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Article

Novel Glycolipid Chips with a Double Layer of Au Nanoparticles for Biological Toxin Detection

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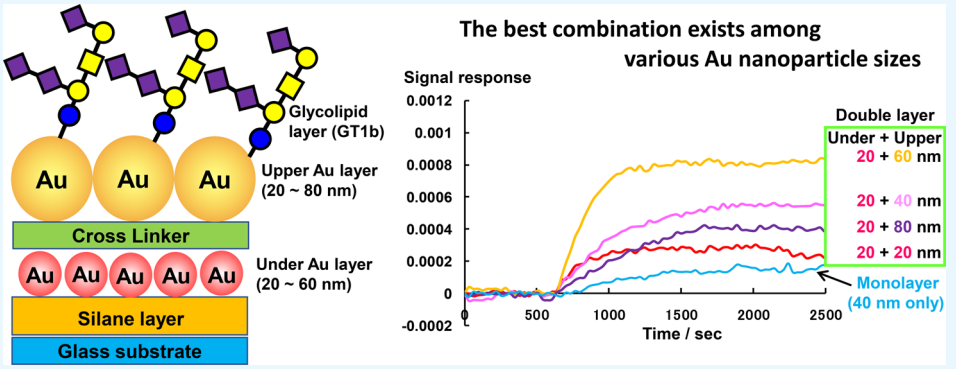

ABSTRACT: Glycolipid chips having a double layer of Au nanoparticles are proposed for detection of biological toxins. The sugar-modified chips constitute an under and an upper layer of Au nanoparticles of 20−80 nm diameter on glass plates, and Au nanoparticles of each layer are linked with 1,8-octanedithiol by a self-assembled monolayer (SAM) technique. A tris-sialo glycosphingolipid, ganglioside GT1b, having lipoic amide at the sphingosine part was immobilized on the Au outside surface of the upper layer, and botulinum toxin (type A heavy chain) was detected by localized surface plasmon resonance (LSPR). The GT1b-Cer-coated chip having a double layer of Au nanoparticles enhanced the toxin detection by LSPR more than those with single monolayers. The LSPR response changed according to the sizes of Au nanoparticles in each under and upper layer. The combination of 60 and 40 nm Au nanoparticles in the under and upper layer, respectively, gave the best result, which enabled the toxin detection at concentrations below 5 ng/mL with the portable LSPR device.

INTRODUCTION

Localized surface plasmon resonance (LSPR) spectrometry provides a useful sensing technology without needing any labeling processes.[1−4] LSPR biosensors utilize a unique plasmonic resonance of Au nanoparticles to analyze intermolecular interactions of various biomacromolecules in real time. They have the merits of portable sizes and low cost, which are often required in biomedical and point-of-care applications. Recent LSPR biosensors are applied to detect different viruses like new SARS coronaviruses,[5,6] dengue virus,[7−9] and rotavirus[10] and also to diagnose Alzheimer's disease.[11] These LSPR detection methods utilize the immune systems involving species-specific antigen−antibody reactions.

On the other hand, mammalian oligosaccharides and glycosphingolipids (GSLs) widely exist on the cell surface, and they are largely related to infection of host cells by pathogenic viruses, bacteria, and toxins.[12,13] For instance, Shiga toxin (Stx) from *Escherichia coli* O157 binds globotriaosyl (Gb_3) ceramide on kidneys. Cholera is caused by cholera toxin (CTX) secreted by *Vibrio cholerae* on intestinal epithelial cells, in which CTX preferentially binds to GM1 ganglioside on the target host cells. These mammalian oligosaccharides and glycosphingolipids are considered to serve as alternative, useful ligands for detecting these toxins. These toxins as well as a plant toxin (ricin) and another deadly bacterial toxin (botulinum toxin) are known as biological toxins, and development of their detection systems is of high significance. Recently, we have developed LSPR biosensors for detection of the biological toxins assuming on-site detection in terror and crime scenes.[14,15] Our studies involve design and synthesis of glycolipids having a disulfide group for attachment on the Au surface. In addition, we have recently proposed a new glycolipid immobilization method applying microwave irradiation, which facilitates the preparation of glycolipid chips with high quality and improves the sensitivity of targeting toxins.[16]

