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High-Sensitivity Analysis of Naturally Occurring Sugar Chains, Using a Novel Fluorescent Linker Molecule

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To analyse the binding of sugar chains to proteins, viruses and cells, the surface plasmon resonance (SPR) technique is very convenient and effective because it is a real-time, non-destructive detection system. Key to this method is linker compounds for immobilization of the sugar chains to the gold-coated chip for SPR. Also, well-designed fluorescent labelling reagents are essential when analysing the structure of trace amounts of sugar chains derived from natural sources, such as glycoproteins on the surface of specific cells. In this report, we developed a novel linker molecule, named 'f-mono', which has both of these properties: simple immobilization chemistry and a fluorescent label. Since the molecule contains a 2,5-diaminopyridyl group and a thioctic acid group, conjugation with sugar chains can be achieved using the well-established reductive amination reaction. This conjugate of sugar chain and fluorescent linker (fluorescent ligand-conjugate, FLC) has fluorescent properties (ex. 335 nm, em. 380 nm), and as little as 1 μg of FLC can be easily purified using HPLC with a fluorescent detector. MS and MS/MS analysis of the FLC is also possible. As a +2 Da larger MS peak ($[M+H+2]^+$ ion) was always associated with the theoretical MS peak ($[M+H]^+$) (due to the reduction of the thioctic acid moiety), the MS peaks of the FLC were easily found, even using unfractionated crude samples. Immobilization of the FLC onto gold-coated chips, and their subsequent SPR analyses were successively accomplished, as had been performed previously using non-fluorescent ligand conjugates.

Key words: immobilization, sugar chain, high sensitivity, analysis, fluorescence, linker molecule, mass spectrometry.

Abbreviations: DMAc, N, N-dimethyl acetoamide; aoWS, N^{α}-((aminooxy)acetyl)tryptophanylarginine methyl ester.

The carbohydrates that make up proteoglycans, glycoproteins or glycolipids are responsible for many biological functions and play crucial roles in cellular binding and signalling (*1*). However, because of their structural complexity, the methods for studying sugar chains are more challenging than that for DNA, RNA or proteins. The numerous isomeric and anomeric configurations of sugar chains, as well as the difficulties in isolating sufficient quantities of naturally occurring sugars, make binding analysis and structure–function studies challenging.

For the structural analysis of naturally occurring sugar chains, fluorescent labelling of the sugars has been one popular technique (*2*). Recently, mass spectrometry (MS) has been used for structural analysis of sugar structures, thanks to the development of structurally well-defined standards (*3*, *4*). Surface plasmon resonance (SPR) methodology is also a very effective method to quantify binding interactions between sugar-chains and lectins or viruses in real time, because it is a non-destructive technology that does not require large quantities of the often scarce materials to be studied (*5–9*). We have previously reported the development of the 'sugar chip', in which defined sugar chains are immobilized on an SPR sensor chip using our specialized linker molecules (*10*, *11*). But the purification of these linker-carbohydrate conjugates for SPR has been difficult when the quantities of the target sugar chains were limited (*i.e.* <1 mg). To overcome this and the other challenges in the analysis of scarce sugar chains, we have developed a novel carbohydrate linker molecule that is also fluorescent (named 'f-mono'). Here we report the successful synthesis of this novel fluorescent linker molecule, and the preparation and purification of conjugates (fluorescent ligand-conjugate or FLC) using as little as 1 μg of sugar chain. These FLCs were then effectively employed in SPR analysis of carbohydrate–protein binding, as well as MS and MS/MS structural analyses.

MATERIALS AND METHODS

General Procedure—All reactions in organic media were carried out with freshly distilled solvents or with

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commercially available extra grade solvents purchased from Kanto Chem. Co. (Tokyo, Japan), Nacalai Tesque (Kyoto, Japan) or Wako Chem. Co. (Osaka, Japan). Silica gel column chromatography was performed using PSQ 60B (Fuji Silysia Chem. Ltd., Aichi, Japan). Electrospray ionization time-of-flight mass (ESI-TOF/MS) spectra were obtained by Mariner™ (Applied Biosystems, Framingham, MA, USA). ^{1}H-NMR measurements were performed with JEOL (Tokyo, Japan) ECA-600.

