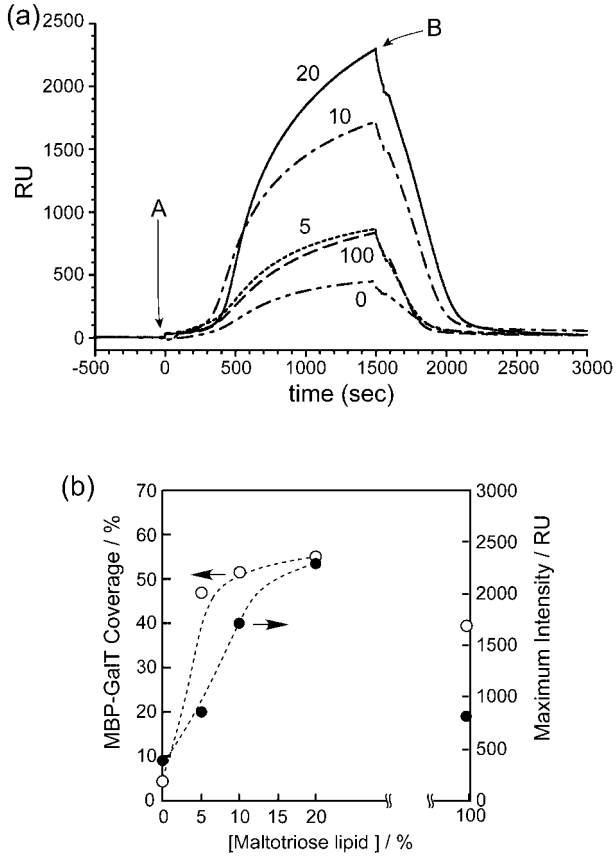


Figure 4. The effect of the sugar density of the LB film on the activity of immobilized MBP-GalT. (**a**) SPR sensorgram upon addition of the mixture of acceptor polymer **3** (42 μM based on GlcNAc) and donor UDP-Gal (0.5 mM). The density of glycolipid **1** in LB film was 0, 5, 10, 20 or 100% as shown in the figure. (**b**) Correlation between the area occupied with MBP-GalT and the maximum intensity obtained by SPR measurement. The area was calculated on the computer simulation. First all AFM images were rearranged to a black and white image with 800 × 800 dots, then dots were substituted by "0" for black and by "1" for white. The protein area corresponds to the total number of "1".

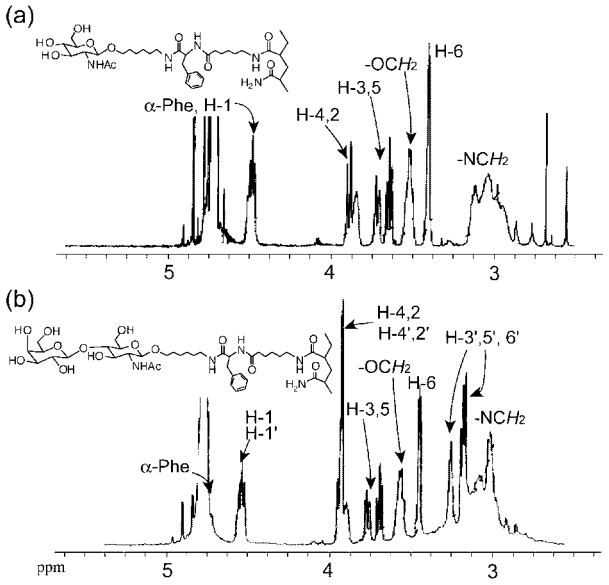


Figure 5. NMR spectra of the acceptor polymer **3** (**a**) and the product after the glycosylation by immobilized MBP-GalT (**b**).

previous observation[6] that acceptor sugar residues highly branched on the water-soluble polymers become excellent substrates for enzymatic glycosylation reactions due to a sort of "cluster effect", which is known that the exponentially amplified interaction between multivalent glycoligands and proteins.[15–17]

To investigate the effect of the sugar density of the LB film on the activity of MBP-GalT immobilized on the sensor chip, sensorgrams observed in the films prepared by using some different monomer ratios were compared as shown in Figure 4. In all cases, characteristic sigmoidal curves were obtained. The maximum intensity of sensorgrams and the protein coverages estimated by AFM images were co-plotted as a function of the sugar density on the film.

