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Immobilization of carbohydrate clusters on a quartz crystal microbalance sensor surface

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ABSTRACT

The immobilization of carbohydrates on gold surfaces is a prerequisite technology for carbohydrate-related studies, including those of carbohydrate–biomolecule interactions. Glycolipid domains in cell membranes, such as lipid rafts, are thought to play an important role in cell biology through their carbohydrate portions. To understand the recognition of glycolipid domains such as receptors for bacterial toxins and viruses, we immobilized clusters of carbohydrates on a gold surface by using polyamidoamine (PAMAM) dendrimers as a scaffold. The PAMAM dendrimers were adsorbed on the gold-coated surface of a quartz crystal microbalance (QCM) sensor and were observed by means of QCM with dissipation (QCM-D). After adsorption of the PAMAM dendrimers, lysoganglioside-GM_1 and 12-aminododecyl-*N*-acetylglucosaminide (GlcNAc–C12–NH_2) were immobilized on the amino groups of PAMAM dendrimers by means of an NH_2 cross-linker. Immobilization of the carbohydrates was confirmed by observation of their specific interaction with anti-ganglioside GM_1 antibody or wheat germ agglutinin (WGA). Surfaces with different GlcNAc–C12–NH_2 cluster sizes and densities were prepared by varying the size of the PAMAM dendrimers or the concentration of GlcNAc–C12–NH_2 immobilized on the dendrimers, respectively. Analysis of the binding between the GlcNAc–C12–NH_2-immobilized surface and WGA revealed that the size of the PAMAM dendrimers influenced the GlcNAc–C12–NH_2–WGA interaction, with larger dendrimers resulting in higher WGA binding constants.

1. Introduction

Carbohydrates consist of various kinds of saccharides and multiple linkage structures of saccharide units. Many carbohydrates on cell surface exist as glycolipids or glycoproteins, that is, complexes with lipids or proteins, respectively. Carbohydrates protect cells against harmful environmental factors [1–3], mediate cell attachment [4,5], participate in signal transduction pathways [6,7], and serve as receptors for microorganisms and their toxins [8–10]. Glycolipids frequently aggregate owing to the hydrophobicity of their lipid moieties, forming carbohydrate-enriched microdomains known as "lipid rafts" on cell surfaces. Recently, these lipid rafts have been suggested to serve as platforms for various cellular events, such as those mentioned above [11].

Numerous studies, including studies of the interactions between carbohydrates and proteins, carbohydrates, and other biomolecules, have been carried out to understand the role of carbohydrates in biological events. The immobilization of carbohydrates on a solid substrate, such as gold sensor chips for quartz crystal microbalance (QCM) and surface plasmon resonance (SPR) [12], are essential techniques for the study of carbohydrate–protein interactions. However, because the lipid moieties of glycolipids are unreactive, glycolipids cannot be readily immobilized on solid substrates. To overcome this problem, various immobilization methods utilizing the lipids' hydrophobicity have been reported, such as attachment of a self-assembled monolayer (SAM) of alkanethiols on a gold surface [13], direct attachment of gangliosides to a conventional CM5 carboxymethyl dextran sensor chip [14], and utilization of vesicles composed of glycolipids and alkanethiols onto gold electrode [15]. In addition, ligand-conjugated saccharides, such as SH-saccharides synthesized by amination between an aminophenyl disulfide and a carbohydrate [16], have been utilized for the modification of a gold surface.

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In general, the binding affinity of an individual carbohydrate motif for a protein receptor is lower than that required for biological function. To increase the binding affinity between carbohydrates and protein receptors, carbohydrates often exist in aggregated (i.e., lipid raft) or polymeric forms. Multivalent linker compounds containing one, three, or four aromatic amines and thioctic acid moieties [17] have been developed to immobilize carbohydrates on SPR sensor chips, and the effect of these compounds on the clustered carbohydrates' binding affinity has been evaluated. Although such linkers allow the immobilization of clustered oligosaccharides on SPR sensor chips, the preparation of the linker and subsequent reaction with the ligand-conjugated saccharide require many complicated synthetic steps.

We have developed a comparatively simple method to immobilize clustered carbohydrates on a gold sensor surface, without the need for complex synthetic steps. In this report, we describe a novel strategy for controlling carbohydrate density on sensor surfaces (Fig. 1). Polyamidoamine (PAMAM) dendrimers are adsorbed strongly on the gold surface of a QCM sensor chip. The immobilization of carbohydrates on the PAMAM dendrimers is shown schematically in Fig. 1A. The immobilization is carried out with 2,4,6-trichloro-1,3,5-triazine (cyanuric chloride), which cross-links the amino group of the PAMAM dendrimer with that of a lysoganglioside (GD_{1a} or GM_1, Fig. 2) or a 12-aminododecyl carbohydrate derivative (GlcNAc–C12–NH_2, Fig. 2). Under a reaction of the chlorine atoms in cyanuric chloride with amine derivatives, sequential substitutions of the first, second, and third chlorine atoms in cyanuric chloride is reported to occur at 0–25, 20–40, and 60–100 °C, respectively [18]. In our immobilization method, firstly, the adsorption of PAMAM dendrimers on the gold sensor surface of QCM-D was carried out. After removing non-adsorbed dendrimers, the reaction mixture of carbohydrates (Fig. 2) and cyanuric chloride was loaded on the PAMAM dendrimers immobilized on the gold sensor surface. In the reaction mixture the cyanuric chloride can bind one or two carbohydrates at room temperature. However, the products bearing two carbohydrates cannot react with any amino residues of PAMAM dendrimer more because the substitution reaction of the third chlorine atom hardly occurs at room temperature. Hence, we suppose that the products bearing one carbohydrate exist on the surface of PAMAM dendrimer (Fig. 1A).

