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Carbohydrate on the cell surfaces are known to interact not only with lectins but also other carbohydrates; the latter process is known as a carbohydrate-carbohydrate interaction. Such interactions are observed in complex oligosaccharides. It would be surprising if these interactions were observed in simple monosaccharide of mannose. In this study, the interaction between glycopolymers carrying monosaccharide of mannose was quantitatively investigated by quartz crystal microbalance measurements. We measured interactions with glycopolymer carrying mannose, galactose and glucose. Surprisingly, the interaction between the glycopolymers with mannose was much stronger than other sachcarides.

1. Introduction

Carbohydrates play important roles in various biological systems such as cell-cell adhesion, cell recognition, immune system, infection disease and cell differentiation.[1-4] In the glycoscience, most of the research focused on their specific interaction with carbohydrates–carbohydrate recognition proteins (lectins).[5,6] Hakomori *et al* found the carbohydarate-carbohydrate interactions (CCI) are also present in the living systems and play important roles in the living systems.[2] However, the role of CCI in biological system is not completely clear, because CCI have been studied less than carbohydrate-lectin interactions. CCI have been reported to occur with specific combination of oligosaccharides, such as Lewis X, G_{g3}, and G_{M3}. It is believed that the CCI are related to specific biological events such as cell recognition of cancer cells, and that CCI are specific to the cell.[2,7,8] If CCI, which is expressed in complex oligosaccharides, is also present in monosaccharides, the implications for biology would be immense. But at the same time, CCI have not been investigated for a wide variety of saccharide

Mannose is frequently found in saccharides in living systems. For example, high mannose-type *N*-glycans function as a quality control system and enable intracellular transportation of glycoproteins.[9] Mannose residues are abundant on the surfaces of pathogenic viruses and fungi and are intimately involved in pathogenicity and the host immune response in diseases.[10-15] Moreover, various immune cells such as macrophages and dendric cells, possess mannose-binding lectins, which play important roles related to immune response. Mannose is of high importance, and in particular, much attention has been given to the specific interaction of mannose with C-type lectins.[16,17] It would be surprising if CCI existed between simple mannose moieties.

The interactions of carbohydrates are usually weak, and the CCI is even weaker than the carbohydrate-lectin interaction, which means they are difficult to measure.[7,18] Glycoclusters, like glycolipids and glycoproteins play important roles in exhibiting CCI in the natural system. The artificial glycoclusters of glycopolymers are reported to much amplify the carbohydrate interaction. It has been reported that glycopolymers can show CCI based on the multivalent effect.[19-21] Recently, we discovered a self-assembling mannose cluster comprised glycopolymers in an aqueous solution, which suggested CCI in glycopolymers.[22] In this report, we prepared the biomimetic interface with glycopolymer-immoblized substrate to measure CCI between simple mannose clusters (a mannose carrying glycopolymer); we measured this quantitatively and compared it with other simple sugar clusters.

2. Experimental section

2-1 Characterization

^{1}H NMR spectra were recorded on a JNM-ECZ400 spectrometer (JEOL, Tokyo, Japan) using $CDCl_3$, d^6-DMSO, or D_2O as a solvent. Gel permeation chromatography (GPC) with organic solvent was performed on a HLC-8320 GPC Eco-SEC system equipped with a TSKgel Super AW guard column and TSKgel Super AW (4000, 3000, and 2500) columns (Tosoh, Tokyo, Japan). GPC with water solvent was performed on a JASCO DG-980-50 degasser equipped with a JASCO PU-980 pump (JASCO Co., Tokyo, Japan), a Shodex OHpak SB-G guard column, a Shodex OHpak LB-806 HQ column (Showa Denko, Tokyo, Japan), a JASCO RI-2031 Plus RI detector. GPC analyses were performed by injecting 20 μL of a polymer solution (1 g L^{-1}) in DMF buffer containing LiBr (10 mM) or $NaNO_3$ aqueous solution (100 mM). The buffer solution was also used as the eluent at a flow rate of 0.5 mL min^{-1}. The GPC was calibrated using a poly(methyl methacrylate) standard (Shodex) for organic solvent GPC and pullulan standard (Shodex) for aqueous GPC. UV-spectra were recorded on an Agilent 8453 spectrometer (Agilent Technologies Inc., Santa Clara, CA, USA). Dynamic light scattering (DLS) was performed on a Zetasizer Nano-ZS (Malvern, UK). DLS analyses were performed by using a 1 mL disposable cell of a polymer solution (1 g L^{-1}) in HEPES buffer solution with Ca^{2+} (pH 7.4). A 27 MHz quartz crystal microbalance (QCM) system (Affnix Q8, Ulvac Inc., Japan) was used to monitor the interaction.