Synthesis of f-mono linker—2,6-Diaminopyridine (1.06 g, 9.70 mmol, Sigma, USA) and thioctic acid (1.00 g, 4.80 mmol, Sigma, USA) were dissolved in anhydrous *N, N*-dimethylformamide (10 ml). Then, 1-hydroxy-7-azabenzotriazole (HOAt, 0.66 g, 4.80 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide monohydrochloride (EDC-HCl, 0.93 g, 4.80 mmol), and diisopropylethylamine (DIEA, 0.84 ml, 4.80 mmol) were added to the solution. After stirring for 6 h under argon gas, the reaction product was extracted into the organic phase using dichloromethane (CH_2Cl_2, 20 ml), and was washed with water (10 ml) three times and then with saturated sodium bicarbonate aqueous solution. The product was then purified by silica gel column chromatography (80 g, eluted with toluene/ethyl acetate = 3/1, v/v) to obtain a yellow solid. Yield: 1.37 g (95%). MS calcd. for $C_{13}H_{19}N_3OS_2$: 297.10, Found: *m/z* 298.12 $[M+H]^+$; ^{1}H NMR (600 MHz, CDCl3), δ7.58 (1H, s), 7.53 (1H, d, J = 7.5 Hz), 7.46 (1H, t, J = 7.6, J = 8.2 Hz), 6.26 (1H, d, J = 8.2 Hz), 4.24 (1H, m), 3.59–3.56 (1H, m), 3.19–3.10 (2H, m), 2.48–2.44 (1H, m), 2.37–2.34 (2H, m), 1.93–1.90 (1H, m), 1.77–1.68 (4H, m), 1.53–1.48 (2H, m).

Preparation of a Conjugate with Lactose—Lactose monohydrate (20 mg, 56 μmol) and f-mono (18 mg, 61 μmol) were dissolved in a 2.2 ml solution of H_2O/AcOH/DMAc = 5/1/5 (v/v/v). After stirring for 5 h, sodium cyanoborohydrate (17 mg, 280 μmol) was added to the solution. The reaction mixture was left standing at 37°C for 1.5 days and lyophilized. The residue was dissolved in water and purified with an ODS column (20 g, 1.8 cmΦ × 46 cm, eluted with water/methanol = 1/1, v/v). The appropriate fraction was lyophilized with water to obtain the desired final product: fluorescent ligand-conjugate (FLC, abbreviated as Galβ1-4Glc-f-mono) as a white powder. Yield: 19 mg (50%). MS calcd. for $C_{25}H_{41}N_3O_{11}S_2$: 623.17, Found: m/z 624.17 $[M+H]^+$; ^{1}H NMR (600 MHz, MeOD), δ 7.31 (1H, d, J = 8.1 Hz), 6.99 (1H, d, J = 2.0 Hz), 6.20 (1H, d, J = 8.0 Hz), 4.28 (1H, d, H-1′), 3.89 (1H, dd, H-4), 3.75 (2H, m, H-2, H-5), 3.63 (3H, m, H-3, H-6a, H-6b), 3.52 (1H, m), 3.45 (1H, m, H-4′), 3.30 (2H, m, H-3′, H-5′), 3.25–3.14 (2H, m, H-1a,H-2′), 3.05–2.94 (3H, m, H-1b), 2.36 (1H, m), 2.26 (2H, t, J = 7.3 Hz), 1.84–1.76 (1H, m), 1.54–1.35 (6H, m).

Preparation of a Conjugate with Maltose—Maltose (20 mg, 56 μmol) and f-mono (18 mg, 61 μmol) were dissolved in a 2.2 ml solution of H_2O/AcOH/DMAc = 5/1/5 (v/v/v). After stirring for 5 h, sodium cyanoborohydrate (17 mg, 280 μmol) was added to the solution. The reaction mixture was left standing at 37°C for 1.5 days, and lyophilized. The residue was dissolved in water and purified with ODS column (20 g, 1.8 cmΦ × 46 cm, eluted with water/methanol = 1/1, v/v). The appropriate fraction was lyophilized with water to obtain Glcα1-4Glc-f-mono as a white powder. Yield: 17 mg (46%). MS calcd. for $C_{25}H_{41}N_3O_{11}S_2$: 623.17, Found: m/z 624.17 $[M+H]$+; ^{1}H NMR (600 MHz, MeOD), δ 7.32 (1H,d), 6.99 (1H, d), 6.20 (1H, d), 4.95 (1H, s, H-1′), 3.89–3.78 (3H, m, H-2, H-4, H-5), 3.63 (3H, m, H-3, H-6a, H-6b), 3.42 (1H, m), 3.38 (1H, d, H-4′), 3.13 (2H, m, H-3′, H-5′), 2.85–2.3 (5H, m, H-1a,H-2′), 3.05–2.94 (3H, m, H-1b), 2.36 (1H, m), 1.74–1.26 (7H, m).