The extent of carbohydrate immobilization on the sensor surface can be controlled by two different means. First, the size of the carbohydrate cluster can be controlled by varying the diameter of the PAMAM dendrimers adsorbed to the sensor surface (Fig. 1B). Second, the density of carbohydrates within an immobilized cluster can be regulated by controlling the amount of cyanuric-chloride-linked carbohydrates that are coordinated to the PAMAM dendrimers (Fig. 1C). Using these methods, we obtained immobilized, clustered carbohydrates that were tightly bound to the PAMAM dendrimers, owing to the multivalent interactions of the carbohydrates, and that mimicked the lipid raft structures found on cell surfaces. Because the lipid rafts exist like scattered dots on a cell surface, we aim to fabricate a concentrative immobilization of the carbohydrates on scattered PAMAM dendrimer surface. Therefore, we attempted efficient immobilization with small amount of carbohydrates rather than the high loading capacity of

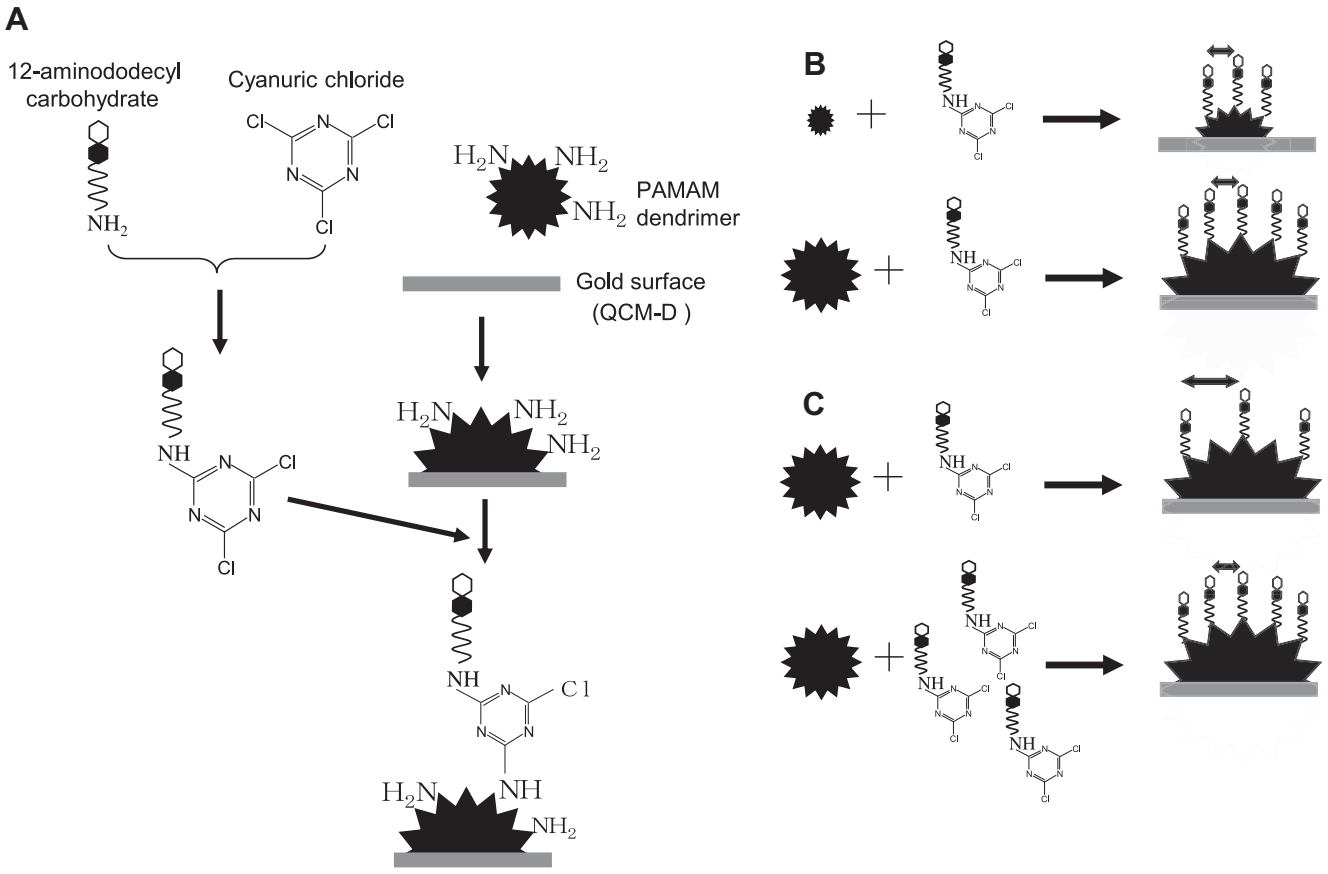

Fig. 1. (A) Schematic depicting the immobilization of a carbohydrate molecule on a PAMAM-adsorbed sensor surface for QCM-D studies. The cluster sizes and densities of carbohydrates immobilized on the sensor surface can be controlled by adjusting (B) the size of the PAMAM dendrimers or (C) the density of carbohydrates immobilized on the dendrimers.