The enzyme activity detected by SPR analyses was strongly dependent on the amount of immobilized proteins as indicated by the surface area covered by this engineered enzyme. Our attention was then directed toward the versatility of the present GalT-immobilized sensor chip to an automated synthetic system of glycoconjugates.

To identify the structure of the product synthesized by the MBP-GalT sensor chip, the same sugar elongation reaction was repeated on a large scale. Instead of the SPR sensor chip, a hydrophobic glass plate (18 × 18 mm) was used to immobilize MBP-GalT as described in the experimental section. To mimic the SPR system, the substrate polymer **3** was incubated with UDP-Gal in the subphase on which the MBP-GalT-immobilized glass plate was placed, and the produced polymer was purified by gel filtration. As expected, the NMR spectrum of the product exhibited a perfect glycosylation on the designated polymer primer **3** (Figure 5). This result suggests that the present method to use a novel type of glycosyltransferase microarray chip might allow the realization of an "automated microsynthesizer" for glycoconjugates.

Conclusion

In conclusion, real-time and direct monitoring of glycosyl transferase activity was performed by using an SPR sensor chip immobilizing an engineered MBP-GalT, in which the LB thin films prepared from the photosensitive glycolipids were key materials to construct a functional protein microarray. By using a

polymeric acceptor substrate, an efficient glycosyl transfer reaction was achieved and large SPR signals were obtained. It should be noted that these glycosyltransferase microarray chips may become a versatile tool both for investigating the mechanism of enzymatic sugar-elongation reactions and for searching for novel inhibitors from synthetic libraries of non-natural sugar nucleotides and related compounds. The present method will also become a key technology for the development of an automated glycosynthesizer associated with a simple SPR monitoring system.

Experimental Section

General Remarks

10,12-Pentacosadiyn-1-yl bromide was purchased from Tokyo Kasei Co. (Japan). 1,2-Bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phodphocholine was purchased from Avanti polar-lipids, Inc. MBP-GalT expressed in *Escherichia coli* was kindly supplied from Toyobo Co., LTD. Reactions were monitored by TLC on 250 μm silica gel plates (E. Merck, 60F254) using UV light and cerium molybdate solution [10% cerium(IV) sulfate, 15% H_2SO_4 aqueous solution]. NMR spectra were recorded on Bruker AMX-500 and AVANCE 600 instruments. All NMR measurements were carried out at room temperature in $CDCl_3$ or methanol-d_4. Molecular weights were determined by using JMS-HX110 (JEOL, Japan) and reflex (Bruker). Lipid monolayers were prepared and π-A isotherms were recorded on the LB trough (U. S. I. system, Kyusyu, Japan). For the photopolymerization of the monolayer, UV (254 nm, 8 W) was irradiated with UVG-54 (UVP, Upland, CA, U. S. A.). The AFM measurement was carried out at 23 °C on a Multimode Nanoscope III with tapping mode in air (Digital Instruments, Santa Barbara, CA, U. S. A.). The imaging was performed by using silicon cantilevers (NanoDevices, Inc. Santa Barbara, CA, U. S. A.) with a resonance frequency 400 ~ 300 Hz and a spring constant of 20 ~ 70 N/m. The scan rate was 0.5 Hz. The SPR measurement was performed with a Biacore-X (Biacore AB, Uppsala, Sweden) and a gold-coated sensor chip (SIA Kit Au).