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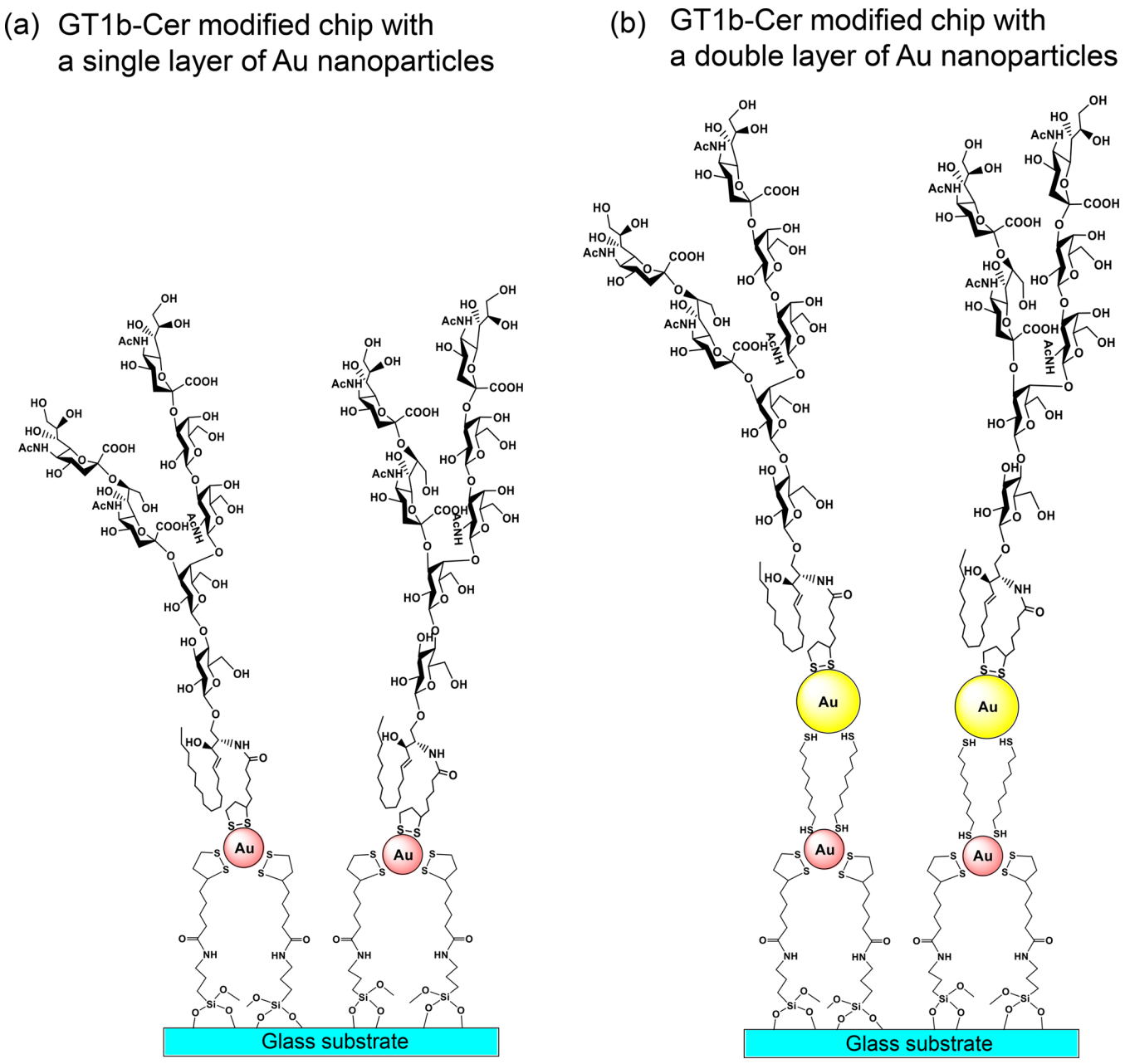

Figure 1. Two types of GT1b-Cer glass chips with Au nanoparticles in (a) a single layer and (b) bilayer. The single layer GT1b-Cer chip was prepared in our preceding study.[16] The illustration in panel (a) was adapted with permission from refs 15 and16. Copyright 2018 Springer Nature and Copyright 2021 American Chemical Society.

In our ongoing projects on development of on-site detection systems, our recent effort is focused on a highly sensitive and facile detection system for the botulinum toxins (BTX) from *Clostridium botulinum* strains, which are grouped into seven different serotypes (A−G).[17] The toxins are listed as the highest dangerous pathogens in "Category A" by the CDC (Centers for Disease Control and Prevention)[18] because the toxins have extremely low LD_{50} (a mean 50% lethal dose) values and may illegally be used in terror and crime scenes. A botulinum neurotoxin, a minimum unit of the toxin, consists of a light (∼50 kDa) and a heavy chain (∼100 kDa), which are linked with a disulfide bonding. The heavy chain (Hc) has a sugar binding domain to gangliosides on neuronal cell membranes, while the light chain (Lc) has a catalytic domain, which inhibits acetylcholine release.[17] Tris-sialo ganglioside, GT1b-Cer, is a major ligand of Hc. In this work, BTX/C and BTX/A/Hc are selected as the target toxins. To achieve their on-site and quick detection with the LSPR device, we previously designed a GT1b-Cer glycochip with Au nanoparticles on glass substrates. In the present study, we propose the other type of GT1b-Cer glycochip which possesses double-layered Au nanoparticles on a glass substate. The structure is apparently different from our previous monolayer study, and the function greatly improved.

Au nanoparticles are known to construct multilayer structures on substrates. In reported methods,[19−26] Au nanoparticles are stacked on substrates with a layer-by-layer method, and optical and electrochemical properties are evaluated in terms of the number (n) of layers by means of a cyclic voltammetry or UV spectroscopy. For formation of the

