Assembly of Glycochips with Mammalian GSLs Mimetics toward the On-site Detection of Biological Toxins

Hirotaka Uzawa,* Satoshi Kondo, Takehiro Nagatsu, Hajime Miyaguchi, Yasuo Seto, Aguri Oshita, Hirofumi Dohi, Yoshihiro Nishida, Masato Saito, and Eiichi Tamiya

ABSTRACT: According to our previously proposed scheme, each of three kinds of glycosphingolipid (GSL) derivatives, that is, lactosyl ceramide [Lac−Cer (1)] and gangliosides [GM1-Cer (2) and GT1b-Cer (3)], was installed onto the glass surface modified with Au nanoparticles. In the present study, we tried to apply microwave irradiation to promote their installing reactions. Otherwise, this procedure takes a lot of time as long as a conventional self-assembled monolayer (SAM) technique is applied. Using an advanced microwave reactor capable of adjusting ambient temperatures within a desired range, various GSL glycochips were prepared from the derivatives (1)−(3) under different microwave irradiation conditions. The overall assembling process was programed with an IC controller to finish in 1 h, and the derived GSL glycochips were evaluated in the analysis of three kinds of biological toxins [a Ricinus agglutinin (RCA120), botulinum toxin (BTX), and cholera toxin (CTX)] using a localized surface plasmon resonance (LSPR) biosensor. In the LSPR analysis, most of the irradiated GSL chips showed an enhanced response to the targeting toxin when they were irradiated under optimal temperature conditions. Lac−Cer chips showed the highest response to RCA120 (an agglutinin with β-D-Gal specificity) when the microwave irradiation was conducted at 30−35 °C. Compared to our former Lac−Cer glycochips with the conventional SAM condition, their response was enhanced by 3.6 times. Analogously, GT1b chips gained an approximately 4.1 times enhancement in their response to botulinum type C toxin (BTX/C) when the irradiation was conducted around at 45−60 °C. In the LSPR evaluation of the GM1-Cer glycochips using CTX, an optimal condition also appeared at around 30−35 °C. On the other hand, the microwave irradiation did not lead to a notable increase compared to the former GM1-Cer chips derived with the SAM technique. Judging from these experimental results, the microwave irradiation effectively promotes the installing process for all the three kinds of the GSL derivatives, while the optimal thermal condition becomes different from each other. Many bacterial and botanic proteinous toxins are composed of such carbohydrate binding domains or subunits that can discriminate both the key epitope structure and the dimension of glycoconjugates on the host cell surface. It is assumed that the optimal irradiation and thermal conditions are required to array these semi-synthetic GSL derivatives on the Au nanoparticles in a proper density and geometry for tight adhesion with each of the biological toxins.

INTRODUCTION

Glycosphingolipids (GSLs) play key roles in mammalian cell lives like those in cell to cell recognition and signal transduction.1−4 They are also involved in the process of the recognition and infection of host cells by viruses, microbes, and their toxins like Escherichia coli O-157 strains and Shiga toxins.5−8 Recently, those glycochips carrying either natural or synthetic GSL derivatives have gained strong interest because they provide useful tools in studies on cell lives including the invasion by pathogens.9−15 Different approaches have been undertaken to introduce the GSL derivatives onto the surface of sensor chips. For example, a species-specific adhesive interaction between Au and thiol groups has been often applied.10−14,16 Diels−Alder coupling reactions,17 1,3-dipolar cycloaddition between azido and alkyne groups,18,19 addition reactions between isocyanate and amino groups,20 Michael-type of 1,4-addition reactions,21 and Staudinger reactions22,23 will be effective, too.

Throughout these chemical approaches, there still remains several concerns. Generally, the installation of oligosaccharides will take a lot of time to complete. A conventional self-
assembled monolayer (SAM) technique takes time more than 15 h particularly at low concentrations such as 0.1 mM substrates. In addition, the ongoing process is hard to follow in real time. These matters make it difficult to set up an optimal reaction condition for the process of installing oligosaccharides to solid or polymer substrates. To address this issue, microwave irradiation may be helpful. The microwave irradiation is reported to accelerate certain types of heterogeneous solid-phase reactions. DNA hybridization is also accelerated and used practically in the genomic analyses targeting Bacillus anthracis, Chlamydia trachomatis, and Hepatitis C.