SPR Analysis—SPR experiments were perfomed with a 12-channel SPR machine (Moritex Co., Yokohama, Japan) using the manufacturer's recommended guidelines with slight modification. Sensor chips used for SPR experiments were prepared as follows. The gold-coated chip was purchased from SUDx-Biotec (Kagoshima, Japan), and washed in ozone cleaner. The chip was soaked in a 10-, 1-, or 0.1-μM solution of Galβ1-4Glc-f-mono or Glcα1-4Glc-f-mono, dissolved in methanol/water = 1/1 (v/v) at room temperature for 2 h or overnight, followed by subsequent washing with a methanol/water (1/1, v/v) containing 0.05% Tween-20, phosphate-buffered saline (PBS) at pH 7.4 containing 0.05% Tween-20, and PBS (pH 7.4). All washings were done with ultra-sonication for 20 min.

Binding studies were performed between test proteins in the aqueous phase and the stated sugars immobilized via fluorescent linkers (f-mono) attached to the sugar chips. The test proteins concanavalin A (Con A, EY Laboratories, San Mateo, CA, USA), RCA120 (Ricinus Communis Agglutinin I, Vector Laboratories, Servion, Switzerland), and bovine serum albumin (BSA, Nakalai Tesque) were perfused in the aqueous phase (PBS with 0.05% Tween-20 at pH 7.4) at a flow rate of 15 μl/min at 25°C.

Fluorescent Spectra—Fluorescent spectra were measured with a Spectro Fluorometer FP-6310 (JASCO, Tokyo, Japan). The concentration of f-mono was 100 μg/ml in $CHCl_3$. For comparison, our previous mono-valent non-fluorescent linker molecule [abbreviated as 'mono' in this paper (*11*)] was dissolved in $CHCl_3$ at 100 μg/ml, and used as a control.

Mass Spectrometry—MS and MS/MS spectra of FLCs were obtained with an AXIMA-QIT (Shimadzu, Kyoto, Japan), which is a quadrupole ion trap and matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (QIT-MALDI-TOF/MS). Acquisition and data processing were controlled by the manufacturer's software (Kratos Analytical, Manchester, UK). For matrix, a purified 2,5-dihydroxybenzoic acid (DHBA) was dissolved in a mixed solvent with double distilled water containing 0.1% TFA/acetonitrile = 3/1 (v/v) at 10 mg/ml. To 1 μl of sample dissolved in the above mixed solvent spotted on a stainless-steel target, an equal volume of matrix solution was placed and allowed to dry.

Preparation of f-mono-Labelled Glycans from Human IgG—One hundred micrograms of human IgG (Institute of Immunology Co., LTD., Tokyo, Japan) was dissolved in 5 μl of H_2O and 5 μl of 1 M aqueous NH_4HCO_3, and 5 μl of 120 mM aqueous dithiothreitol were added. The reaction solution was heated at 60°C for 30 min. Then, 10 μl of 123 mM aqueous iodoacetamide was added. After incubation in the dark at room temperature for an hour, 10 μl of

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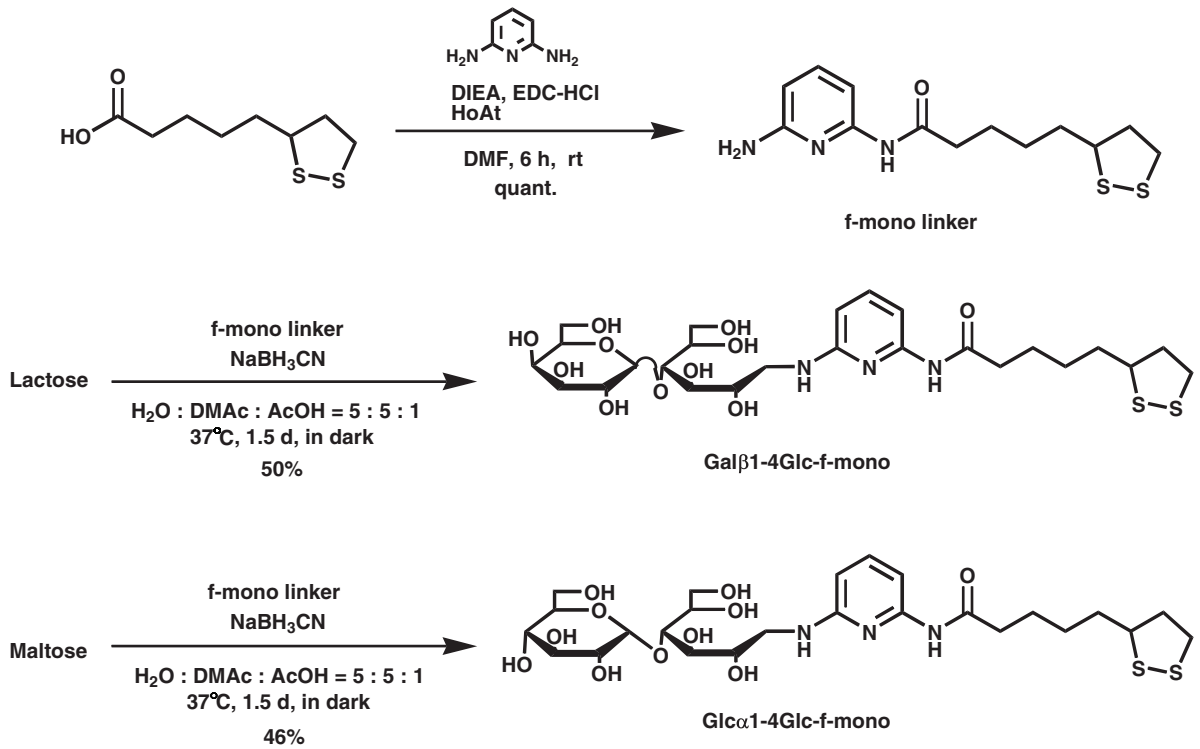
trypsin (Sigma-Aldrich, 40 U/μl, dissolved in 1 mM HCl) was added. After an hour, trypsin was inactivated by heating at 90°C for 5 min. Then, 10 U of PNGase F (Roche, Switzerland) was added to the solution (*12*). After incubating at 37°C for 12 h, the solution was lyophilized.