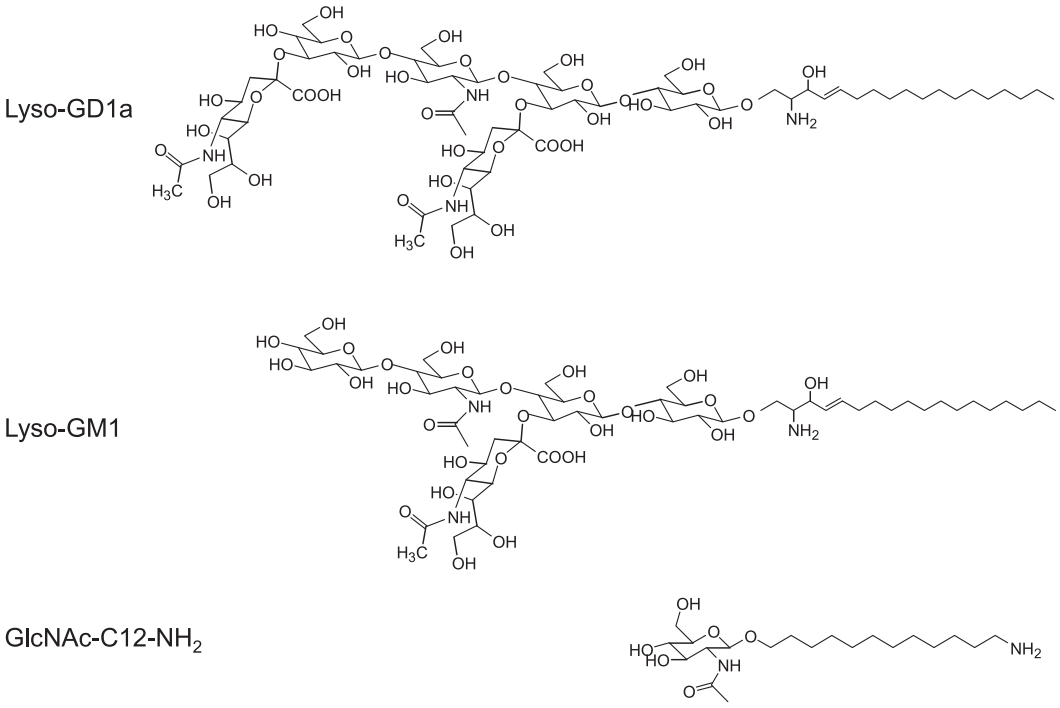

Fig. 2. Chemical structures of the carbohydrates used in this study.

the carbohydrates on a sensor surface. A size of the PAMAM dendrimers used in this study was 4–8 nm, and is comparable with that of middle-sized proteins. Moreover, a cluster size of the carbohydrates that immobilized on the PAMAM dendrimer as a scaffold is similar to that of the lipid raft (<70 nm) [19]. These facts suggest that the use of the PAMAM dendrimers has the advantage of the study on a function of lipid raft. We then used a quartz crystal microbalance with dissipation (QCM-D) to investigate the influence of the clustered condition of the immobilized carbohydrates on a lectin–carbohydrate interaction using the lectin wheat germ agglutinin.

2. Materials and methods

2.1. Materials

Polyamidoamine (PAMAM) dendrimers (generations 4, 5, and 7) and lysoganglioside-GM_1 from bovine brain were obtained from Sigma–Aldrich (St. Louis, MO, USA). Lysoganglioside-GD_{1a} was purchased from Takara Bio Inc. (Ootsu, Shiga, Japan). 2,4,6-Trichloro-1,3,5-triazine (cyanuric chloride) was purchased from Wako Pure Chemical Industries (Chuou-ku, Osaka, Japan). Anti-ganglioside GM_1 rabbit pAb was obtained from EMD Biosciences, Inc. (La Jolla, CA, USA). Wheat germ agglutinin (WGA) was obtained from Seikagaku Corporation (Chiyoda-ku, Tokyo, Japan). 12-Aminododecyl-*N*-acetylglucosaminide was synthesized by the Pd/C-catalyzed reduction of 12-azidododecyl-*N*-acetylglucosaminide [20,21].

2.2. Immobilization of carbohydrates on a QCM sensor surface

AT-cut, gold-coated quartz crystals (QSX301, Q-sense AB, Gothenburg, Sweden, diameter 14 mm, thickness 300 μM) with a resonance frequency of 5 MHz were used both as the immobilizing substrate and as sensing elements for QCM-D analysis. To clean the quartz crystals, they were immersed in a 1:1:5 (v/v) mixture of 30% aqueous H_2O_2:25% aqueous ammonia:purified water heated at 80 °C for 10 min and subsequently were rinsed with purified water and dried under atmospheric conditions. After treatment with an ultraviolet ozone cleaner (Bioforce Nanoscience, Ames, IA, USA) for 10 min, the cleaned quartz crystals were mounted on the flow module of the QCM-D system (Q-sense E4, Q-sense AB, Gothenburg, Sweden). QCM measurements were conducted in the flow module at the location where the loading solution contacted the gold surface of the quartz crystal. After establishing a stable baseline with a loading buffer solution (0.1 M carbonate-bicarbonate buffer, pH 9.2) in the flow module, several concentrations (0.056–5.6 nmol/mL) of various PAMAM dendrimer generations (4, 5, 7) in the loading buffer solution were loaded onto the gold surface of the quartz crystals for 8 min at a flow rate of 0.1 mL/min. After 30 min, the gold surface was washed with pure loading buffer to rinse away non-adsorbed dendrimers. Rinsing was performed at the same flow rate as that used to establish the baseline and was continued until a steady-state signal was observed.