In our research projects, we have tried to develop a practical technology for detecting biological toxins on site and remove them from the polluted scene. Previously, we proposed our way applying a localized surface plasmon resonance (LSPR) biosensor for this purpose. Similarly to the sensing methodology using an SPR instrument, our LSPR approach needs to prepare those glycochips that can make a quick response to target biological toxins. Our way involves an enzymatic de-N-acylation in a GSL ceramide moiety followed by chemical replacement with α-lipoic acid to give a lipoic GSL derivative having a disulfide group in the ceramide moiety. As outlined in Figure 1 with a lactosyl ceramide derivative Lac−Cer (1), the semi-synthetic GSL is installed onto a glass surface, which is ornamented with Au nanoparticles. Each of the Au-modified glycochips (Figure 1) can serve as a ligand of both RCA120 and ricin (RCA60) that have a binding site with selectivity for the β-D-Gal epitope. The GM1-Cer and GT1b-Cer derivatives (2 and 3, Figure 2) can serve as a ligand of cholera CTX and botulinum BTX, respectively. GM1 and GT1b are grouped in the GSL ganglioside family possessing O-sialyl linkages (Figure 2).

Given the complicated oligosaccharide structures of GM1 and GT1b, the preparation of their installed glycochips should be a challenging and worthwhile matter.

**RESULTS AND DISCUSSION**

The three kinds of GSL derivatives (1)−(3) were prepared in the same way as those previously reported. Glass plates (12 × 26 mm size, 2 mm thick) are processed with Au nanoparticles (20−40 nm φ) in our reported way to provide Au-modified glass chips (Figure 1). Each of the Au-modified glass chips is ornamented first with Au nanoparticles (20 nm φ or 40 nm φ) and then with the Lac−Cer derivative (1) to afford a Lac−Cer glycochip. The process is assumed to complete in Stage-III (high sugar density) taking en route Stage-I (low) and Stage-II (middle). A chemical structure Lac−Cer (1) was adapted with permissions from ref 34. Copyright 2008 Elsevier. Others are adapted with permissions from ref 33. Copyright 2013 American Chemical Society.
plates is placed in a reactor vessel containing each of the three GSL derivatives (1)−(3) in methanol solution and irradiated under a N₂ atmosphere. The reactor is equipped with a cooling device in addition to the microwave oscillator and inlet/outlet gas tubes.

The irradiation conditions are programmed with a controller and run under the IC control. Actually, the irradiation sequence is programed and repeated in an on/off manner so as to maintain the prescribed temperature in a desired range (Figure 3). One irradiation cycle is composed of an irradiation period (sec) and an interval one (sec). These two periods are key determinants for the ambient temperature to be controlled. The total running time (sec) is given by the repeated number (n) of the irradiation cycle. In the present study, the running time is fixed at 1 h (3,600 s) for all the conditions examined here. As described along with this paper, we notice that the microwave irradiation works out so effectively that a prolonged running time more than 1 h makes no sense.

Microwave Irradiation for the Assembly of Lac–Cer Glycoclips from the GSL Derivative (1). The Lac–Cer derivative (1) was installed on the surface of the Au-glass plates under various conditions (A)−(C) and evaluated with the LSPR sensor using RCA120 as the biological toxin. The Lac–Cer derivative (1) serves as a semi-synthetic ligand of ricin (RCA60) and its homologous agglutinin (RCA120) from beans of *Ricinus communis*.37−41 These two botanic toxins are of carbohydrate binding proteins with selectivity for β-D-Gal epitope. Ricin has been used for crimes in the past.42 The results are summarized in Table 1 and Figure 4. In the condition A, the conventional SAM technique is applied at 30 °C without irradiation.34 In the condition B, microwave irradiation is conducted under temperature control without a cooling device. In conditions C1−C5, a cooling device is applied during waiting periods. Owing to this device, the surrounding temperature is effectively controlled around a prescribed temperature (±1.5 °C) in the mechanically allowed range between 10 °C (cooling limitation) and 65 °C (boiling point of the used solvent, methanol). Owing to the cooling device, the interval waiting time can be shortened and the repeated numbers increased.

The LSPR response to RCA120 significantly varies among the Lac–Cer chips applied and evaluated here as sensor chips (Figure 4). This result means that the surrounding temperature is of a significant factor to be controlled. The Lac–Cer chips with the C3 condition (30−35 °C) make the highest LSPR response to this proteinous toxin. Their responses are 3.6 times as high as those of the standard chips derived with the SAM condition keeping the surrounding condition around 30 °C without irradiation (condition A). Judging from the decreasing response of the Lac–Cer chips with the conditions C4 and C5 (45−65 °C), a prolonged irradiation at elevated

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Figure 2. Two kinds of semi-synthetic GSL (ganglioside) derivatives, GM1-Cer (2) and GT1b-Cer (3), are also examined in the present study. They were chemoenzymatically derived together with Lac–Cer (1).33,34 These two gangliosides are characterized with the presence of sialic acid (SA) residues in the terminal position, which govern both of their biological and physicochemical properties. A chemical structure GM1-Cer (2) was adapted with permissions from ref 33 (Copyright 2013 American Chemical Society). A chemical structure GT1b-Cer (3) was adapted with permissions from ref 43 (Copyright 2018 Springer Nature).