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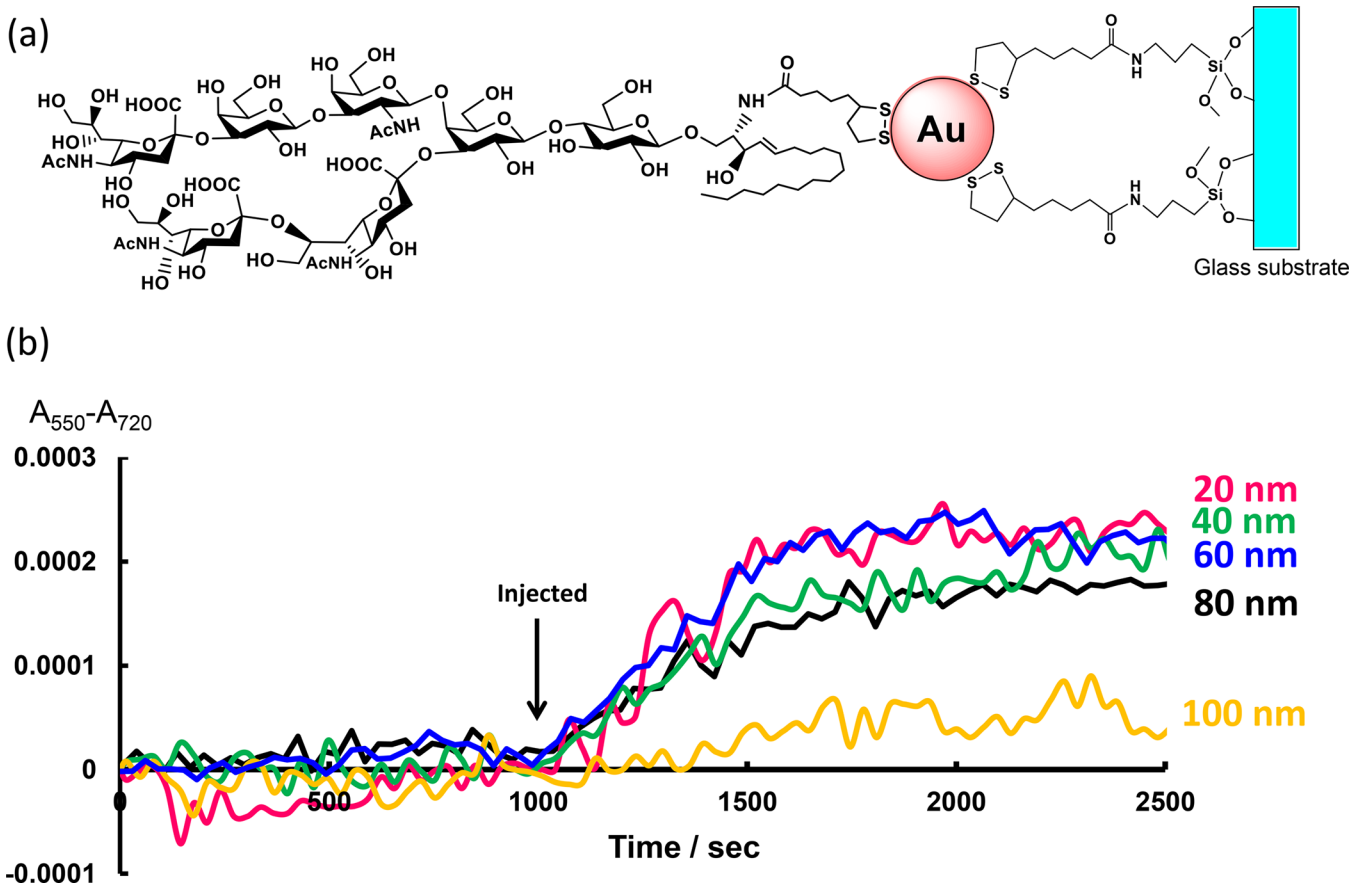

Figure 2. Glycolipid chip having a single layer of Au nanoparticles and the LSPR response. (a) GT1b-Cer modified chip having different diameters of Au nanoparticles (20, 40, 60, 80, and 100 nm). The chip of the single layer was prepared by our established method.[16] (b) Effects of Au nanoparticle diameter sizes on an LSPR detection of BTX/C. [BTX/C] = 50 ng/mL. All samples were injected at 1000 s. Details are given in the Experimental Section. The structural illustration in panel (a) was adapted with permission from refs 15 and16. Copyright 2018 Springer Nature and Copyright 2021 American Chemical Society.

Au-nanoparticle multilayers, different cross-linkers are applied like polyelectrolytes,[19] polyaniline,[20] mercaptoethylamine,[21] porphyrin,[22] and α,ω-alkane dithiols.[23−26] In the evaluation study, either optical property or electrochemical behavior is correlated with the number or thickness of the Au multilayers. However, a factor of diameter sizes of Au nanoparticles has been little examined in the past. In the present study, we have prepared the GT1b-Cer glycochips with Au nanoparticles in different sizes and evaluated the products in terms of LSPR response to the botulinum toxin. The novel glycolipid chips with Au nanoparticles in optimal sizes enable us to detect the deadly toxin at concentrations below 5 ng/mL.

RESULTS AND DISCUSSION

Preparation of GT1b Glycolipid Chip with a Single Layer of Au Nanoparticles and Effects on Au Nanoparticle Sizes in LSPR Detection Using Botulinum Toxin Type C (BTX/C). The GT1b-Cer glycolipid chip having a single layer of Au nanoparticles[16] is depicted in Figure 1a. There, Au nanoparticles bind with lipoic acids having silanol amide groups on a glass substrate, making the single-layered Au nanoparticles. The chip with the single layer is further modified with a synthetic glycolipid GT1b-Cer homologue[16] having a lipoic amide group by a self-assembled monolayer (SAM) technique.