The lyophilized residue was dissolved in 20 μl of H_2O, and concentrated by Blot Glyco (Kit No. MALDI type BS-45601S, Sumitomo Bakelite Co., Ltd. Tokyo, Japan) (*13*, *14*). At the final stage, using the manufacturer's guideline, N-glycans from IgG were released in H_2O, lyophilized and transformed to fluorescent ligand conjugates as follows. The mixture of naturally occurring lyophilized N-glycans and f-mono (100 mg, 340 μmol) were dissolved in 1.0 ml of a mixed solvent (H_2O/AcOH/DMAc = 5/1/5, v/v/v). After 5 h, sodium cyanoborohydrate (62 mg, 1.0 mmol) was added to the solution. The reaction mixture was left standing at 37°C for 1.5 days and lyophilized. To the residue, 200 μl of H_2O was added. Then, the aqueous phase was washed three times with 200 μl of phenol/$CHCl_3$ (1:1, v/v). The aqueous layer was concentrated in vacuo, and excess f-mono and other chemical reagents were removed using an ODS short column attached to the kit. For comparison, the N-glycans of IgG were transformed to the 'sugar-aoWRs condensation product (*15*)' according to the manufacturer's manual. The labelled N-glycans were examined by HPLC (Pump: L-6200, HITACHI, Tokyo, Japan; Detector: FP 2020, JASCO, Tokyo, Japan; Column: COSMOSIL 5C_{18}-PAQ Waters, Nacalai Tesque; Elution: H_2O/MeOH = 1/1, v/v), and by mass spectrometry as described above.

RESULTS AND DISCUSSION

Introducing fluorescence into the linker was accomplished by replacing the 2,6-diaminobenzene unit of our original linker molecule (mono) (*11*), with a 2,6-diaminopyridine moiety (Scheme 1). The labelling of sugar chains using 2-aminopyridine (PA) reported by Hase *et al*. (*16*) was a pioneering advance for the analysis of trace amounts of sugar-chains, and has been applied to 2- or 3-dimensional mapping by Takahashi *et al*. (*17*, *18*) for the conventional structural identification of sugar chains from natural sources, such as glycoproteins. The high fluorescence of the 2,6-diaminopyridine moiety has also been well established, and its use for the biotinylation or immobilization of sugar chains has been reported (*19*, *20*). As expected, our novel f-mono linker molecule showed fluorescence at an excitation (ex) maximum of 335 nm and an emission (em) maximum of 380 nm. Since the sensitivity of detection of fluorescence is about 1,000 times higher than that of UV/VIS, a small quantity (~1 nmol) of sugar chain can be effectively derivatized by using this f-mono linker molecule. In addition, the molecular absorption coefficient (ε value) of f-mono was five times higher than that of the original linker molecule from which it was derived, indicating increased sensitivity even with a standard UV/VIS detector.