GM1-Cer (2)

GT1b-Cer (3)
temperatures makes no sense but even affects the LSPR response.

As a control experiment to check a species-specific RCA120/Lac−Cer interaction, we used a bovine serum protein (BSA), which is hydrophobic and useful to assess a nonspecific background interaction with the Au surface. Though a nonglycosylated or ceramide-bound Au-chip may give another approach to this issue, the nonglycosylated Au nanoparticle simply causes the hydrophobic and −SH interaction with proteins. The specificity of RCA120 and ricin proteins to Lac−Cer Au-nanoparticles was already checked with different plant lectins.33 In the presence of excessive BSA (300 ng/mL), the Lac−Cer chips derived with conditions C1 and C5 show a weak response to this hydrophobic protein (C1/BSA and C5/BSA in Figure 4a). Because we have already confirmed that the glass surface is fully covered with Au nanoparticles,33 some of the Au nanoparticles seem to afford an opened space on their surface to cause the nonspecific hydrophobic interaction with BSA. We judge that the installation under the conditions C1 and C5 gives Lac−Cer glycochips at Stage I, as shown in Figure 1.

Compared to the other conditions (C3−C5), the overall irradiation period is shortened in the conditions C1 and C2 to keep the temperature below 25 °C. In the condition B (entry 2 in Table 1), the surrounding temperature is increased to 45−60 °C with keeping nearly the same irradiation period as those of C1. The LSPR response of the derived Lac−Cer chips is increased thereby. With increasing temperatures in C1−C3, the LSPR response increases progressively and gives a peak around the conditions C3 (30−35 °C) and C4 (40−45 °C). Probably, the installation of the condition C3 has reached the final stage (Stage III in Figure 1). To our interest, the LSPR signals come down in the conditions C4 and C5 with a prolonged irradiation period at rising temperatures. In the C5 condition, their LSPR signal falls down to the level of the conditions C2 and A (SAM) and come back to showing the nonselective response to BSA (C5/BSA in Figure 4a).

These observations indicate that the irradiation sequence of the condition C (30−35 °C) provides an optimal condition. The condition C3 can induce more than 3.6 times stronger LSPR response to RCA120 than those by the conventional SAM technique. In addition, the condition C3 causes no apparent response to BSA (C3/BSA in Figure 4a) even if an excessive amount of BSA (3 times higher concentration than that of RCA120) is applied in the LSPR analysis. Microwave irradiation is known to have both heating and vibration effects on activating solid-phase reactions.25 We assume that microwave irradiation enhances the reactivity of the lipid disulfide group

Table 1. Microwave-Promoted Installation of Lac−Cer Derivative (1) onto the Surface of the Glass Chip under Various Temperature Conditions

| entry | types | net irradiation period (sec)/total incubation period (hr) | temperatures (°C) | irradiation (sec) | interval (sec) | repetition number (n) | relative LSPR response to RCA120 |
|-------|-------|----------------------------------------------------------|-------------------|------------------|----------------|---------------------|---------------------------------|
| 1 A   |       | 1/18 h                                                   | 30                | 5                | 82             | 41                  | 1.00                            |
| 2 B   |       | 205 s/1 h                                                | 45−60             | 5                | 7              | 27                  | 1.78                            |
| 3 C1  |       | 315 s/1 h                                                | 10−15             | 5                | 7              | 45                  | 0.46                            |
| 4 C2  |       | 970 s/1 h                                                | 20−25             | 10               | 7              | 97                  | 1.09                            |
| 5 C3  |       | 1300 s/1 h                                               | 30−35             | 13               | 23             | 100                 | 3.56                            |
| 6 C4  |       | 2304 s/1 h                                               | 45−50             | 18               | 10             | 128                 | 2.68                            |
| 7 C5  |       | 2394 s/1 h                                               | 60−65             | 14               | 7              | 171                 | 1.07                            |

Without microwave irradiation (conventional SAM technique at 30 °C for 18 h). With microwave irradiation without using a forced cooling device. With microwave irradiation using a forced cooling device. LSPR response is set at 1.0 for that derived with the conventional SAM technique (condition A = 1.0).