Previously, we reported that the size of Au nanoparticles used in a single layer is an important factor for detection sensitivity:[14] For detection of ricin, lactose on 20 nm Au nanoparticles showed the highest LSPR response, while Gb_3 and GM1 glycoconjugates on 40 nm Au nanoparticles showed the highest responses to Shiga toxin and cholera toxin. Recently, we preliminarily reported LSPR responses of botulinum toxin type C (BTX/C) with a GT1b glycolipid chip having a monolayer of 40 nm Au nanoparticles.[16] For the BTX detection, however, optimization of size of Au nanoparticles of the single monolayer have not been achieved. At the beginning of this study, we tried to determine an optimal Au size for detection of the botulinum toxin type C as shown in Figure 2.

The GT1b-Cer chips with Au nanoparticles in different sizes (20−100 nm) were prepared by our previously reported method[16] and used in the LSPR detection of botulinum toxin type C (BTX/C) (Figure 2). Different from our expectation, LSPR responses were almost independent of Au nanoparticle sizes in a range between 20 and 60 nm, and the response decreased with increasing sizes in a range between 60 and 100 nm (Figure 2b). This observation is different from what we previously observed for other biological toxins (ricin, Shiga toxins, and cholera toxins). GT1b-Cer has a branched, sialo oligosaccharide structure with high flexibility in molecular conformations compared to sphingosine oligosaccharides with

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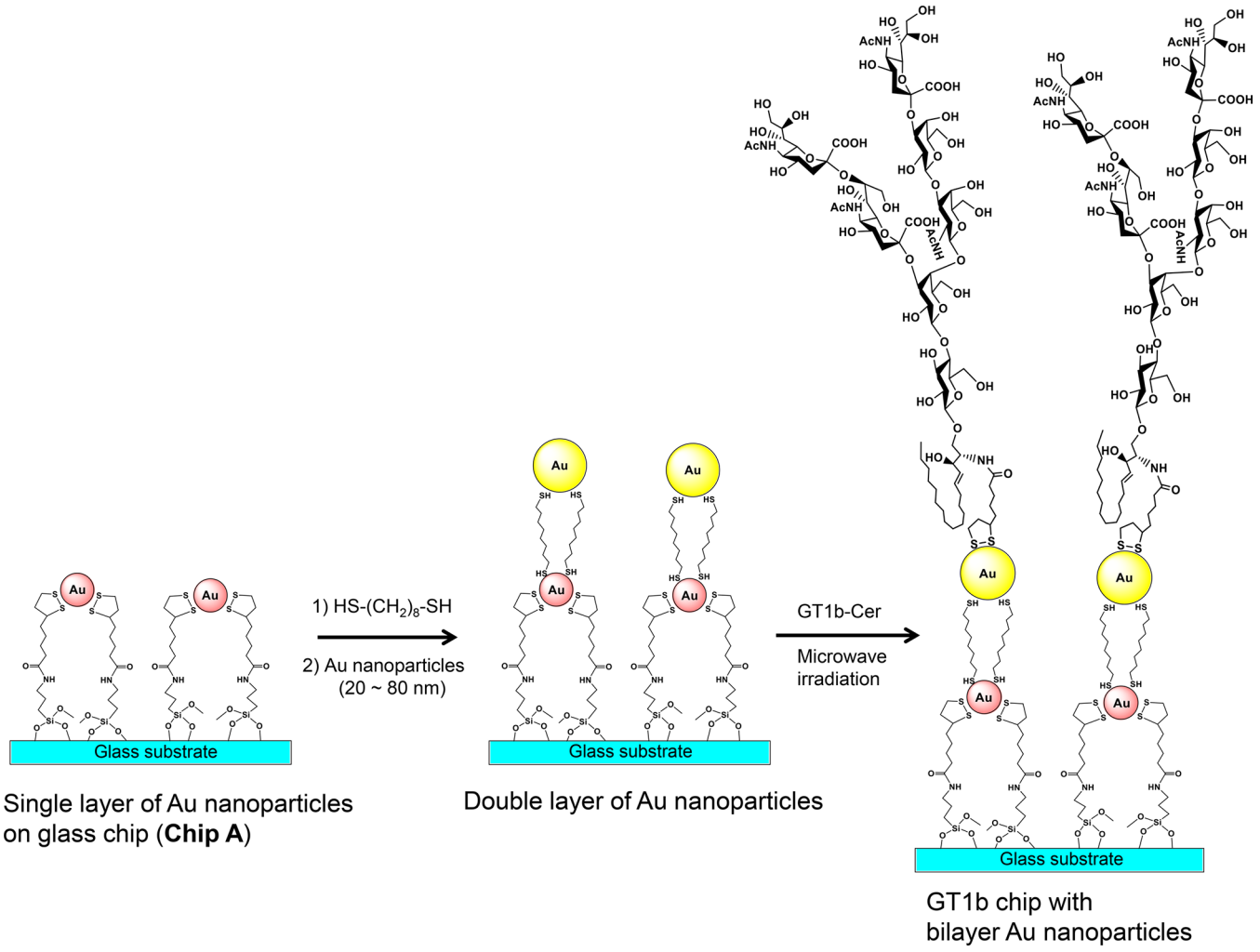

Figure 3. Preparation of glycochip with Au nanoparticles in bilayers. The chip of the single layer (Chip A) and the GT1b-Cer analogue were prepared by our established method.[16] Figure of Chip A was adapted from ref 16. Copyright 2021 American Chemical Society.

lactose for ricin (RCA_{60}) and RCA_{120}, Gb_3 for Shiga toxins, and GM1 for CTX. Due to the enlarged molecular size and variations in molecular conformations, the GT1b-Cer chips may maintain a consistent surface terrain during the heating process under microwave irradiation to make the BTX response almost independent of the sizes of Au nanoparticles between 20 and 60 nm ϕ.