After rinsing with loading buffer, the number of PAMAM dendrimers adsorbed on the QCM sensor surface (per 1 cm^2 of surface area) was determined from the adsorbed mass (Δm, ng/cm^2), which was calibrated from the Voigt model with Q-sense software. Subsequently, the surface coverage per 1 cm^2 of the sensor surface was calculated by multiplying the area of a dendrimer (at its widest circumference) by the total number of dendrimers adsorbed on the sensor surface.

To immobilize carbohydrates on the adsorbed dendrimers, several ratios and concentrations of 2,4,6-trichloro-1,3,5-triazine (cyanuric chloride) mixed with lysoganglioside or GlcNAc–C12–NH_2 (0.35–22.4 nmol/mL and 3.6–139.2 nmol/mL, respectively) were prepared, and each mixture was loaded onto the QCM sensor surface though the flow module for 1.5 min at a flow rate of 0.3 mL/min. After 2.5 h, the QCM sensor surface was washed with the loading buffer at a flow rate of 0.1 mL/min until a steady-state signal was observed. The changes in the frequency (ΔF) and the dissipation (ΔD) at 25 °C caused by dendrimer adsorption and carbohydrate immobilization were monitored for the fundamental

frequency and six overtones (n = 3, 5, 7, 9, 11, 13). Data at each overtone were recorded as the fundamental value divided by the overtone number (e.g., $\Delta F_7/7$ for n = 7) to compare the changes in overtone frequency with ΔF. From the results of ΔF and ΔD, the adsorption mass (Δm) and thickness of the adsorbed layer on the QCM sensor surface were determined with Q-sense software, which is based on the Voigt model [22–24].

2.3. Observation of morphology on dendrimer-adsorbed sensor surfaces

PAMAM dendrimer-adsorbed sensor surfaces were prepared as described above and were then removed from the flow chamber and rinsed gently with purified water. After drying under atmospheric conditions, the morphology of the dendrimer-adsorbed sensor surface was observed using an atomic force microscope (AFM; S-image/NanoNavistation, SII NanoTechnology Inc., Tokyo, Tsukiji) with a silicon cantilever (SI-DF20) tip operating in dynamic force microscopy mode.

2.4. Binding of a protein to carbohydrates immobilized on the sensor surface

After the unreacted chloride groups, which derived from the immobilized cyanuric chloride, were blocked with an ethanolamine solution (0.1 M carbonyl buffer, pH 9.2), the PAMAM, the cyanuric chloride, and the carbohydrate-immobilized sensor surface were washed with phosphate-buffered saline solution containing 0.005% Tween-20 (TPBS) at a flow rate of 0.1 mL/min until a steady-state signal was obtained. Several concentrations of WGA (25–200 μg/mL) in TPBS solution were applied to the carbohydrate-immobilized surface at a flow rate of 0.1 mL/min, and interaction of the surface with WGA was monitored by observing ΔF and ΔD at 25 °C for the fundamental frequency and six overtones (n = 3, 5, 7, 9, 11, 13). The different overtone data were compared to the fundamental value, and Δm was determined as described above.

The saturation binding kinetics of WGA to the carbohydrate-immobilized sensor surface are expressed by Eq. (1) as linear reciprocal plots of [WGA]/Δm versus [WGA].

$$\frac{[\mathrm{WGA}]}{\Delta m} = \frac{[\mathrm{WGA}]}{\Delta m_{max}} + \frac{1}{\Delta m_{max} K_a} \quad (1)$$

The binding constant (K_a) and maximum binding amount (Δm_{max}) are calculated from the slope and the intercept of Eq. (1), respectively.

3. Results and discussion

3.1. Adsorption of PAMAM dendrimers on the QCM sensor surface for QCM-D

In our experimental approach, PAMAM dendrimers are used as a scaffold to immobilize clusters of carbohydrates. Stable adsorption of the dendrimers on the QCM sensor surface was achieved by simply flowing an aqueous PAMAM dendrimer solution onto the sensor surface. The QCM-D can monitor adsorption/binding events by a frequency change of the QCM sensor. Increase in the frequency value considering desorption of dendrimers on the gold surface of QCM sensor was not observed during the processes of the immobilization of carbohydrates and the interaction with WGA. Our preliminary experiments also indicated that the amount of adsorbed PAMAM dendrimers on the surface increased with decreasing buffer pH (the range of pH 8.7–10.0, Fig. S1 in Supporting Information). In contrast, when the dendrimers were solvated in an ethanol solution, only a few dendrimers were adsorbed on the gold surface. Since the intrinsic pK_a for the 64 primary amino groups in the periphery of a G4 PAMAM dendrimer is 9.20 ± 0.05 [25], the proportion of charged primary amines is expected to have increased with decreasing pH of the buffer solution. Although the exact adsorption mechanism of the PAMAM dendrimers on the gold surface has not yet been determined, the charged primary amines would have played an important role in the adsorption process.