Figure 1 shows the SPR data of Con A, RCA120 and BSA binding to α-glucose or β-galactose immobilized *via* FLCs prepared with the f-mono linker to the sensor chip. BSA was used as a negative control, because our previous investigation (*21*) showed that it does not bind to



Scheme 1. **Synthesis of f-mono linker and preparation of ligand conjugates, Galβ1-4Glc-f-mono and Glcα1-4Glc-f-mono.**

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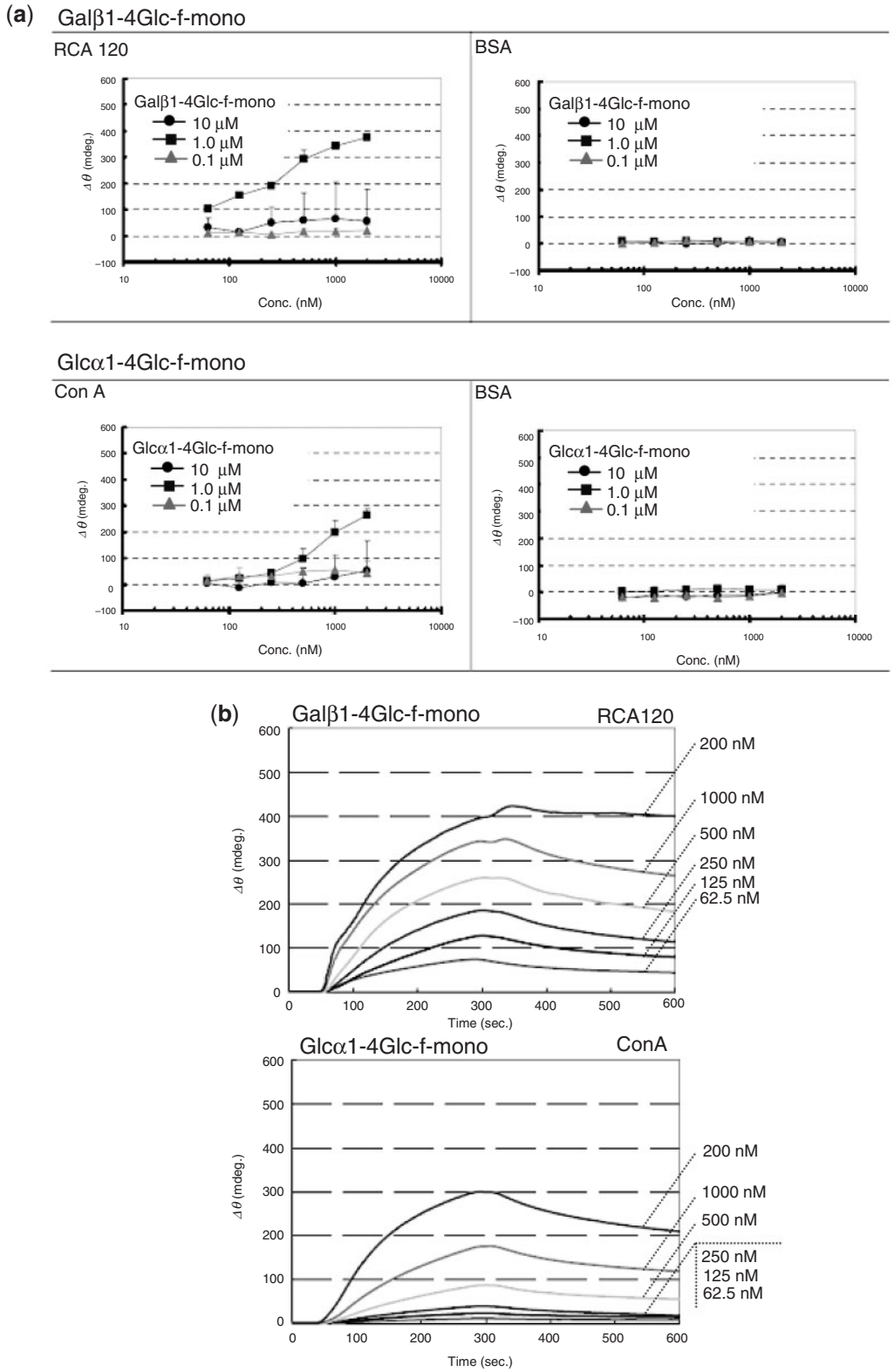

Fig. 1. **SPR analysis of lectins (Con A and RCA120) binding to defined sugars.** Galβ1-4Glc-f-mono or Glcα1-4Glc-f-mono were immobilized on gold-coated chips (see MATERIALS AND METHODS section for details). The test proteins were perfused in the aqueous phase (PBS with 0.05% Tween-20 at pH 7.4) at a flow rate of 15 μl/min at 25°C using a 12-channel SPR machine (Moritex Co., Tokyo, Japan). (a) Dependency of the lectin binding on concentration of FLCs immobilized on the chip. (b) SPR sensor grams of RCA120 for the Galβ1-4Glc-f-mono chip, and Con A for the Glcα1-4Glc-f-mono chip, immobilized at 1 μM.