2-2. General procedure for polymer preparation

The glycopolymers were prepared by the polymerization with reversible addition fragmentation chain transfer (RAFT) reagent (Scheme 1).[22] The polymers were prepared via polymerization of 3-(trimethylsilyl)prop-2-yn-1-yl methacrylate (TMS-PrMA) with RAFT reagent of CPADB and polyethyelene glycol (PEG) terminated chain transfer reagent. The saccharides of mannose (Man), galactose (Gal) and glucose (Glc) were added to the

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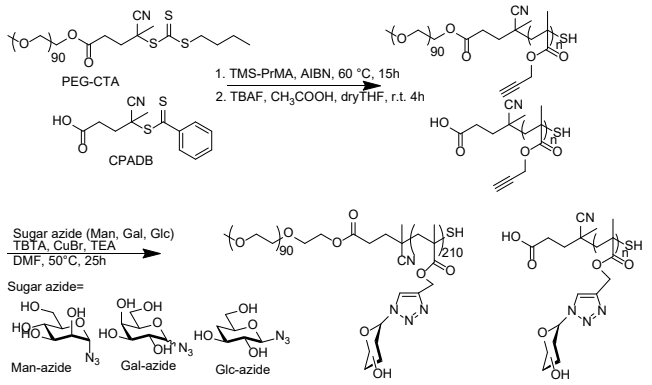
obtained polymer by Huisgen reaction. The polymers prepared were homo-glycopolymers and polyethylene glycol (PEG) terminated glycopolymers. The polymer terminals were converted to thiol to immobilize the gold substrate. The detail of the syntheses was described in supporting information.



Scheme 1 Preparation of glycopolymers in this study via RAFT polymerization and Huisgen reaction.

2-3 Glycopolymer immobilization on the QCM substrate

A 27 MHz QCM system was used to monitor the interaction. The QCM cell was cleaned with piranha solution (4 µL), and the solution was left for 10 mins and washed with Milli-Q (three cycle). In the last cycle, the gold surface was replaced with Milli-Q 10 times. The QCM cell was placed in the QCM apparatus and monitored with 100 µL of Milli-Q until the frequency was constant. Once the frequency was constant, 100 µL of each glycopolymer was added to bring the concentration in the cell to 10 g/L, and the cell was kept at 15 °C for 2 hours. To remove unfixed glycopolymer from the cell, the cell was washed with Milli-Q three times, then washed again with HEPES buffer and monitored until the frequency became constant.

2-4 Interaction measurement by QCM.

A 27 MHz QCM system was used to monitor the interaction. Glycopolymer was immobilized on QCM substrate as the previous section. After the frequency became constant, each PEG-terminated glycopolymer or PEG solution was added successively at 15 °C, and the cell was measured until the frequency became constant.

2-5 Turbidity measurement

Each glycopolymer was dissolved in HEPES buffer (HEPES: 10 mM, NaCl: 137 mM, KCl: 2.7 mM, $CaCl_2$: 1.8 mM) at 1 g $L^{-1.}$ After incubation at 15 °C for 48 h, the absorbance of 600 nm (was recorded.

2-6 X-ray photoelectron spectroscopy (XPS) measurement

XPS measurement was performed on a Shimadzu/Kratos AXIS-ultra (Kratos Analytical Ltd., Manchester, UK, Shimadzu Co., Kyoto, Japan) for the analysis of the glycopolymer immobilized. All XPS spectra energy referenced to the C (1s) photoemission peak at 285.0 eV. The substrates for XPS measurements were prepared by incubate aqueous solution (10 g/L) of glycopolymers for 2 h on the glass substrates coated Au (50 nm).

