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ChemComm
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Enzymatic preparation of biotinylated naturally-occurring sialylglycan and its molecular recognition on a quartz-crystal microbalance†

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Received (in Cambridge, UK) 20th July 2004, Accepted 11th October 2004
First published as an Advance Article on the web 29th October 2004

A biotinylated sialylglycan was prepared enzymatically by *endo*-M, and binding behavior of an SSA lectin was studied on a different coverage of a sialylglycan-immobilized 27 MHz quartz-crystal microbalance (QCM).

Glycoconjugates such as glycolipids and glycoproteins on cell membranes play an important role of receptor to lectins, toxins and viruses in living systems, and are significantly related to the formation of organized systems such as cell adhesion, differentiation and morphogenesis.[1,2] In order to elucidate the function of glycoconjugates on the cell surface, it is necessary to design a suitable glyco-microarray. However, the immobilization of carbohydrates on a substrate or chip due to complexed structures of glycoconjugates is still unexploited compared with that for nucleic acids and proteins.[3,4] The simple way for this purpose is to immobilize directly naturally-occurring glycoconjugates such as glycoproteins and glycolipids on a substrate; however, this is not suitable for studying the role of the carbohydrate itself such as in molecular recognition. It was developed to immobilize only the synthetic sugar chains on a substrate by using chemical methods such as Diels–Alder reactions and activated esterification;[5,6] however, it required complicated chemical treatments such as the selective protection and de-protection of certain functional hydroxyl groups. It is also common to achieve the immobilization through a Schiff's base linkage using an aldehyde group of a reducing end of a carbohydrate with an amine or hydrazide group;[2] however, this method has a disadvantage of collapsing the reducing end.

In this communication, we have prepared a biotinylated naturally-occurring sialylglycan by enzymatic transglycosylation of *endo*-β-acetylglucosaminidase from *Mucor hiemalis*, as shown in a step (a) of Fig. 1. The biotinylated sialylglycan was immobilized on an avidin-covered 27 MHz quartz-crystal microbalance (QCM) [step (b)]. SSA lectin from *Sambucus sieboldiana* could recognize specifically the sialyl(α2-6)Gal/GalNAc sequence independent of the surface coverage (5–80%) of the carbohydrate [step (c)]. The 27 MHz QCM is known as a mass measuring device, which has been calibrated as the 1 Hz frequency decrease corresponding to the 0.62 ng cm^{-2} mass increase on the QCM electrode in the aqueous solution as well as in the air phase.[7–9] The calibration of the 27 MHz QCM in the aqueous solution is described in the ESI†, as compared with in the air phase.

In the preparation of the biotinylated sialylglycan, we chose *endo*-β-*N*-acetyl glucosaminidase from *Mucor hiemalis* (EC 3.2.1.96) (*endo*-M) as an enzyme, which is a unique endoglycosidase for the hydrolysis of *N*,*N*′-diacetylchitobiosyl linkages in oligosaccharides bound to asparaginyl residues of various glycoproteins to leave one *N*-acetylglucosamine residue on the protein moieties.[10–12] *Endo*-M was found to hydrolyze not only the high mannose type but the complex type and the hybrid type of asparagine-linked oligosaccharides.[12,13] Furthermore, *endo*-M also showed transglycosylation activity.[12–18] Sialylglycopeptide (SGP) was separated from hen's egg yolk according to a previous method.[18] SGP (340 μg, 12 mM) was reacted with biotinylated GlcNAc (38 μg, 2.1 mM) in the presence of *endo*-M (30 mU in 30 μL) in 10 mM phosphate buffer, pH 6.0 at 37 °C [step (a) in Fig. 1]. The biotinylated sialylglycan could be prepared in 55–60% yield after 5 h of reaction, and it was purified by HPLC (C_{18} column, eluent: CH_3CN containing trifluoroacetic acid) and confirmed by MALDI-TOF MS (matrix: DHB, $+Na^+$: 2619.4, calibrated with insulin). The yield was comparable to that of the transglycosylation to glucose and aliphatic alcohols as acceptors (30–50%).[10]