In addition, we found that the amount of adsorbed G4 dendrimers on the gold surface could be regulated by loading varying concentrations of dendrimer solution onto the surface. Fig. 3A shows the relationship between the surface coverage of the G4 dendrimers adsorbed on the QCM sensor surface and the loading concentration of the dendrimer solution. The surface coverage was calculated from the number of PAMAM dendrimers which remain on the gold surface of QCM sensor after washing and rinsing with loading buffer. The linear relationship between coverage rate

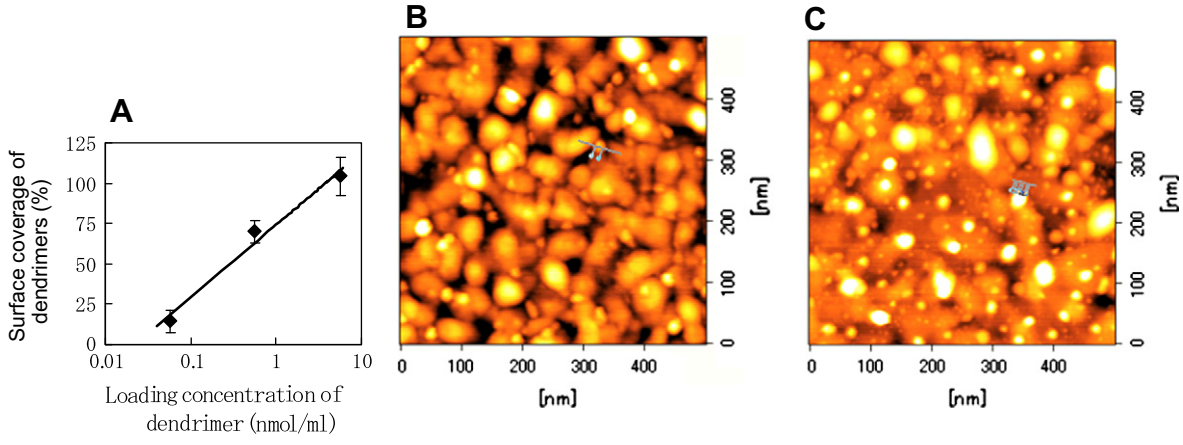

Fig. 3. Adsorption of G4 PAMAM dendrimers on the sensor surface for QCM-D. (A) Relationship between surface coverage of dendrimers adsorbed on the QCM sensor surface and loading concentration of the dendrimer solution onto the sensor. The number of PAMAM dendrimers on the QCM sensor surface was determined from the adsorption mass (Δm) obtained by Q-sense software based on the Voigt model, and the surface coverage of the dendrimers was calculated as described in the Experimental section. The error bars represented the standard error with $n \geqq 4$. (B) Atomic force microscope (AFM) image of the bare QCM sensor surface. (C) AFM image of the sensor surface after PAMAM dendrimer adsorption. For preparation of the dendrimer-adsorbed sensor surface, a 0.28 nmol/mL PAMAM dendrimers (G4) in the loading buffer solution was loaded onto the gold sensor surface though the flow module for 8 min at a flow late of 0.1 mL/min.

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and loading concentration demonstrates our ability to control the amount of dendrimers adsorbed on the sensor surface.

The surface morphology and distribution of the adsorbed G4 dendrimers on the QCM sensor surface were investigated by comparing an AFM image of a bare QCM sensor surface (Fig. 3B) with that of a dendrimer-adsorbed sensor surface (Fig. 3C). In this experiment, a 0.28 nmol/mL PAMAM dendrimers (G4) in the loading buffer solution was used to prepare the dendrimer-adsorbed sensor surface. To fabricate the carbohydrate-immobilized cluster surface, the adsorption coverage on the gold surface should be much lower than 100% (7–8 nmol/mL). The loading concentration of 0.28 nmol/mL dendrimers was corresponding to about 50% of adsorption coverage on the gold surface. According to the random sequential adsorption (RSA) model [26], adsorbing spheres can only reach a maximum degree of coverage of 54% because of excluded area effects. In our study, the adsorption coverage reached 100% by loading a high concentration of dendrimers (>8 nmol/mL). The difference of adsorption profile between the RSA model and our result might be arisen from the pH-induced conformational change of PAMAM dendrimers [27].

In Fig. 3C, small granular substances with diameters of 10 nm or smaller, which are assumed to be PAMAM dendrimers, were observed, whereas the small granular substances were not observed on the bare gold surface (Fig. 3B). These AFM images clearly confirm the adsorption of the dendrimers onto the sensor surface.

3.2. Immobilization of carbohydrates on PAMAM dendrimers

Carbohydrates were immobilized on the dendrimer-adsorbed gold surface. The immobilization of lysoganglioside-GM_1 on the G4 dendrimer-adsorbed gold surface was monitored in real time by simultaneously measuring ΔF and ΔD (Fig. 4, t_3–t_5). The immobilization of lysoganglioside-GM_1 on the dendrimer-adsorbed gold surface was confirmed by observing the decrease of ΔF and the increase of ΔD at time t_5 in comparison with the respective ΔF and ΔD values at time t_3, that is, before the cyanuric chloride/lysoganglioside-GM_1 mixture was loaded onto the sensor surface.