3. Results and Discussion

Fig. 1 (a) illustrates the method of measuring the interaction between glycopolymers by quartz crystal microbalance (QCM) measurements, the chemical structures and the abbreviations of the polymers used in this investigation (Fig. 1 (b)). **$pGal_{200}$**, **$pMan_{200}$**, and **$pGlc_{200}$** stand for glycopolymer carrying galactose, mannose, and glucose with degree of polymerization (D. P.) of 200, respectively. **$P_{90}Gal_{200}$**, **$P_{90}Man_{200}$**, and **$P_{90}Glc_{200}$** stand for PEG-terminated glycopolymers. Glycopolymers were synthesized via reversible addition fragmentation chain transfer (RAFT) polymerization (**TMS200)** and a Huisgen reaction. PEG-terminated block glycopolymers were prepared by the macro-RAFT with PEG (Fig. S4-4 and S4-6). After deprotection of the side chain of TMS group (Fig.S4-5 and S4-7), the sugar azide (Fig. S4-1 to S4-3) were incorporated into the polymer main chain by a Huisgen reaction (**$pMan_{200}$**, **$pGal_{200}$**, **$pGlc_{200}$**, **$P_{90}Man_{200}$**, **$P_{90}Gal_{200}$**, and **$P_{90}Glc_{200}$**).[22] All of the alkyne group at side chain was converted to sugar triazoles, which was confirmed by ^{1}HNMR (Fig.S4-8, and S4-9). The polymer terminals were converted to thiol at the deprotection of TMS group, which was confirmed by the decrease of UV absorbance at 305 nm (Fig. S6-1). Molecular weights were estimated by GPC measurements (S5-1 and S5-2).

The glycopolymer thin layer with **$pGal_{200}$**, **$pMan_{200}$**, and **$pGlc_{200}$** was formed by incubation on the gold electrode of QCM, and the substrate was rinsed with MilliQ water to remove the non-specific adsorbed polymer. The formation of glycopolymer layer was monitored by QCM and X-ray photoelectron spectroscopy (XPS) (Fig.2, S7-1, S8-1, S8-2 and S8-3).[23] Time course of frequency change in QCM indicated the adsorption of glycopolymer on Au-S, where the adsorption behaviour was different depending on the kind of polymer (Fig.2 and S7-1). The XPS was measured after the formation of polymer layer and the rinsing with water. In the XPS spectra, the decrease of Au(4f) and the strength of C(1s) peaks showed the formation of glycopolymer layer formation (Fig.2, S8-1, S8-2,. Although there were differences in the adsorption behaviour of each polymer by QCM, the XPS peaks were almost the same for all three polymers, suggesting that the thickness of the polymers on the gold substrate is almost the same regardless of the polymer. The weakly adsorbed glycopolymers on the substrate were removed by vigorous water rinsing. The thiol-terminated polymers were considered to form pancake like polymer monolayers due to the interaction between the gold substrate and the polymer. The thickness of the

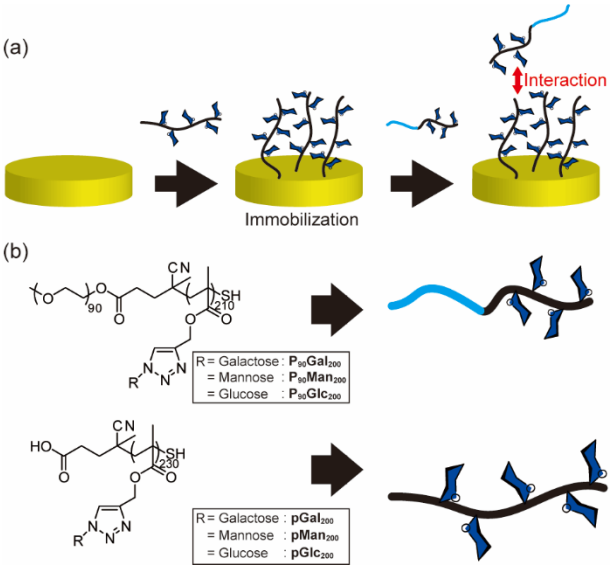
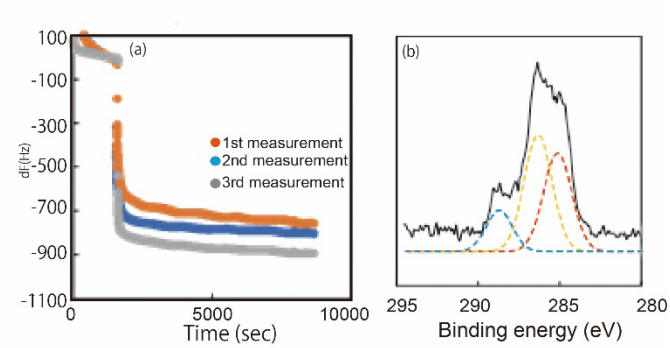


Figure 1 (a) Schematic illustration of QCM measurement, (b) structure and abbreviation of glycopolymer and DHBG used in this study.

polymer layer was estimated around 2-5 nm based on the previous report.[23,24]

Figure 2 Glycopolymer layer formation by adsorption of thiol terminated pMan200. (a) Time course of frequency change of three different measurements in QCM by adsorption of $pMan_{200}$ with 10 g L^{-1} at 15 C for 2h. (b) XPS C(1s) spectrum of $pMan_{200}$ immobilized substrate after adsorption of the glycopolymer and water rinsing.