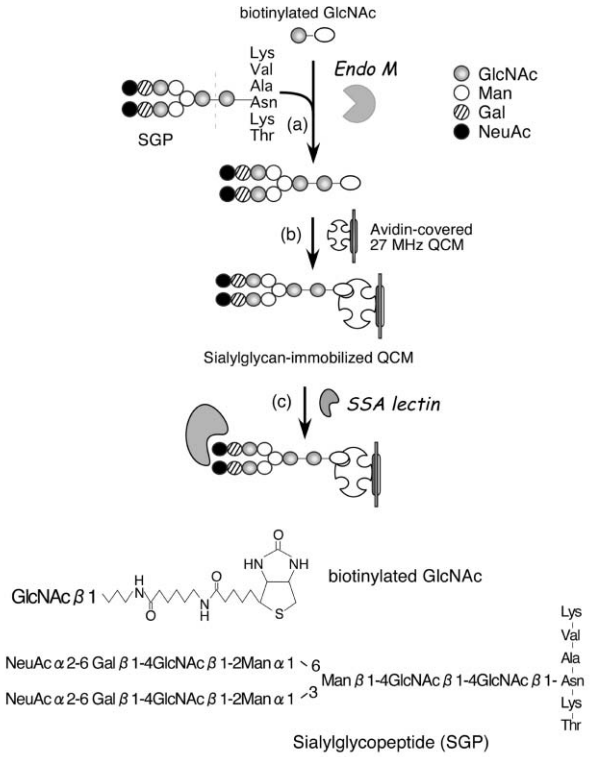

Fig. 1 Schematic illustrations of: (a) preparation of a biotinylated sialylglycan from a biotinylated GlcNAc and sialylglycopeptide (SGP) catalyzed by *endo*-M; (b) immobilization of the biotinylated sialylglycan on a NeutrAvidin-covered 27 MHz quartz-crystal microbalance (QCM); and (c) binding of SSA lectin to the sialylglycan surface on the QCM.

On an Au electrode (4.9 mm^2) of a 27 MHz QCM plate (8 mm diameter) of AffinixQ,[4] NeutrAvidin was immobilized as a monolayer coverage through an amine coupling with dithiopropionic acid according to previous methods.[7–9] NeutrAvidin was chosen as a biotin-binding protein because carbohydrates on the avidin have been removed to reduce non-specific binding. When an aqueous solution of sialylglycan was injected to the

† Electronic supplementary information (ESI) available: 27 MHz QCM setup and its calibration, and Fig. S1 showing time-courses of sialylglycan binding experiments. See http://www.rsc.org/suppdata/cc/b4/b411082j/

DOI: 10.1039/b411082j

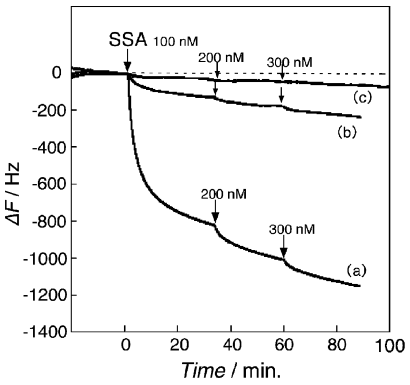

Fig. 2 Typical time-courses of frequency decreases (mass increases) of: (a) the sialylglycan-, (b) GlcNAc-, and (c) NeutrAvidin-immobilized QCM, responding to continuous additions of SSA lectin (*ca.* 600 nM). [Sialylglycan] = 410 ng cm^{-2}, [GlcNAc] = 410 ng cm^{-2}, 10 mM phosphate buffer, pH 7.2, 150 mM NaCl, 25 °C.

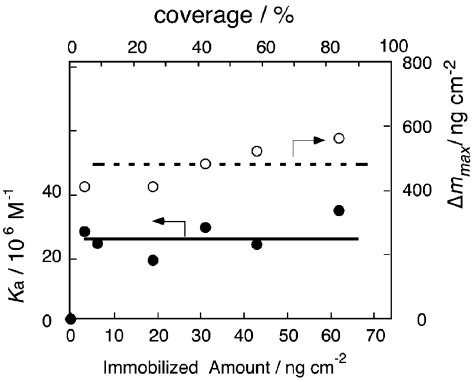

Fig. 3 Maximum binding amount (Δm_{max}) and binding constant (K_a) of SSA lectin to the sialylglycan-immobilized QCM, depending on the immobilized amount (surface coverage). 10 mM phosphate buffer, pH 6.0, 150 mM NaCl, 25 °C.