Stability of the immobilized carbohydrate clusters on the gold surface was verified by the ΔF and ΔD measurement in Fig. 4. After loading with the cyanuric chloride/lysoganglioside-GM_1 mixture for 2.5 h (Fig. 4 t_3–t_4), the gold surface was washed continuously with a loading buffer for 40 min at a flow rate of 0.1 mL/min (Fig. 4 after t_4). During the washing process, non-reactive molecules of cyanuric chloride/lysoganglioside-GM_1 and unstable clusters should be removed from the gold surface. Because the values of ΔF and ΔD reached a steady-state in the washing process and the value of ΔF at time t_5 decrease that at time t_3 (the value of ΔD at time t_5 increase that at time t_3), the stability of the carbohydrate clusters on the gold surface was confirmed.

In addition, biochemical evidence for carbohydrate immobilization on the sensor surface was obtained by applying an anti-GM_1 antibody to the lysoganglioside-GM_1-immobilized surface. As a control, the anti-GM_1 antibody was also applied to a lysoganglioside-GD_{1a}-immobilized sensor surface. The resulting decrease in ΔF for lysoganglioside-GM_1 was 1.8 times that observed for lysoganglioside-GD_{1a} (Fig. 5A). These results indicate that the amount of anti-GM_1 antibody bound on GM_1 was higher than the amount bound on GD_{1a}. Hence, the carbohydrates were shown to retain their binding affinities after immobilization onto the sensor surface by our preparation method.

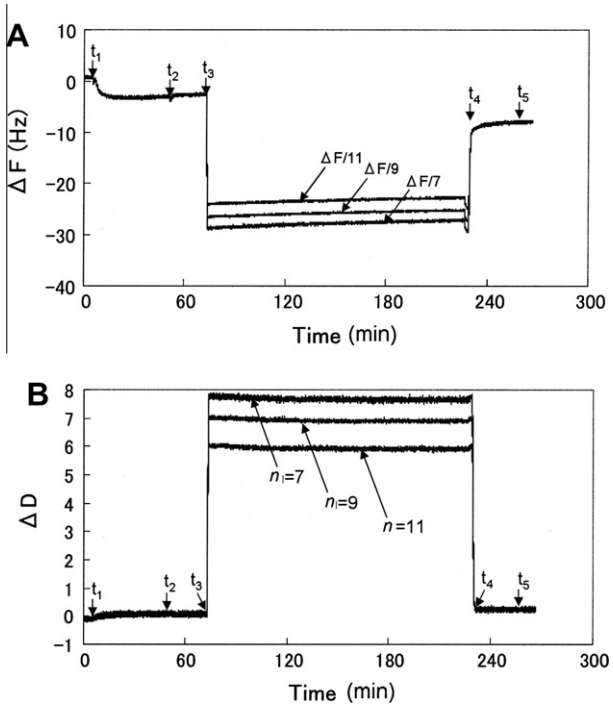

Fig. 4. Typical time courses of (A) frequency shift (ΔF) and (B) dissipation shift (ΔD) during the immobilization of lysoganglioside-GM_1 on a QCM sensor surface. After achieving a steady-state signal with loading buffer, G4 PAMAM dendrimers in loading buffer were applied (t_1) on the gold surface, following by washing (t_2) to remove non-adsorbed dendrimers. For immobilization of carbohydrates, a mixture of 2,4,6-trichloro-1,3,5-triazine and lysoganglioside-GM_1 was loaded on the dendrimer-adsorbed sensor surface (t_3). After 2.5 h, the sensor surface was washed with the loading buffer (t_4) until a steady-state signal was observed (t_5). The changes in the frequency (ΔF) and the dissipation (ΔD) at three different overtones (n = 7, 9, and 11) were recorded as the fundamental value divided by overtone number as described in the Experimental section.

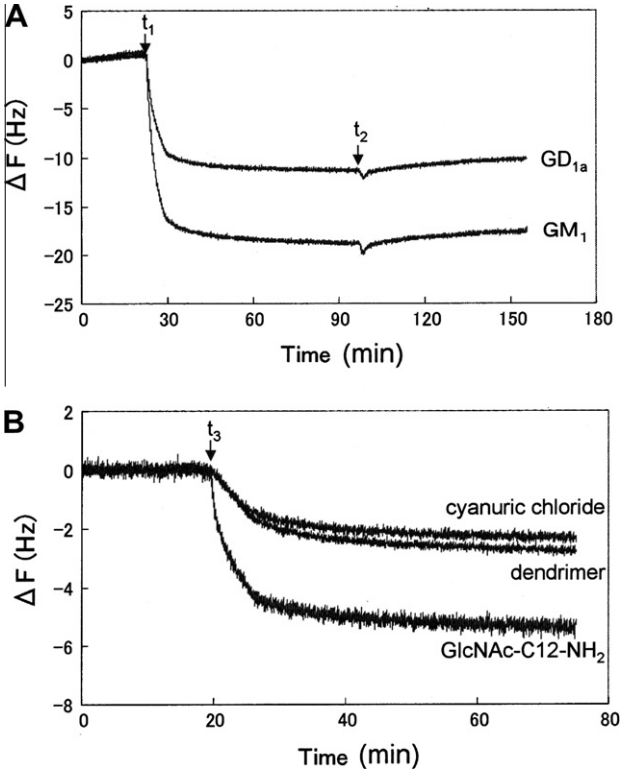

Fig. 5. Binding of proteins to carbohydrates immobilized on the sensor surface. (A) Anti-GM_1 antibody binding to lysoganglioside-GM_1 and lisoganglioside-GD1a immobilized on sensor surfaces. (B) WGA binding to sensor surfaces with adsorbed PAMAM dendrimers and with immobilized cyanuric chloride and GlcNAc–C12–NH_2. (t_1) Injection of anti-GM_1 antibody solution. (t_2) Washing with loading buffer. (t_3) Injection of WGA solution.