Preparation and Evaluation of GT1b Glycolipid Chip with Double Layered Au Nanoparticles for LSPR Detection of Botulinum Toxin (Type A Heavy Chain, BTX/A/Hc). Next, we propose another type of glycolipid chips which possess double-layered Au nanoparticles (Figure 1b). There, Au nanoparticles of an under and upper layer are linked with 1,8-octanedithiol as a cross-linker. On the outer Au surface, the synthetic GT1b-Cer homologue[16] is immobilized. With the GT1b-Cer chip having the double-layered Au nanoparticles, the detection response is examined by changing the diameter sizes of Au nanoparticles in each of the two layers.

We prepared a series of GT1b chips having the double-layered Au nanoparticles in different sizes in a common way as shown in Figure 3, in which Chip A having 20 nm Au nanoparticles in the first layer is shown as an example. After immobilization of 20 nm Au nanoparticles on an activated glass surface, each of the different Au nanoparticles (20, 40, 60, and 80 nm) was stacked with 1,8-octanedithiol to make the second layer. Finally, GT1b-Cer was installed onto the second Au nanoparticles by the self-assembled monolayer (SAM) technique under microwave irradiation according to our established method.[16]

As illustrated in Figures 4 and 5, the two Au nanoparticle layers are stacked on the glass substrate. In Figure 4, the diameter of Au nanoparticles in the first layer is fixed at 20 nm ϕ, while the size is changed in the second phase at 20, 40, 60, and 80 nm. Upon addition of BTX/A/Hc, the LSPR device showed a quick response with various intensities depending on the Au nanoparticle sizes (Figure 4b). A combination of 20 nm (the first layer) and 60 nm (the second) brings the highest response. When Au nanoparticles in 20 nm ϕ size are applied in both of the two layers, the LSPR response becomes the lowest. In Figure 5, the size of Au nanoparticles in the lower layer is fixed at 60 nm ϕ. In this case, the combination of 60 and 40 nm ϕ brings the highest LSPR response, followed by the combination of 60 and 60 nm. On the other hand, the combination of 60 nm with 20 nm ϕ or 80 nm ϕ fails to gain a high LSPR response to BTX (Figure 5b).

These results indicate that the bilayer Au nanoparticles can notably enhance the LSPR response when Au nanoparticles with sizes are optimized at each of the two layers. Applying Au nanoparticles in the bilayer, an apparent distance from the glass surface is doubly elongated. The distance from the glass surface

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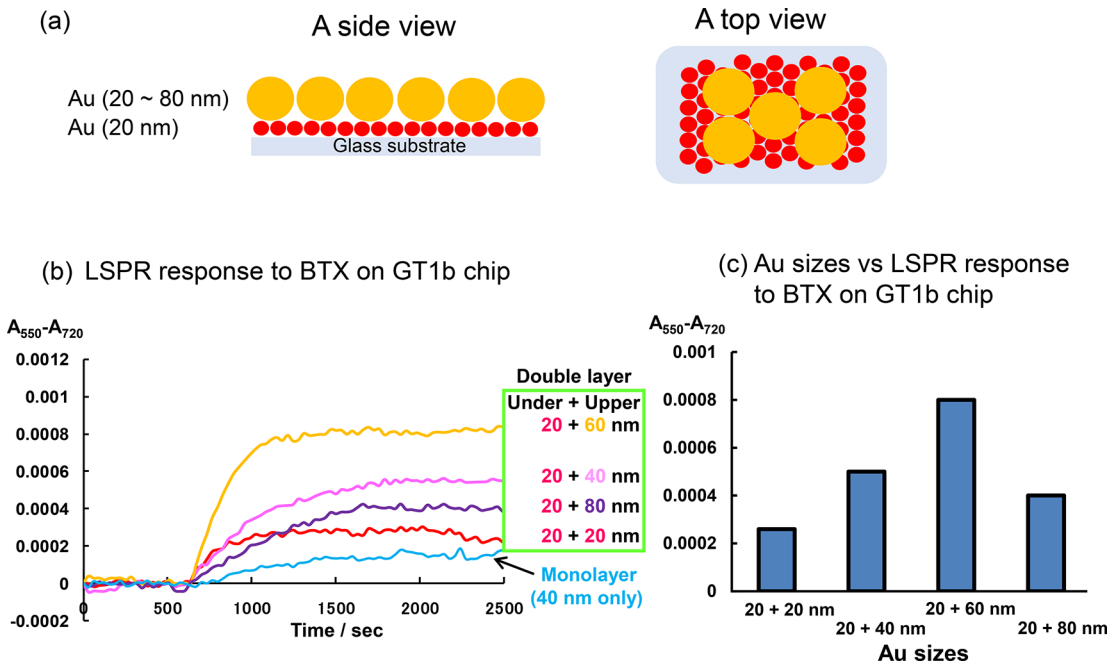

Figure 4. Evaluation of GT1b-Cer glycochips with double-layered Au nanoparticles: (a) Illustration of each side and top view of the Au nanoparticles in double layers. (b) Effect of Au nanoparticle diameter sizes in the upper layer. (c) Relation between LSPR responses and the diameter sizes of Au nanoparticles in the upper layer. In panel (a), two different Au nanoparticle layers are stacked on the glass substrate without showing a silanol attachment, a cross-linker (1,8-octanedithiol), and GT1b-Cer. Au nanoparticles in the under layer have ca. 20 nm diameter size. Au nanoparticles in the upper layer may have larger sizes up to 80 nm. In (b), data from a glycochip with single-layered Au nanoparticles (40 nm) is also shown. Each sample was injected at 500 s. [BTX/A/Hc] = 50 ng/mL.