Figure 4. LSPR response of Lac−Cer glycochips to RCA120. (a) LSPR sensorgrams compared among the Lac−Cer glycochips from the different assembled conditions. (b) Their responses to RCA120 relative to those of the SAM process (condition A = 1.0) with a ca. 5% margin of error. Concentrations: RCA120 = 100 ng/mL and BSA = 300 ng/mL. Samples were injected at 500 s. Details are described in the Experimental section.
of the semi-synthetic GSLs more effectively than those by authentic heating systems. The irradiation may also increase the molecular vibration of the GSL reactants and facilitate the GSL installation on the Au surface under the heterogeneous solid-phase conditions.

In the condition C5, either thermal or chemical disorder is induced on the surface terrain even though all the installation reactions are conducted in a N₂ atmosphere under the careful control of surrounding temperatures. Actually, the microwave irradiation is useful to facilitate the installation process and improve the quality of the derived Lac−Cer chips as sensor chips. In these results, we have also recognized that the irradiation should be carefully approached with optimal irradiation sequences. Otherwise, the excessive irradiation may downgrade their performance.

Microwave Irradiation for the Assembly of GM1-Cer Glycochips from the GSL Derivative (2). Next, the LSPR assessment is conducted for the GM1-Cer chips using CTX as one of the biological toxins with binding activity for human GM1-Cer. The CTX protein may cause a serious foodborne illness and occur worldwide particularly in tropical regions. The results shown in Figure 5 show that the GM1-Cer chips obtained from the C3 condition (30−35 °C) make the highest response to this toxin in the LSPR analysis. Though this result matches with the observation for the Lac−Cer chips, the LSPR response is not so enlarged in the GM1 glycochips derived under the optimal condition C. Probably, the SAM technique also efficiently works out though it takes a prolonged processing time. Different from the Lac−Cer glycochips, every GM1-Cer chip displays a nearly consistent LSPR response to CTX regardless of the irradiation conditions examined here. Because they make no apparent response to BSA, the surface of each Au nanoparticle should be fully covered with GM1-Cer molecules. The sugar density may be different from each other.

Figure 5. LSPR responses of GM1-Cer glycochips to CTX. (a) LSPR sensorgrams with the strength of response in the vertical axis and the sensing time (sec) in the horizon. (b) Relative LSPR response (condition A = 1.0) of each GM1-Cer glycochips derived at various temperatures prescribed in each of the C1−C5 conditions with a ca. 5% margin of error. Concentrations: CTX = 100 ng/mL and BSA = 300 ng/mL. Samples were injected at 500 s. See the Experimental Section.

Figure 6. LSPR responses of the derived GT1b-Cer glycochips to a BTX/C. (a) Comparison of LSPR sensorgrams among those derived under various irradiation and temperature conditions (conditions B and C). (b) Comparison of their relative responses to BTX/C, in which the response is set at 1.0 for those derived with the conventional SAM technique (condition A = 1.0). The microwave irradiation conditions B1 and B2 without using a forced cooling device are as follows: B1: total irradiation = 130 s/1 h at 30−45 °C and B2: total irradiation = 205 s/1 h at 45−60 °C. The relative LSPR responses may allow ca. 5% margin of errors. Concentrations: BTX/C = 100 ng/mL and BSA = 300 ng/mL. Samples were injected at 500 s. See the Experimental Section.
by the irradiation conditions undertaken similar to the preceding case of the Lac–Cer glycochips.

Probably, these results may be rationalized with apparent molecular sizes that are different between Lac–Cer (1) and GM1-Cer (2). In addition to the branched and extended chemical structure of GM1-Cer, the terminal SA residue enlarges its apparent molecular size having water molecules around the carboxylic acid. The ionic and enlarged structure of GM1-Cer (2) is difficult to assemble themselves in high density on the Au nanoparticles (Figure 1). On the other hand, the tiny Lac–Cer derivative (1) yields a series of Lac–Cer glycocohips in various sugar densities and hence displays the LSPR sensorgrams widely fluctuating (Figure 4).

Microwave Irradiation for Assembly of GT1b-Cer Glycocohips from the GSL Derivative (3). The GT1b-Cer derivative (3) serves as a ligand of botulinum toxins (BTX) from *Clostridium botulinum*. The BTX provide seven subtypes (BTX/A ~ G), some of which are listed in "Category A" by the CDC (Centers for Disease Control and Prevention). GT1b-Cer (3) possesses an even more extended structure bearing three SA residues (Figure 2). A series of GT1b-Cer glycochips were prepared under different irradiation conditions and evaluated with a botulinum C-type toxin (BTX/C) in the same way as those just described to give the results, as summarized in Figure 6.