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α-glucose or β-galactose. Figure 1(a) illustrates the dependency of protein binding on the density of immobilization of the sugar chain *via* FLCs on the chip. The data suggest that the optimal density for immobilization of both FLCs appears to be ~1 μM. At higher concentrations, steric hindrance due to the high concentration of ligands may occur and prevent the binding of protein. At 0.1 μM of FLC the ligand sugar chains may be too diluted on the chip to effectively bind protein, because of non-clustered ligands.

Using the chip immobilized with Galβ1-4Glc-f-mono, it was detected that RCA120 bound, but Con A and BSA did not. In contrast, using the chip with Glcα1-4Glc-fmono, Con A bound, but RCA120 and BSA did not. The binding of BSA to the sensor chips was negligible.

Figure 1(b) shows typical sensorgrams of RCA120 and Con A binding to the appropriate sugar chip. The calculated binding parameters were in agreement with those in the literatures (*22*, *23*) and with our previous data using a non-fluorescent linker molecule. The kinetic parameters were; RCA120 *vs*. Galβ1-4Glc-f-mono, $k_{on} = 6.3 \times 10^3\ M^{-1}\ s^{-1}$, $k_{off} = 4.1 \times 10^{-3}\ s^{-1}$, $K_D = 0.66\ \mu M$; Con A *vs*. Glcα1-4Glc-f-mono, $k_{on} = 2.5 \times 10^3\ M^{-1}\ s^{-1}$, $k_{off} = 3.1 \times 10^{3}\ s^{-1}$, $K_D = 1.2\ \mu M$.

The results of MS and MS/MS analyses of Galβ1-4Glc-f-mono are shown in Fig. 2. A set of two unique peaks was detected. In addition to the regular $[M+H]^+$ and

Fig. 2. **MS and MS/MS analyses of Galβ1-4Glc-f-mono.** A quadrupole ion trap and matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (AXIMA-QIT) was used (see MATERIALS AND METHODS section for details). (a) MS spectrum of Galβ1-4Glc-f-mono; (b) MS/MS analysis of Galβ1-4Glc-f-mono.

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$[M+Na]^+$ ion, 2-Da bigger peaks ($[M+H+2]^+$ and $[M+Na+2]^+$) were found [Fig. 2(a)]. These later peaks were derived from the reduction of the disulphide bond in the thioctic acid moiety of f-mono, since DHB (the matrix for MALDI) tends to reduce samples with the laser energy (*24*). This property of the f-mono linker was very useful for distinguishing MS peaks of f-mono-labelled glycans from contaminating peaks. In the MS/MS analysis, peaks lacking a galactose unit and thioctic acid from the precursor ion (m/z 624) were observed [Fig. 2(b)]. The cleavage here was as simple in the MS/MS analysis as that using PA-labelled sugar chains (*3*), facilitating structural analysis. For analysing structure and identifying specific sugars, the f-mono linker greatly enhanced the ability to recognize the labelled glycans. From these results, it is suggested that our f-mono linker is a highly effective reagent for MS analysis, at least in a system employing MALDI-QIT and DHBA.

Next, the N-glycans of human IgG were analysed using f-mono. As described in MATERIALS AND METHODS section, N-glycans were extracted from human IgG, concentrated, and then reacted with f-mono. Figure 3 shows the HPLC profile. Two fractions were collected and analysed using MS and MS/MS to confirm f-mono-labelled N-glycans (Fig. S1). From the MS and MS/MS, 162 or 203 different peaks were obtained, suggesting the carbohydrate-derived compounds. In addition, the f-mono labelled glycans were quite easily visualized as +2-Da differentially larger peaks in MS. From the calibration curve (Fig. S2) prepared with Galβ1-4Glc-f-mono, 518 pmol of labelled compounds were estimated to obtain from 100 μg of IgG using the HPLC results, and the detection limit in our HPLC system was estimated to be 5 pmol in 10 μl of injected sample solution.

For comparison, the released N-glycans were also labelled with a reagent (aoWRs) from the kit for MS.