Table 1
Maximum binding amounts (Δm_{max}) and binding constants (K_a) of WGA for GlcNAc–C12–NH_2 immobilized on a QCM sensor surface.

Dendrimer	Size (Å)	Number of surface amino groups on dendrimer (I)	Concentration of GlcNAc–C12–NH_2[a] (μM) (II)	II/I[b]	Δm_{max} (ng cm^{-2})	K_a ($\times 10^6$ M^{-1})
G4	40	64	34.8	0.54	588	1.16
G5	53	128	17.4	0.14	435	1.76
			34.8	0.27	476	2.59
			69.6	0.54	588	2.30
G7	80	512	278.4	0.54	588	4.72

[a] Concentration of cyanuric chloride/GlcNAc–C12–NH_2 mixture solution used for GlcNAc–C12–NH_2 immobilization on PAMAM dendrimers.
[b] Ratio of concentration of GlcNAc–C12–NH_2 to number of dendrimer amino groups.

Carbohydrate immobilization also was confirmed by applying WGA (0.93×10^{-7} M) to the GlcNAc–C12–NH_2-immobilized sensor surface (Fig. 5B). For control experiments, WGA also was applied to both a dendrimer-adsorbed sensor surface and a cyanuric chloride–immobilized sensor surface. The GlcNAc–C12–NH_2-immobilized sensor exhibited a definite decrease in ΔF owing to the WGA binding, whereas the dendrimer- and the cyanuric chloride-immobilized sensor surfaces exhibited only small decreases in ΔF, which were attributed to nonspecific binding (Fig. 5B). This result suggests that the GlcNAc–C12–NH_2 were shown to retain their binding affinities after immobilization onto the sensor surface by our preparation method.

3.3. Dependence of carbohydrate cluster size on WGA interaction

The diameters of G4, G5, G6, and G7 PAMAM dendrimers are 40, 53, 67, and 80 Å, respectively [28]. By adjusting the size of the PAMAM dendrimers adsorbed on the sensor surface, different sizes of carbohydrate clusters were subsequently immobilized. Furthermore, the density of carbohydrates immobilized on the sensor surface was regulated by controlling the concentration of linker/carbohydrate solutions applied to the surface, as demonstrated in Fig. 1C for cyanuric chloride/GlcNAc–C12–NH_2. For G5 dendrimers, the concentration of cyanuric chloride/GlcNAc–C12–NH_2 was adjusted to be twice as high as that used for the G4 dendrimers (69.6 μM and 34.8 μM, respectively; Table 1), because the G5 dendrimer possesses twice as many terminal amine groups as the G4 dendrimer. When the WGA solution was injected over both of these GlcNAc–C12–NH_2-immobilized surfaces, simple saturation curves were obtained by plotting the binding amount of WGA (m) versus the concentration of the WGA solution ([WGA], Fig. 6A). The maximum binding amount of WGA (m_{max}) and binding constant (K_a) were calculated from the slope and the intercept of reciprocal plots between [WGA]/m and [WGA] (Fig. 6B) according to Eq. (1). Table 1 summarizes the dendrimer size (which approximately corresponds to the cluster size of GlcNAc–C12–NH_2), m_{max}, and K_a of WGA for GlcNAc–C12–NH_2 immobilized on a QCM sensor surface. Each experiment in Table 1 was repeated twice and described only one result, because the resulted K_a vales from the duplicate data indicated good repeatability. For example, the typical other K_a values of G4, G5 (GlcNAc–C12–NH_2:69.6 μM), and G7 were 0.95×10^6 M^{-1}, 2.26×10^6 M^{-1} and 5.00×10^6 M^{-1} respectively. WGA contains four unique carbohydrate binding sites, and crystallographic studies have revealed that all four are functional, although two of them appear to have affinities too weak to be detectable in solution [29]. Notably, the kinetics data presented here cannot be used to establish an exact constant, since an accurate kinetics model for the varying and partly undefined carbohydrate-WGA interactions that are likely to occur has not yet been developed; construction of such a model is probably impossible. Therefore, the kinetics data were calculated as a means of indirect comparison. Though the m_{max} values listed in Table 1 are independent of dendrimer size, the K_a values increase with increasing dendrimer size over the range of dendrimer generations studied here. Therefore, the affinities of WGA for the carbohydrate cluster size were as follows: G7 (80 Å) > G5 (53 Å) > G4 (40 Å). Because the cyanuric chloride/GlcNAc–C12–NH_2 concentrations were regulated to immobilize the same density of GlcNAc–C12–NH_2 on all three types of dendrimers, the observed difference in K_a among these three dendrimers is attributed to differences in the number of immobilized GlcNAc–C12–NH_2 molecules that were able to interact with WGA.