When the conventional SAM technology (condition A) is applied, the LSPR analysis of the derived GT1b-Cer chips show a rather complicated response to BTX/C, as shown in Figure 6a. The LSPR signal increases soon after injection of this toxin, while it progressively goes down after 1500 s. This suggests that the SAM condition, which keeps the methanol solution at 30 °C for 18 h without microwave irradiation, can not fully set up a proper terrain on the Au nanoparticles. The LSPR response is improved when the GT1b-Cer chips are prepared under the irradiation conditions C2 and C3. Different from Lac–Cer (1) and GM1-Cer (2), the GT1b-Cer chips from the condition C2 (20–25 °C) make a slightly higher response than those of the condition C3 (30–35 °C). It is also notable that a prolonged irradiation under the condition C4 (45–50 °C) seriously downgrade their quality as sensor chips at least for detecting the BTX. The irradiation condition B2 (45–60 °C, total irradiation = 205 s/1 h) without using the cooling apparatus brings the highest response in the present LSPR analyses. The LSPR response of the condition B2 is 4.1 times greater than that of the conventional SAM condition A. When the surrounding temperature is lowered to 30–45 °C in the condition B1 (total irradiation = 130 s/1 h), the LSPR response of the derived GT1b-Cer chip goes down to the level lower than that with the conditions C2.

These observations are more complicated than the cases of Lac–Cer (1) and GM1-Cer (2). The GT1b-Cer derivative (3) taking on three SA residues in its structure is not easily accessible to the Au surface. Under the conventional SAM condition (condition A), some of the GT1b-Cer molecules may overlay on the surface without a rigid covalent bond. Upon binding with the toxin, those molecular species are released from the sensor surface together with the toxin to display the unusual LSPR signals. This kind of vague interactions around the surface of the Au nanoparticles can be removed during microwave irradiation, as can be seen in the signals of the conditions C2 and B2. In other words, microwave irradiation plays an essential role to form a rigid terrain during the process of installing the enlarged GSL molecules. On the other hand, the results of the condition C4 imply that we have to approach the microwave irradiation with caution because the associated thermal or chemical factor may irritate the Au surface and deform the established topology on it.

The conditions C2 and B2 bring a higher quality to the derived GT1b-Cer chips than those by the prolonged irradiation conditions C3 and C4. We assume that the BTX/C may favor a sparse GT1b-Cer array in Stage-II (Figure 1) rather than the dense one in Stage-III. Reversely, the botanic toxin (RCA120) favors a dense Lac–Cer array in Stage III more than the sparse one in Stage-I. This kind of inclination for the density of GSL clusters seems weakened in CTX, which bind GM1-Cer clusters in a wide range of densities (Figure 5). Among the three biological toxins examined here, the CTX possesses a unique AB₃ type of subunit structure, in which the B-subunits serve as a GM1-Cer binding protein. The B₅-subunits are symmetrically arranged underneath the A-subunit in the AB₃ structure. The unique molecular configuration seems to help the CTX toxin establish tight adhesion onto the host cell GSL microdomains expressing GM1-Cer arrays.

Surface Terrains of the GSL Glycochips Using Au Nanoparticles as Hinge. GSLs per se are of amphiphilic biomolecules that are hardly soluble in aqueous solutions. In nature, they are localized in mammalian cytoplasmic membranes constructing microdomains called “GSL rafts” together with cholesterol and other lipid elements. Similar to viruses, some biological toxins are able to discriminate the GSL rafts and finally internalize into the host cells by endocytosis. Our GSL glycochips are designed referring to these naturally occurring events. We use the Au nanoparticles as hinge to conjugate each of the GSL derivatives on glass plates. The Au nanoparticles also play an essential role in the present LSPR analysis to amplify resonance signals. An optimal size of the Au nanoparticles is 20 nm ϕ for Lac–Cer (1) and 40 nm ϕ for both of GM1-Cer (2) and GT1b-Cer (3). The larger one works better for the GSL derivatives with molecular sizes enlarged by SA (NeuSac) residues. An AFM (atomic force microscopy) image of an Au-modified glass plate indicates that the glass surface is fully covered with Au nanoparticles.