Synthesis of Photopolymerizable Glycolipids

Compound 6: A mixture of thioglycoside **5** (0.5 g, 0.49 mmol), *N*-iodosuccinimide (0.7 g, 3.3 mmol), 2,2-dimethyl-1,3-dioxolane-4-methanol (0.2 g, 1.52 mmol) and molecular sieves 300AW (50 mg) in anhydrous CH_2Cl_2 (5 mL) was stirred for 1 h under a nitrogen atmosphere at −20 °C. Trifluoromethanesulfonic acid (10 μL) was added to the mixture and stirred for 1 h at −20 °C and 4 hr at room temperature. The reaction was quenched by addition of TEA, diluted with CH_2Cl_2, and filtered over celite. The filtrate was washed with aqueous NaS_2O_3 and brine. The organic layer was dried (Na_2SO_4), filtered and the filtrate was concentrated under vacuum. The residue was purified by silica gel chromatography (5:5 CH_2Cl_2-EtOAc) to give white solid **6**; yield: 70%. ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 5.42 (m, 1H), 5.39 (d, 1H, J = 8.82 Hz), 5.36 (t, 1H, J = 10.4 Hz), 5.29 – 5.24 (m, 2H), 5.08 (t, 1H, J = 9.78 Hz), 4.87 (dd, 1H, J = 3.79, 10.1 Hz), 4.85 – 4.80 (m, 1H), 4.745 (dd, 1H, J = 4.1, 10.4 Hz), 4.63 (dd, 1H, J = 1.58, 7.89 Hz), 4.50 – 4.45 (m, 2H), 4.34 – 4.3 (m, 1H), 4.28 – 4.17 (m, 3H), 4.08 – 3.92 (m, 6H), 3.8 – 3.76 (m, 1H), 3.75 – 3.70 (m, 2H), 3.67 – 3.60 (m, 1H), 2.18, 2.16, 2.11, 2.06, 2.04, 2.03, 2.02, 2.01, 2.00, 1.99 (s, 3H × 10), 1.41 (s, 3H), 1.352 (s, 3H); ESI-MS (pos): calcd. for $C_{44}H_{62}O_{28}Na$: m/z = 1061.2 $[M + Na]^+$; found: m/z = 1061.3.

Compound 7: Amberlite IR-120 (H^+) resin was added to a solution of compound **6** (0.3 g) in methanol (10 mL). The suspension was stirred for 2 days at room temperature, and filtered. The filtrate was concentrated and purified by silica gel column chromatography (4:1 EtOAc-MeOH) to give the compound **7**; yield: 87%. ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 5.39 (d, 1H, J = 3.8 Hz), 5.36 (dd, 1H, J = 7.57, 1.26 Hz), 5.32 (d, 1H, J = 9.78 Hz), 5.27 – 5.22 (m, 2H), 5.046 (t, 1H, J = 10.4 Hz), 4.83 (dd, 1H, J = 4.1, 10.72 Hz), 4.80 (m, 1H), 4.72 (dd, 1H, J = 3.78, 10.09 Hz), 4.58 – 4.53 (m, 2H), 4.46 (dd, 1H, J = 2.2, 12.29 Hz), 4.27 – 4.20 (m, 2H), 4.17 (dd, 1H, J = 3.15, 12.61 Hz), 4.03 (dd, 1H, J = 2.53, 12.29 Hz), 3.97 – 3.88 (m, 3H), 3.85 – 3.70 (m, 4H), 2.17, 2.14, 2.08, 2.03, 2.00, 1.983, 1.978, 1.97 (s, 3H × 8), 2.01 (s, 6H); TOF-MS: calcd. for $C_{41}H_{59}O_{28}$: m/z = 999.3 $[M + H]^+$; found: m/z = 999.0.

Photopolymerizable Glyceroglycolipid (1): Sodium hydride in oil (60%, 40 mg) was added to a mixture of 10,12-pentacosadiyn-1-yl bromide (300 mg, 0.71 mmol) and compound **7** (200 mg, 0.2 mmol) in an anhydrous DMF. The mixture was stirred for 2 days at room temperature, and concentrated under vacuum. The residue was dissolved in CH_2Cl_2, washed with water and brine. The solvent was evaporated and the residue was dissolved in dry methanol. Sodium methoxide (10.8 mg, 0.2 mmol) was added to the solution and stirred for 12 h. After neutralized with Amberlite IR-120 (H +) resin, the solvent was evaporated. The residue was purified by silica gel column chromatography (4:1 $CHCl_3$-MeOH) to give compound **1** as white solid; yield: 45%. ^{1}H NMR ($CDCl_3$/CD_3OD, 7/3, 500 MHz): δ = 5.12 (d, 1H, J = 3.47 Hz), 5.11 (d, 1H, J = 3.47 Hz), 4.32 (d, 1H, J = 7.83 Hz), 3.95 – 3.20 (m, 17H), 3.09 (dd, 1H, J = 7.78, 9.77 Hz), 2.22 (t, 8H, J = 8.20), 1.65 – 1.20 (m, 64H), 0.85 (t, 6H, J = 6.93 Hz); ESI-MS (pos): calcd for $C_{71}H_{122}O_{18}Na$: m/z = 1285.8 $[M + Na]^+$; found: m/z = 1285.6.