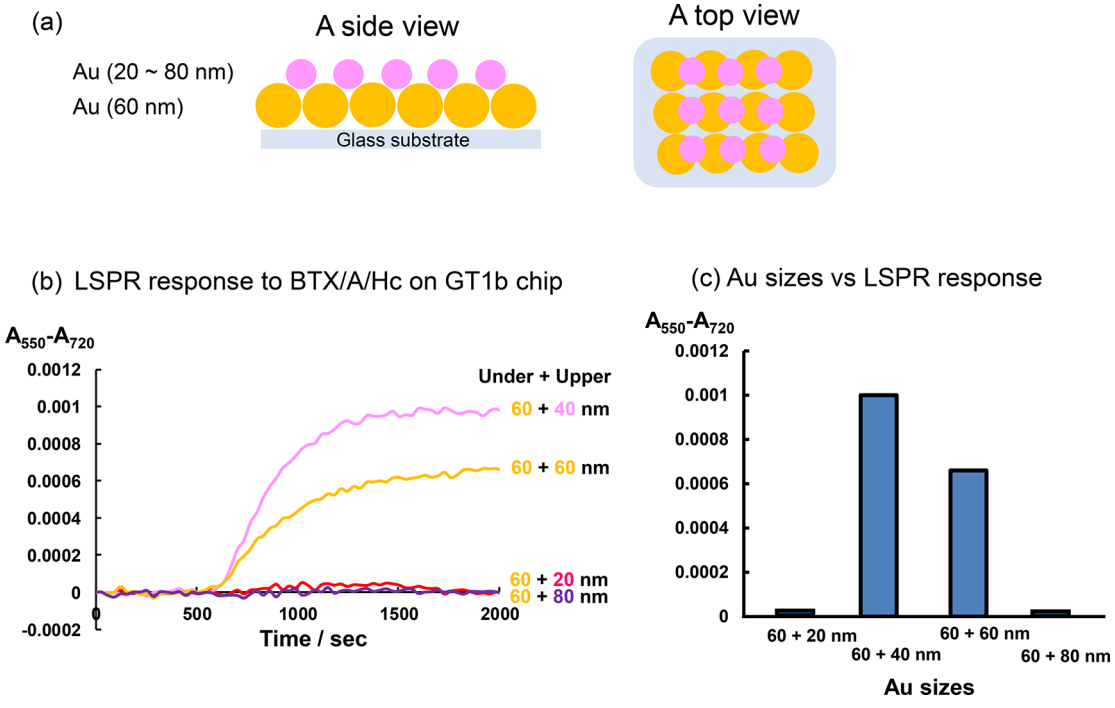

Figure 5. Effect on the double layer of Au nanoparticles. (a) Illustration of each side and top view of double-layer Au nanoparticles. (b) Effect of sizes of the Au nanoparticles in the upper layer. In this case, the size of Au nanoparticles in the first layer was kept constant at 60 nm. The second Au nanoparticles were varied in sizes (20−80 nm ϕ). Samples were injected at 500 s. [BTX/A/Hc] = 50 ng/mL. (c) A relation between LSPR responses and the sizes of Au nanoparticles in the upper layer.

as well as the surface terrain seem to be important for the biological toxin to recognize and made tight adhesion on the LSPR glycochip. There are, however, optimal Au sizes at each of the first and the second layer as summarized in Table 1. Otherwise, the following combinations may fail to detect BTX: 60 nm + 20 nm, 40 nm + 80 nm, and 60 nm + 80 nm. In the current study, the combination of 60 and 40 nm brings the highest sensitivity, followed by the combination of 20 and 60

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Table 1. Effects of Au Sizes in the Two Layers on the LSPR Response to BTX[a]

	Au in the second layer (nm ϕ)			
Au in the first layer (nm ϕ)	20	40	60	80
20	2.6	5.0	8.0	4.0
40	3.0	7.0	5.0	~0
60	~0	10.0	6.5	~0

[a]Relative LSPR responses ($A_{550} - A_{720}$) from the combination of 60 nm + 40 nm (10.0) are given.

nm. With these two combinations, the sensitivity is increased by a factor of 8−10 compared to the LSPR analysis using the monolayer glycochip (Figure 4b).

Possible double-layered structures in Figures 4a and 5a are illustrated, referring to the AFM images of LSPR chips with monolayer Au nanoparticles.[15,16] We had already shown the AFM images for the monolayered one, in which the first (lower) layer is packed with Au nanoparticles as illustrated in these figures. The double-layered structure is almost promised by the bottom-up chemical manipulation to pile up the two phases using established chemical reactions. Extinction (absorption) spectra of each mono- and double-layered chip of Au nanoparticles are shown in the Supporting Information (Figure 1S). The LSPR chips made of Au nanoparticles (20, 40, and 60 nm ϕ) in the mono layer showed the absorption band with a maximum wavelength (λ_{max}) around 530 nm, whereas the chips with the double-layered Au nanoparticles (20 nm + 60 nm ϕ and 60 + 40 nm ϕ) gave the second band in a longer wavelength region (820−830 nm). The second characteristic band arises from an interparticle plasmon coupling,[27,28] which is known to occur when Au nanoparticles make a packed bilayer or multilayer structures. This means that the presence of the second band by itself supports the double-layered structure of the LSPR chips. Compared to the first absorption band at 530 nm, the second band at 820−830 nm has a lower intensity by 1/3−1/4 and becomes more broadened. The observed difference also agreed with the double-layered structures, in which the Au nanoparticles in the lower layer are packed more rigidly[15,16] compared to those in the upper layer. On the other hand, it is difficult to rationalize why the combination of 60 and 40 nm can bring the best LSPR response to the biological toxin. Including experimental deviations, we may need further microscopic or spectroscopic evidence to correlate the LSPR response with the Au sizes.