As illustrated in Figure 1, polar oligosaccharides direct upward to the water layer and interact with each of the biological toxins. Each of the 20 nm ϕ Au nanoparticle has ca. 315 nm² area on a quarter top surface. In our calculation for Gal-Cer glycochips with X-ray photoelectron spectroscopy and ellipsometry, almost 3.1 pieces of Gal-Cer molecules occupy a 1.0 nm² area on an Au-plate surface. This means that the 20 nm ϕ Au nanoparticle is able to accommodate 970 pieces of Gal-Cer molecules in a 1/4 top surface area. The 40 nm ϕ Au nanoparticle can accept 3900 Gal-Cer molecules on its 1/4 top surface (1260 nm²). These numbers will decrease for the GM1-Cer and GT1b-Cer derivatives with larger molecular sizes and higher polarity than the Gal-Cer derivative. The polar groups in NeuSac residues make H-bond networks with water molecules and thereby make the GSL surroundings mucus. Albeit these possible regulatory factors in these ganglioside GSL derivatives, we assume that the 40 nm ϕ Au nanoparticle gives an open space to array them in the final stage (stage-III) via the two developing stages (stages-I and II), as shown in Figure 1. The surface terrain of the Au nanoparticles in the stage-III covered with GSL derivatives in cluster may be analogous to those of the GSL rafts in mammalian cell surfaces. The three stages (I–III) are,
however, hypotheses. If the glycosylation amount on each of the GSL glycochips can be correctly quantified, a precise discussion will become possible on their surface terrains.

**CONCLUSIONS**

Mammalian GSLs give a family of gangliosides, which carry SA such as Neu5Ac at the nonreducing end of oligosaccharide chains (Figure 2). They show a structural diversity regarding the total number of SA residues and their positions. Each of the SA residues has an acidic carboxylate group, and the O-sialyl linkage is labile under acidic conditions. Moreover, their reactive functional groups. The overall amphiphilic molecular structure makes gangliosides and other GSLs hardly soluble in aqueous medium forming a self-assembled supramolecule. For the purpose of installing such highly functionalized GSL biomolecules onto glass plates, we have figured out the general scheme using the Au nanoparticles as hinge (Figure 1) and tried completing the process in 1 h under microwave irradiation.

To our interest, the optimal irradiation condition appeared variably among the three kinds of the GSL derivatives (1)–(3), as summarized in Table 2. This practically means we have to select the optimal irradiation for each of the GSL derivatives if we need those qualified GSL glycochips that can make the highest response to a target toxin. This can be easily done with the advance irradiation device capable of programming irradiation sequences and keeping them in the IC memory. At the same time, it is of significance to control the surrounding temperature within a prescribed range. To achieve this matter, a forced cooling device is available as an option, which works out well to shorten the interval time and increase the repetition numbers within the limited running time (1 h in the present study). However, we have seen that this option may not be always required like the case of the GT1b-Cer (3) (entries 2 and 3 in Table 2). The condition B may also afford a comparable condition without using the forced cooling device.

Our goal of this study is to establish a set way of detecting biological toxins on-site. We have continuously applied our synthetic glycotechnology for this purpose. This is because most of infection events start from a species-specific interaction with the host glycoconjugates. Mammalian GSLs are involved in a central part of infections not only by the bacterial and botanic toxins but also by viruses and bacteria themselves. The synthetic glycotechnology has been rapidly developing these years and making aggressive approaches to solve the problem of contagious diseases spreading worldwide. We hope that our proposed scheme could contribute in some measure.

**EXPERIMENTAL SECTION**

**Materials and Instruments.** *R. communis* agglutinin (RCA120) was purchased from Vector Laboratories Inc. (Burlingame, CA, USA). BTX/C and CTX were supplied from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and BTX/C was safely handled with approval of the Ministry of Health, Labour and Welfare of Japan. Bovine serum albumin (BSA) was obtained from Kokusan-kagaku (Tokyo, Japan). D-lactosyl-β1-1′-2′-erythro-sphingosine (d18:1) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Ganglioside GT1b and GM1 were obtained from IsoSep AB (Tullinge, Sweden). Sphingolipid ceramide N-deacetylase (SCDase, E.C. 3.5.1.69) was obtained from TAKARA Bioscience (Shiga, Japan). Lac−(1) and GM1-Cer (2) were prepared according to our previous reports. Au nanoparticles (20 nm ϕ and 40 nm ϕ) were supplied from Tanaka Precious Metals (Tokyo, Japan). Microwave irradiation was conducted with a Wave Magic MWO-1000S (2.45 GHz, EYELA Co. Ltd., Tokyo, Japan). A tungsten halogen light source (LS-1), 400 μm core diameter optical fibers (P400-1-UV/VIS), and a spectrometer (QE65000 or QE65 Pro, bandwidth: 475–851 nm) with a long-path filter (OF1-GG475) were purchased from Ocean Optics (Dunedin, FL, USA). A flowing pump (TE-361N) was obtained from Terumo (Tokyo, Japan). A Petlir-controlled cuvette holder (qpod) with a Z-height of 15 mm (Quantum Northwest, Liberty Lake, WA, USA), a sample injector (Rheodyne 9725 or 7725, IDEX Health & Science LLC, Rohnert Park, WA, USA), and a flow cell (FLAB50-UV-02, GL Science, Tokyo, Japan) were obtained from commercial sources.