NeutrAvidin-covered QCM plate, the frequency decreased (mass increased) depending on the concentration of sialylglycan (10–600 nM), and the immobilized amount could be controlled to be 5–60 ng cm^{-2} (1.9–23 pmol cm^{-2}) on the QCM through biotin–avidin linkages [step (b) in Fig. 1]. This corresponds to approximately 3–60% coverage of the NeutrAvidin surface on the QCM. In the presence of an excess amount of biotin in the solution, the biotinylated sialylglycan did not bind to the NeutrAvidin-immobilized QCM, and the non-biotinylated sialylglycan also hardly bound to the NeutrAvidin surface (see ESI Fig. S1†).

The state and orientation of the sialylglycan on the QCM chip was confirmed by binding of SSA lectin from *Sambucus sieboldiana*, which is known to recognize specifically the NeuAc(α2-6)Gal/GalNAc sequence of carbohydrate chains of glycoconjugates [step (c) in Fig. 1].[19–21] As shown in Fig. 2, when SSA lectin was injected to the sialylglycan surface (5–80% coverage) on the QCM, the frequency decreased (mass increased) with saturation behavior. SSA lectin bound weakly to the GlcNAc surface and NeutrAvidin surface due to the non-specific binding to the surface. Thus, SSA lectin specifically recognizes the surface of NeuAc(α2-6)Gal/GalNAc of the immobilized sialylglycan.

From saturation binding measurements, the maximum binding amount (Δm_{max}) and binding constant (K_a) could be obtained according to eqn. 1,[19–21]

$$\frac{[SSA]_0}{\Delta m} = \frac{1}{\Delta m_{max}}[SSA]_0 + \frac{1}{\Delta m_{max} K_a} \quad (1)$$

where $[SSA]_0$ is the initial concentration of SSA lectin. Values for Δm_{max} and K_a were obtained at different immobilized amounts of sialylglycan on the QCM, and the results are shown in Fig. 3. Both K_a and Δm_{max} values were independent of the immobilized amount of sialylglycan (5–80% of surface coverage). K_a value was obtained as $2.5 \pm 0.4 \times 10^7$ M^{-1}, which is similar to $K_a = 6.7 \times 10^6$ M^{-1} obtained for SSA lectin binding to the NeuAc(α2-6)Gal(β1-4)Glc moiety by a surface plasmon resonance method,[19] and $K_a = 9.5 \times 10^6$ M^{-1} for SSA lectin binding to glycolipid monolayers having NeuAc(α2-6)Gal headgroups.[20] The slightly low K_a value of 3.4×10^5 M^{-1} was obtained in the bulk solution.[21]

The maximum binding amount of SSA lectin was estimated to be 410 ng cm^{-2} as a monolayer adsorption of globular proteins of M_w = 160 kDa, which is consistent with the obtained value of $\Delta m_{max} = 480 \pm 40$ ng cm^{-2}. Thus, SSA lectin can recognize the NeuAc(α2-6)Gal(β1-4)GlcNAc sequence of sialylglycan independent of the surface coverage of 5–80%. SSA lectin is an A_2B_2-type tetramer and a sectional area is roughly calculated to be 16 nm^2. This is very large relative to that of sialylglycan (0.6–0.8 nm^2), and *ca.* 30 sialylglycan molecules are calculated to be covered under one SSA lectin binding. This means that the existence of less than 3% of sialylglycan is enough to bind SSA lectin as a monolayer adsorption.

In conclusion, we can prepare the biotinylated naturally-occurring sialylglycan by enzymatic transglycosylation in a high yield, and the state or orientation of sialylglycan can be assayed by using a QCM technique. We believe that the combination of the biotinylation of naturally-occurring carbohydrates by the *endo*-M enzyme and the QCM chip assay will be very useful for the elucidation of roles of carbohydrates and studies on glycomics.

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