Detection Limit of BTX/A/Hc Using the Glycolipid Chip with the Double-Layered Au Nanoparticles with Optimal Sizes (60 nm + 40 nm). Using the GT1b chip with the bilayer Au nanoparticles in optimal sizes (60 and 40 nm), we tried to estimate a detection limit of BTX/A/Hc in the LSPR analysis. Figure 6 shows that the LSPR response almost linearly changes in a range between 5 and 50 ng/mL. Though an exact quantitative assay was hard to perform with a limited amount of the deadly toxin available in our hands as a test sample, the detection limit was estimated to be approximately 5 ng/mL or less as judged from the S/N ratio (>3) of LSPR sensorgrams. The LSPR signal ($A_{550} - A_{720}$) of BTX at 5 ng/mL gains an order of 2.4×10^{-4} that is much higher than noise level of baseline with an order of 1.2×10^{-5}. At this concentration, the LSPR response looks to be barely keeping the linear relation with toxin concentrations. Experimental deviations estimated from the fluctuation of sensorgram were within ±3% even if the minimal amount of the BTX toxin (5 ng/mL) was applied. This result also supports that the LSPR analysis using the GT1b glycochips on the double layers of Au nanoparticles provide us with a promising approach to detect the deadly toxin.

As a control experiment to check a specificity in the BTX/GT1b-Cer interaction, we used a plant lectin (RCA_{120}) as a negative control. Similar to BSA used in the preceding study,[14−16] RCA_{120} is a hydrophobic protein with low toxicity and is useful for assessing a nonspecific background interaction with the Au surface. The RCA_{120} protein binds with an *exo*-terminal βGal residue in Lac or LacNAc oligosacccharides.[14] Though the GT1b-Cer chip also has a Lac unit at an *endo*-terminal linked to ceramide, the LSPR sensorgram has shown no response to RCA_{120} even at concentrations over 100 ng/mL (Figure 6a). This result means that the present sensor system is based on a specific toxin/oligosaccharide binding interaction. Though the use of a nonglycosylated or a simple alkanethiol-bound Au-chip may give another approach to this issue, the nonglycosylated Au-nanoparticle simply causes the hydrophobic interaction and −SH interaction with proteins.

CONCLUSIONS

We have demonstrated that the detection sensitivity is greatly improved by using the bilayer Au nanoparticles for the

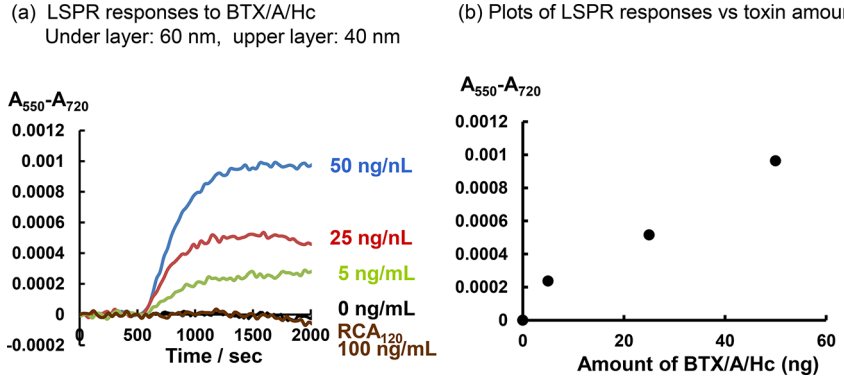

Figure 6. LSPR sensorgrams and plots of LSPR responses against toxin amounts under the optimized conditions. (a) LSPR responses to BTX/A/Hc with bilayer Au nanoparticles (60 nm + 40 nm ϕ). (b) Plots of LSPR responses against the amounts of BTX/A/Hc. Samples were injected at 500 s. [BTX/A/Hc] = 0, 5, 25, and 50 ng/mL. [RCA_{120}] = 100 ng/mL. The LSPR detection signals may involve experimental deviations within ca. ± 3% on estimation from the fluctuation of sensorgrams.

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preparation of GT1b glycolipid chips. In our preceding studies,[16] we already noticed that the surface geometry is very important for preparing those glycolipid chips showing a potent binding activity with a targeting biological toxin in the LSPR analysis. In the present study, we have again recognized the importance of surface geometry. As illustrated in Figures 4 and 5, a surface terrain is predicted to extensively vary by the size of the Au nanoparticles introduced at each of the two layers. This also means that the glycolipid chips give us a double chance for optimizing the surface to enhance the sensitivity for detection of biological toxins. For the deadly botulinum toxin, we have found that the combination of 60 nm (under layer) and 40 nm (upper layer) enables the quick (<20 min) detection of its type-A heavy chain (BTX/A/Hc) at concentrations lower than 5 ng/mL. The sensitivity is 10 times increased from that of the glycolipid chips made of a single-layer Au nanoparticle which required ca. 50 ng/mL of BTX/A/Hc.[15] The optimized combination significantly improves the LSPR detection sensitivity for this toxin compared to our preceding analysis[15] using GT1b-glycolipid chips on monolayer Au nanoparticles.