**Synthesis of GT1b-Cer (3).** Ganglioside GT1b (5 mg) with C18 and C20 sphingosine homologues was dissolved in 100 mM AcONa-AcOH buffer (pH 5.8, 0.5 mL) and treated with 1.3 mL of water and 8 mg/mL taurodeoxycholate (TDC, 0.2 mL). To this mixture was added a 25 μL aqueous solution of sphingolipid ceramide N-deacetylase (SCDase, 5 μI/μL), and the reaction mixture was covered with 20 mL of n-decane and incubated at 37 °C for 6 days. The reaction mixture was frozen at −20 °C, and the n-decane layer was removed in vacuo. The water layer was thawed and applied to a column (4.6 mm I.D. × 25 cm) packed with X-Bridge C18 resin (Waters). The column was eluted with 60% aqueous methanol to remove the TDC and then 80% aqueous methanol to give lyso-type GT1b (4.0 mg, 92%). ESI-MS: calc for lyso-GT1b (C_{18}): [M-2H]^2− 929.9, found: [M-2H]^2− 929.8, and calc: [M-3H]^3− 619.6,

| entry | types | net irradiation period (sec)/total incubation period (hr) | temp (°C) | RCA120/Lac-Cer (1) | CTX/GM1-Cer (2) | BTX/C/GT1b-Cer (3) | relative LSPR response |
|-------|-------|----------------------------------------------------------|---------|-------------------|----------------|-------------------|-----------------------|
| 1 A   | 1     | -18 h/18 h                                               | 30      | 1.00              | 1.00           | 1.00              | 1.00                  |
| 2 B1  | 2     | 130 s/1 h                                                | 30–45   | 1.07              | 0.80           | 0.51              | 2.39                  |
| 3 B2  | 3     | 205 s/1 h                                                | 45–60   | 1.78              | 1.00           | 2.39              | 4.05                  |
| 4 C1  | 3     | 315 s/1 h                                                | 10–15   | 0.46              | 0.53           | 0.16              | 3.29                  |
| 5 C2  | 4     | 970 s/1 h                                                | 20–25   | 1.09              | 0.68           | 2.39              | 0.51                  |
| 6 C3  | 5     | 1300 s/1 h                                               | 30–35   | 3.56              | 1.06           | 1.93              | 0.51                  |
| 7 C4  | 6     | 2304 s/1 h                                               | 45–50   | 2.68              | 0.80           | 0.51              | 0.51                  |
| 8 C5  | 7     | 2394 s/1 h                                               | 60–65   | 1.07              | 0.80           | 0.51              | 0.51                  |

**Table 2. Summary of the Optimal Condition to Install Each of the GSL Derivatives onto the Glass Surface**

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A solution of the **lyso-**GT1b (4.0 mg), *N*-hydroxysuccinimidy 1000 MHz), and 682.7. Glass Chips (Chips 1) Modified with Au Nanoparticles. The Au-modified glass chips (Figure 1) were prepared by our established method. Briefly, glass plates (W 26 mm × D 12 mm × H 2.0 mm) were cleaned with Piranha solution (H₂O₂/H₂SO₄ = 1/3, v/v) at 80 °C for 40 min and treated with 10% aminopropyltrimethoxysilane (APTMS) in ethanol for 15 min. Caution: A piranha solution reacts violently with organic materials and should be handled with extreme care. The glass plates were washed gently with ethanol and dried at 50 °C for 3 h under vacuum. After cooled, the plates were treated with 16.5 mM NHS-activated lipoic acid in DMF (20 mL) at room temperature for 48 h under a N₂ atmosphere. As an alternative method, lipoic acid was reacted with the aminosilane surface with 8.3 mM of NHS-activated lipoic acid under microwave irradiation (100 W, 70 °C, 600 s) to give the glass chip arraying lipoic disulfide groups on the surface.

For the preparation of the GM1-Cer chip, a commercially available suspension of Au nanoparticles (40 nm ϕ, 600 μL) was centrifuged using a centrifuge (Chibitan II, Merck Millipore, Darmstadt, Germany) at 10,000 rpm (5,200 G) for 5 min, and the supernatant was discarded. The precipitates were collected and mixed with another commercially available suspension of Au nanoparticles (40 nm ϕ, 600 μL) to make a doubly condensed Au nanoparticle suspension. The derived glass plate was then treated with the doubly condensed suspension of Au nanoparticles for 6 h to give the Au-modified glass chip shown in Figure 1. For the preparation of Lac−Cer and GT1b-Cer chips, a commercially available Au nanoparticle suspension was used without making the doubly condensed suspension. According to the preceding results from size optimization, 20 nm ϕ Au nanoparticles were applied for Lac−Cer (1) and the larger ones (40 nm ϕ) for both of GM1-Cer (2) and GT1b-Cer (3).