Preparation of the GalT Microarray

Glycolipid **1** and matrix lipid **2** at various concentrations (**1**/**2** = 1/0, 5/1, 9/1, 19/1 and 0/1) in chloroform (0.1 ~ 1 mM) was spread on the surface of an aqueous subphase. After waiting for 10 min to vaporize the chloroform, the mixed lipids were compressed to the pressure of 30 mN/m at a rate of 0.1 nm^2/molecule/min to form a monolayer. The monolayer was equilibrated for 10 min under the same surface pressure and polymerized by UV irradiation (254 nm, 8 W) for 10 min. The distance between the lamp and the monolayer surface was 12 cm. The polymerized monolayer was transferred onto the OTS (octadecyltrichlorosilane) coated glass plate or the SPR sensor chip carrying monolayer of alkanethiol (*n*-octadecylmercaptan) using horizontal deposition technique. Then the glass plate carrying polymerized glycolipid film was placed on the buffer solution containing 0.01 mg/mL MBP-GalT (50 mM Tris-HCl, pH 7.4) for 1 h. The surface of the glass plate was

rinsed with buffer solution and pure water (Milli-Q) for AFM observation.

Monitoring the Substrate Binding to the MBP-GalT Array on SPR Sensor Chip

SPR sensor chips, of which one side is covered with gold, were immersed in an ethanol solution of 1 mM *n*-octadecylmercaptan for 12 h. The hydrophobic surface was washed with ethanol and benzene and dried under a nitrogen stream. MBP-GalT was bound to the glycolipid LB films of **1/2** (1/0, 5/1, 9/1, 19/1 and 0/1) on the modified SPR sensor chips. The sensor chip adsorbed MBP-GalT was set to the SPR instrument. The sensor chip was equilibrated with 10 mM Tris-HCl buffer containing 150 mM NaCl and 10 mM $MnCl_2$. The buffer solution containing substrates for the enzyme was injected to the MBP-GalT immobilized SPR sensor chip and the signal was recorded. The concentrations of glycosyl donor (UDP-Gal) and glycosyl acceptor (compound **3** or **4**) substrates were 0.5 mM and 0.042 mM, respectively.

Identifying the Product of Glycosylation on the Surface of Sensor Chip by Immobilized MBP-GalT

To identify the structure of the product by NMR spectroscopy, the sugar elongation reaction was repeated on a large scale. The enzymatic reaction achieved on the sensor chip was repeated on the basis of MBP-GalT immobilized on a hydrophobic glass plate (18×18 mm) prepared in the same manner as described in the text (the ratio of **1:2** = 1:4). The plate was placed and incubated with the mixture of GlcNAc polymer **3** (5 mg) and UDP-Gal (10 mg) in 10 mM Tris-HCl buffer (pH 7.6, 50 mM NaCl, 10 mM $MnCl_2$) for 17 h at 25 °C. The mixture was subjected to purification by Sephadex G-25 column chromatography eluted with 10 mM ammonium acetate and the polymer-containing fraction was lyophilized (5 mg). Selected spectral data: ^{1}H NMR (D_2O, 600 MHz): δ = 7.25 (m, 5H, Ph), 4.7 (D_2O and Phe-αH), 4.43 (m, 2H, H-1 and H-1′), 3.86 – 3.80 (d, 4H, H-4, H-2, H-4′ and H-2′), 3.70 (dd, 1H, H-3′), 3.60 (t, 1H, H-5′), 3.48 (m, 2H, OCH_2), 3.37 (2, 2H, H-6), 3.12 – 2.94 (br, 10H, H-3′, H-5′, H-6′ and NCH_2 and $PhCH_2$), 2.27 – 2.13 (m, 19H), 1.96 (s, 3H, $NHCH_3$), 1.70 – 1.04 (m, 47H).

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