For detection of BTX, many methods are proposed:[17] mouse bioassay (10−100 pg/mL, 1−4 days),[17] fluorescent/FRET detection (10−150 ng/mL, 1−3 h),[29,30] mass spectrometric assay (49−375 ng/mL, 8−14 h),[17] lateral flow immunoassays (10−20 ng/mL, 20 min),[31] etc. Every method has its merit and demerit. The overall LSPR system used in the present study has a portable size (30 × 30 × 30 cm),[15] which adapts the on-site detection of the deadly biological toxin. The use of chemically homogeneous and stable glycolipids may be also a notable advantage of our present approach, and the glycochips themselves have versatile utilities in biological and medicinal studies on glycoconjugates besides sensing and analytical studies.

Although multiple steps for preparation are required in the double-layered chips, detection sensitivity is increased by a factor of ca. 10 compared to the monolayered one. From the viewpoint of simplicity in handling and consuming time, an immunoassay assay[31] is commercially available as an assay kit for the safety assessment. We aim at developing a facile and simple approach comparable with the immunoassay. Different from the immunological method, our approach utilizes the cell surface oligosaccharide (GT1b) which is thought to bind the BTX protein and assist the internalization of this toxin into the host cell. We envisage that the present approach can be extended to other glycolipid chips for detection of pathogenic viruses and plant or bacterial toxins, and the result will be reported elsewhere in a due course.

EXPERIMENTAL SECTION

Materials and Instruments. All chemicals were received from commercial suppliers and used without further purification. 1,8-Octanedithiol was supplied from ProChimia Surfaces (Sopot, Poland). Ganglioside GT1b was purchased from IsoSep AB (Tullinge, Sweden). Botulinum toxin type C (BTX/C) was obtained 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. Botulinum toxin type A heavy chain (BTX/A/Hc) was obtained from List Biological Laboratories, Inc. (Campbell, CA, USA) under regulations of US Department of Commerce, and BTX/A/Hc was used under the Civil Protection Law in Japan. *Ricinus communis* agglutinin (RCA_{120}) was purchased from Vector Laboratories, Inc. (Burlingame, CA, USA). The GT1b-Cer derivative having a lipoic amide group was prepared according to our previous reports.[16] Au nanoparticles (20, 40, 60, 80, and 100 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 Peltier-controlled cuvette holder (qpod) with 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.

Glass Chip (Chip A) Modified with Au Nanoparticles. Au-modified glass chips (Chip A, Figure 3) were prepared by our established method under microwave irradiation.[16] Then, the Chip A having a single layer of Au nanoparticles (20−80 nm in diameters) was treated with 50 μM of 1,8-octanedithiol in MeOH under microwave irradiation (100 W, 45−60 °C, 600 s) under N_2 atmosphere to give the glass chip arraying 1,8-octanedithiol groups on the surface. The glass plate was washed gently with MeOH, and the chip was treated with Au nanoparticles (20−80 nm in diameters) for 6 h to give the double-layer Au nanoparticles. In Figure 2, 100 nm Au nanoparticles are also used in the first layer and used for detection of BTX/C.

Preparation of GT1b-Cer Chip. GT1b-Cer was installed on the outer upper surface of Au nanoparticles on a glass substrate under microwave irradiation as follows: The chip having the double layer of Au nanoparticles was immersed in a MeOH solution of GT1b-Cer (49 μM, 2 mL), and the installation was carried out with the microwave reactor. The microwave irradiation (250 W) was conducted for 60 min with the sequence program in the PC controller without the cooling system (see the condition B2 in ref 16). The derived GT1b-Cer chip was extensively washed with methanol and water. In particular cases where a nonselective response is so serious, the GT1b chip was treated with lipoic acid in methanol (1 mM) for 1 min before use in LSPR analyses.

LSPR Detection of Biological Toxins. Using the biological toxins (BTX/C or BTX/A/Hc) and the glycolipid chips functionalized with double layers having different Au nanoparticle sizes (20−80 nm), detection responses were examined by our LSPR method as previously reported.[14,16] 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 1 mL, a disassemble flow cell (optical length, 0.025 mm) for attachment of the prepared chips, a temperature-control sample compartment with a Peltier-controlled cuvette holder, a spectrometer for 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. Each 1 mL of

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BTX/C (50 ng/mL) or BTX/A/Hc (5, 25, and 50 ng/mL) was injected for 15 min at a flow rate of 66.6 μL/min. As the negative control, RCA_{120} (100 ng/mL) was also injected. The temperature was 25 °C. The integration time was 8 ms, and the number of accumulation times was 2000. LSPR responses were monitored at $A_{550} - A_{720}$ with time. All LSPR data were analyzed with OOIBase32 software (Ocean Optics, version 2.0).

Safety Considerations. BTX/C is highly toxic if inhaled or digested. Toxicity of BTX/A/Hc 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.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c07976.

Extinction spectra of mono- and double-layer chips, their measurement conditions, and related references (PDF)

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Notes

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