3.4. Dependence of carbohydrate density on WGA interaction

To estimate the effect of the density of immobilized GlcNAc–C12–NH_2 on the carbohydrates' interaction with WGA, GlcNAc–C12–NH_2-immobilized surfaces with different GlcNAc–C12–NH_2

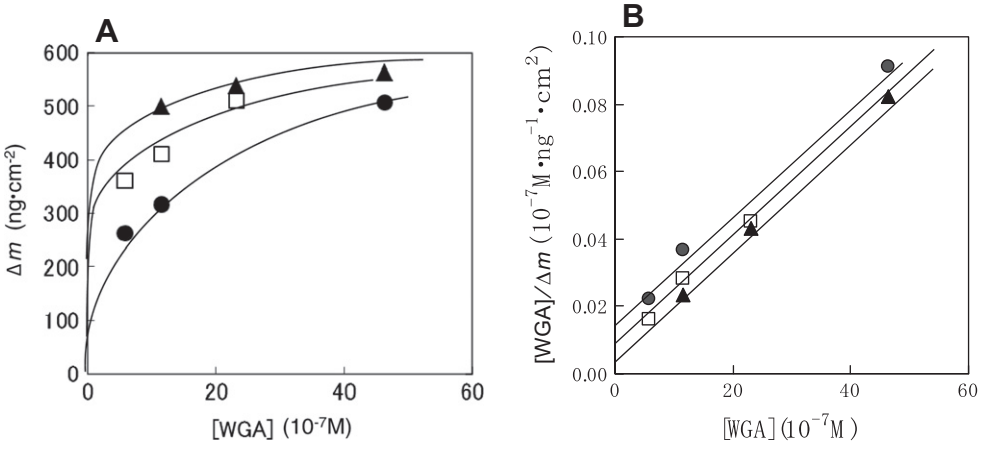

Fig. 6. Binding affinity of WGA for GlcNAc–C12–NH_2-immobilized QCM sensor surfaces with varying carbohydrate cluster sizes. (A) Binding behavior for GlcNAc–C12–NH_2-immobilized sensor surfaces with G4 (●), G5 (□), and G7 (▲) dendrimers. (B) Reciprocal plots [WGA]/Δm versus [WGA] for G4 (●), G5 (□) and G7 (▲) dendrimers.

densities were prepared by applying the several different concentrations of cyanuric chloride/GlcNAc–C12–NH_2 solution to the G5 dendrimer-adsorbed sensor surface (Fig. S2 in supporting information and Fig. 1C), and the WGA binding affinity of these surfaces was investigated. When the cyanuric chloride/GlcNAc–C12–NH_2 solutions containing 17.4, 34.8, and 69.6 μM GlcMAc-C12-NH_2 were loaded on the dendrimer surfaces, 9.6, 12.4, and 35.9 pmol/cm^2 carbohydrates were immobilized, respectively. Mole ratios of carbohydrate to dendrimer were also calculated from the immobilized carbohydrate density divided by the adsorbed dendrimer density, calibrated from ΔF. The mole ratios in a case of the concentration condition of 17.4, 34.8, and 69.6 μM GlcNAc–C12–NH_2 were 9.6:1, 12.4:1, and 35.9:1, respectively. Taking account of 128 primary amines/one G5 dendrimer, the rates of the primary amines on the dendrimer, used for the immobilization of GlcNAc–C12–NH_2, were 7.5%, 9.7%, and 28.0%, respectively. A portion of the remaining free primary amines is thought to contribute to the adsorption on the QCM-D sensor surface. Because the carbohydrates densities increased with increasing carbohydrate concentration in the loading solution, an influence of the steric hindrance on the carbohydrate-immobilization reaction on the surface of PAMAM dendrimer is suggested to be little. In Table 1, m_{max} decreases with decreasing GlcNAc–C12–NH_2 concentration. The WGA binding constants observed for the surfaces with the highest and second-highest GlcNAc–C12–NH_2 densities are larger than the binding constant observed for the surface with the lowest density. However, the differences between these observed binding constants were small, indicating that the surfaces' affinity for WGA was largely unaffected by the GlcNAc–C12–NH_2 density. This result can be explained by considering the difference between the size of WGA and the size of the carbohydrates: since WGA is much larger than the carbohydrates, an increase in the density of carbohydrates on the surface would not necessarily correlate to a greater number of WGA molecules being adsorbed.

4. Conclusions

A new method for immobilizing clusters of carbohydrates on a gold surface has been developed. The lysoganglioside GM_1 and GlcNAc–C12–NH_2 were immobilized via a cyanuric chloride cross-linker on PAMAM dendrimers adsorbed to a gold-coated QCM sensor. The cluster size and density of carbohydrates on the dendrimers' surface were controlled by using different sizes of dendrimers (G4, G5, and G7) and different concentrations of carbohydrate/cyanuric chloride solution. Analysis of the binding between the GlcNAc–C12–NH_2-immobilized surface and WGA revealed that the size of the PAMAM dendrimers influenced the GlcNAc–C12–NH_2–WGA interaction, with larger dendrimers resulting in higher WGA binding constants.

This immobilization method allows for simple introduction of amino groups on the QCM sensor surface. It is a useful technique for molecular interaction and biosensing studies. In addition, this method allows for the fabrication of carbohydrate clusters that mimic those found on cell membrane surfaces, and it offers the potential to elucidate the effect of multivalent carbohydrate–protein interactions on a cell membrane surface at a molecular level.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jcis.2012.10.056.

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