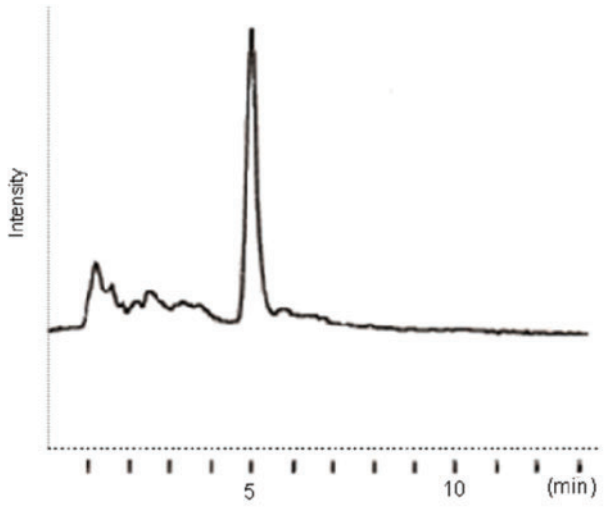

Fig. 3. **HPLC profile of f-mono labelled N-linked sugar chains from IgG.** The extraction of N-glycan and its labelling are described in the MATERIALS AND METHODS section. The conditions of HPLC were as follows. Column: COSMOSIL $5C_{18}$-PAQ Waters (Nacalai Tesque, 4.6 × 150 mm); elution: methanol/water = 1/1 (v/v).

By HPLC analysis, aoWRs-labelled N-glycans did not reveal any detectable peaks because aoWRs had no fluorescence. The MS data are summarized in Fig. 4. The MS spectrum of aoWRs-labelled N-glycans showed six glycan peaks clearly [Fig. 4(a)]. But in the case of the f-mono-labelled glycans, four additional glycans were clearly detected in the MS spectrum [Fig. 4(b)]. Furthermore, the larger 2-Da ions were observed as described above. Figure 4(c) shows the MS/MS spectrum of m/z 1947.1 ions of f-mono-labelled N-glycans. A wealth of structural information could be obtained from this spectrum. For example, from this peak the structure of the glycan containing four hexosamines and five hexoses was easily disclosed. In contrast, the MS/MS analysis of aoWRs-labelled glycan was not possible (data not shown).

The fluorescent intensity of the f-mono reagent was about 1/3 compared to that of 6-aminopyridine (PA) at the same concentration. Therefore, as judged by fluorescent intensity alone, the f-mono reagent is less sensitive. PA-derivatized sugar chains can be analysed by MS, but the f-mono derivatized one can be more easily detected because +2 Da differentially larger peaks are seen every time. Therefore, the novelty of this f-mono reagent is not the improvement of fluorescent sensitivity, but the significant improvement in MS peak identification. Moreover, the derivatized and analysed sugar chain with f-mono can be immobilized on SPR sensor chip sequentially. That is, the extensive merit of this f-mono reagent is that a more-comprehensive and integrated analysis of sugar chains can be sequentially performed using HPLC, MS and SPR.

The difficulty of synthesizing rigorously, structurally defined sugar chains remains a significant challenge to structure–function studies of carbohydrates. Narimatsu and colleagues (*25*) have made important advances in the preparation of sugar-chains using glycosyltransferases, and this synthetic approach holds promise. However, at this time, it is still challenging to prepare large, complex sugar chains. Therefore, for structure–function analyses, approaches that are economical in their use of scarce sugar chains have advantages. One solution is the SPR sugar-chip approach we have illustrated here. By immobilizing the sugar chains on the chip one can use it multiple times. In this case, it is a key to have an efficient linker molecule for immobilization of trace amounts of the defined sugar chain, of which the f-mono linker molecule is a good example.

In conclusion, the f-mono linker molecule is easily synthesized and its ligand-conjugates are easily purified. The labelled glycans are able to be traced with HPLC because of their fluorescence, making this a good application for trace amounts of glycan (~1 pmol/μl). Furthermore, the labelled glycan can be used for binding experiments using SPR, as we have previously demonstrated with non-fluorescent linker-conjugates. MS and MS/MS analyses of f-mono-labelled glycan were possible and effective in determining the glycan's primary structure, because the larger 2-Da ion peaks could be used to distinguish the labelled glycan-derived peaks. With this novel linker molecule, both the structural and functional binding analysis of trace amount of glycans are greatly facilitated, suggesting that this fluorescent linker