**GSL Glychips from the Derivatives (1)−(3).** Lac−Cer (1) was installed on the Au-modified glass chips (Chip 1) under microwave irradiation as follows: Chip 1 was immersed in a MeOH solution of Lac−Cer (1) (62 μM, 2 mL), and the installation was carried out with the microwave reactor. The microwave irradiation (250 W) was conducted repeatedly for 60 min with the sequence program in the PC controller. The ambient temperature was controlled for the sequences in the conditions C with the cooling system (MWC-1000, EYELA Co. Ltd., Tokyo, Japan) or in the condition B without the cooling system. The derived Lac−Cer−chip was extensively washed with methanol and water. In a similar way, the other GSL glychips were derived from each of GM1-Cer (2, 68 μM, 2 mL) and GT1b-Cer (3, 49 μM, 2 mL). In particular cases that a nonselective response is so serious, these GSL chips were treated with lipoic acid in methanol (1 mM) for 1 min before use in LSPR analyses.

Separately, the installation with the conventional SAM method was conducted in the same way as those described before without microwave irradiation. The Au-modified glass chip (Chip 1) was immersed in the MeOH solution of each of the GSL derivatives (1)−(3) (49−68 μM, 2 mL) and incubated 30 °C for 18 h. The derived glychips was extensively washed with methanol and water. They are soaked in pure water and stored at 4 °C in the refrigerator.

**LSPR Detection of Biological Toxins.** Using the different biological toxins and the glycolipid chips functionalized with the GSL derivative (1)−(3), detection responses and specificity were examined by our LSPR method as previously reported. The LSPR system is composed of a tungsten halogen lamp as a light source, a portable pump for flowing, an injection valve with a sample loop for 250 μL or 1 mL, a disassemble flow cell (optical length, 0.025 mm) for attachment of the prepared chips, a temperature-controlled sample compartment with a Peltier-controlled cuvette holder, a spectrometer for the detection of absorbance changes, and a PC for data analysis. The running buffer used in the experiments was 10 mM HEPES (pH 7.5) containing 150 mM NaCl, filtered with a 0.22 μm filter, and degassed before use through all the LSPR experiments. The LSPR system detects and measures absorbance changes when the analytes (the samples being analyzed) start to bind and dissociate from the chip surfaces. The buffer was run in the LSPR system until the baseline was made stable. 250 μL of RCA₁₂₀ at a concentration of 100 ng/mL was then injected into the LSPR system for 7.5 min at a flow rate of 33.3 μL/min. Similarly, 1 mL of BTX/C (100 ng/mL) or CTX (100 ng/mL) was injected for 15 min at a flow rate of 66.6 μL/min. Concentrations of BSA were 300 ng/mL in this study. Detection temperature was 25 °C. Integration time was 8 msec, and the number of accumulation times was 2000. LSPR responses were monitored at A₉₀−A₂₀ with time. All LSPR data were analyzed with OOIBase32 software (Ocean Optics, version 2.0).

**AFM Imaging.** An AFM image of Au nanoparticles (20 nm ϕ) on a glass plate was obtained at ambient temperature on a Nanoscope IIIa (Digital Instrument, Inc.) operated in a tapping mode using silicon cantilevers (125 μm, tip radius ca. 8 nm). A 10 μm × 10 μm scanner was used for imaging. The scanning speed was at a line frequency of 0.5 Hz, and the original images were sampled at a resolution of 512 × 512 points.

**Safety Considerations.** BTX/C and the CTX are highly toxic if inhaled or digested. Toxicity of RCA₁₂₀ to humans has not been fully determined. These toxins and the related protein should be handled with special care. After examination, they must be decomposed using sodium hypochlorite or an autoclave.

**AUTHOR INFORMATION**

**Corresponding Author**

Hirotaka Uzawa — Nanomaterials Research Institute, Tsukuba Center, Tsukuba Central, National Institute of Advanced Industrial Science and Technology, Tsukuba 305-8565, Japan; orcid.org/0000-0002-3834-8243; Email: h.uzawa@aist.go.jp

**Authors**

Satoshi Kondo — Nanomaterials Research Institute, Tsukuba Center, Tsukuba Central, National Institute of Advanced Industrial Science and Technology, Tsukuba 305-8565, Japan; orcid.org/0000-0002-3834-8243; Email: h.uzawa@aist.go.jp
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