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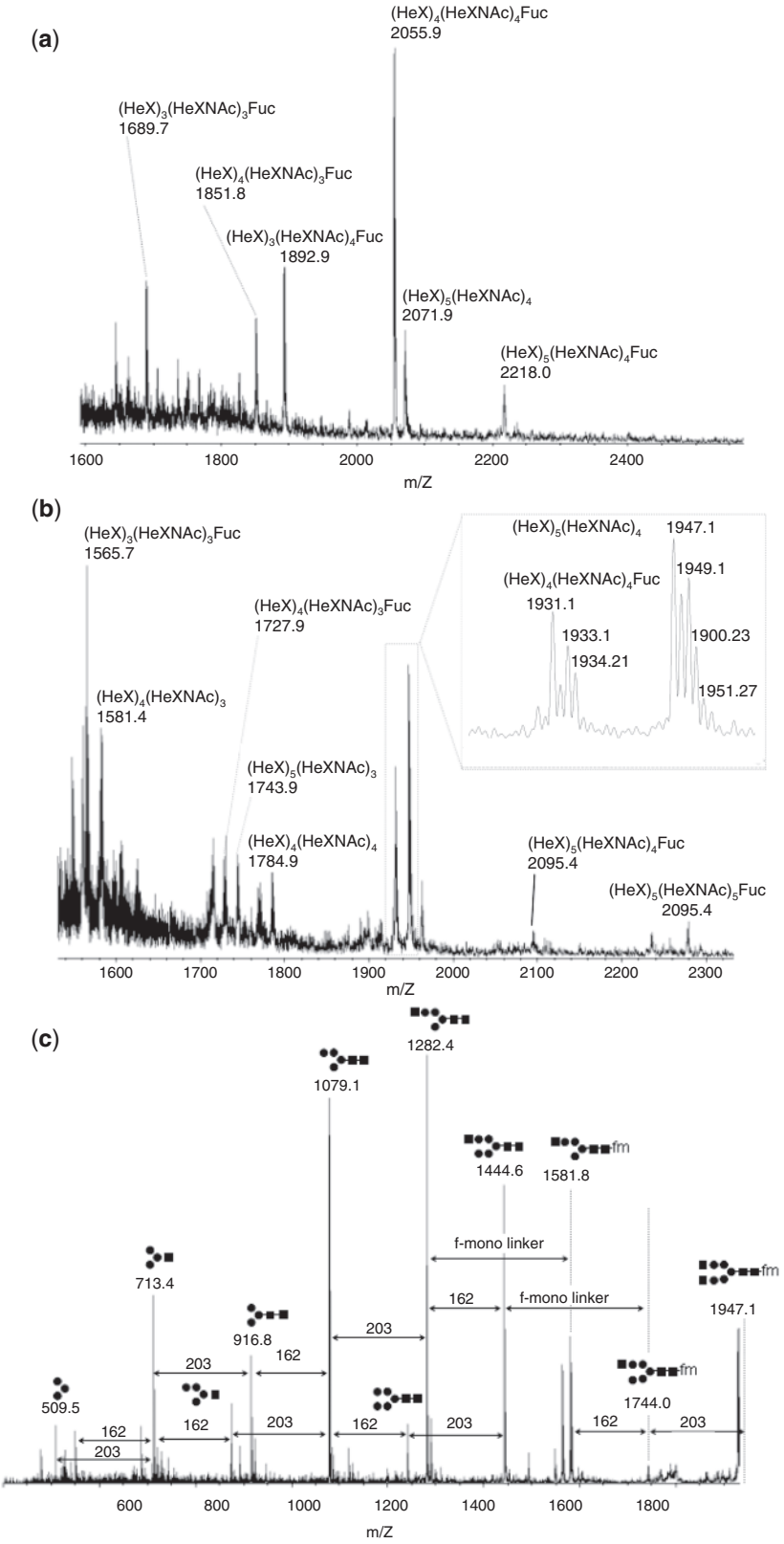

Fig. 4. **MS and MS/MS analyses of labelled N-linked sugar chains from IgG.** From 100 μg of human IgG, N-linked sugar chains were liberated by PNGase F, and concentrated by Blot Glyco (Sumitomo Bakelite Co., Ltd. Tokyo, Japan). The N-glycans were then released in H_2O, lyophilized and transformed to f-mono labelled or aoWRs conjugates as described in MATERIALS AND METHODS section. The labelled N-glycans were examined with mass spectrometry as described above. (a) aoWRs-labelled N-glycans. (b) f-mono labelled N-glycans. (c) MS/MS analysis of a peak ($m/z = 1497.1$) from the MS of f-mono labelled N-linked sugar chain from IgG.

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technology should be a useful tool in the study of proteoglycomics.

SUPPLEMENTARY DATA

Supplementary data are available at *JB* online.

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CONFLICT OF INTEREST

Nature of conflict of interest: Financial

Name of the author with conflict of interest: Y.S.

Entity and nature of the holdings: SUDx-Biotec Corporation

Amount of the holdings: 160/810 (19.753%)

Nature of conflict of interest: Management/Advisory Affiliations

Name of the author with conflict of interest: Y.S.

Nature of the relationships and financial arrangements: President and CSO of SUDx-Biotec Corporation. No financial arrangement.

Nature of conflict of interest: Patent

Name of the author with conflict of interest: M.S., Y.I., N.A., M.B., M.W. and Y.S.

Details and status: Japan Patent Submission #2008-108561 [Pending].

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