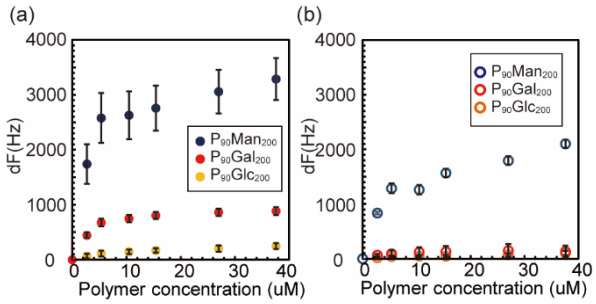


Figure 3 The frequency change of QCM measurement at each PEG-terminated glycopolymer concentration. (a) solvent condition; HEPES buffer with Ca^{2+}(HEPES: 10 mM, NaCl:137 mM, KCl: 2.7 mM, $CaCl_2$: 1.8 mM), (b) HEPES buffer without Ca^{2+}.

Table 1. The apparent binding constants (K_a)between glycopolymers estimated by Langmuir isotherm.

Combination of glycopolymers	K_a(M^{-1})
$pMan_{200}$-$P_{90}Man_{200}$	4.7 ×10^5
$pGal_{200}$-$P_{90}Gal_{200}$	4.9 ×10^5
$pGlc_{200}$-$P_{90}Glc_{200}$	1.5 ×10^5
$pMan_{200}$-$P_{90}Man_{200}$ without Ca^{2+}	2.0 ×10^5
$pGal_{200}$-$P_{90}Gal_{200}$ without Ca^{2+}	n. d.[a]
$pGlc_{200}$-$P_{90}Glc_{200}$ without Ca^{2+}	n. d.[a]

[a]not determined

The interaction between the glycopolymers was measured by the frequency change of QCM (Fig. 3 (a)). In the case of **$pMan_{200}$** immobilized on the gold surface, the glycopolymer of **$pMan_{200}$** stacked up on the surface (Fig. S7-2). Therefore, it was difficult to observe the amount of saturated adsorption of glycopolymer **$pMan_{200}$** on the **$pMan_{200}$** thin layer. The PEG-terminated glycopolymers were prepared to prevent aggregation of the glycopolymers.[25] The maximum frequency change of **$P_{90}Man_{200}$** was more than ten times that of **$P_{90}Glc_{200}$** and about four times that of **$P_{90}Gal_{200}$** (Fig. 3(a) and Fig.S7-3). The amount of glycopolymer bound followed the order: **$pMan_{200}$-$P_{90}Man_{200}$** >> **$pGal_{200}$-$P_{90}Gal_{200}$** > **$pGlc_{200}$-$P_{90}Glc_{200}$**, which suggests a strong interaction between mannose clusters. Langmuir fitting was performed, with the assumption that the interaction between the glycopolymer moieties was a one-to-one binding, though the interaction could occur cooperatively.[26] The apparent binding constant of **$pMan_{200}$** - **$P_{90}Man_{200}$** was 4.7 × 10^5 M^{-1}, and that of **$pGal_{200}$-$P_{90}Gal_{200}$** was similar (Table 1, Fig. S7–7). The interactions of **$pMan_{200}$** with different glycopolymers (**$P_{90}Gal_{200}$**, and **$P_{90}Glc_{200}$**) was much weaker than that between **$pMan_{200}$** and **$P_{90}Man_{200}$**, though a certain level of interaction was observed between **$pMan_{200}$** and **$P_{90}Gal_{200}$** (Fig. S7-6). In addition, interaction of **$pMan_{200}$** with PEG (PEG 4000) was negligible (Fig.S7-5). These results indicate the interaction of glycopolymer with mannose is specific to mannose cluster.

Fig. 3 (b) shows the frequency change at each polymer concentration in the absence of calcium ions. The amount of glycopolymer adsorbed on the substrate is reduced in the absence of calcium ions. The amount of glycopolymer bound followed the order of **$pMan_{200}$-$P_{90}Man_{200}$** >> **$pGal_{200}$-$P_{90}Gal_{200}$** and **$pGlc_{200}$-$P_{90}Glc_{200}$**, though the amount is reduced from that in the presence of calcium. The interaction between glycopolymer with mannose (**$pMan_{200}$-$P_{90}Man_{200}$**) was obvious, and other polymer interactions were small and negligible**.** The apparent binding constant is 2.0 ×10^5 M^{-1}.The results indicate that the interaction between mannose clusters is significantly stronger than that between glucose and galactose,

suggesting the interaction between mannose was contributed significantly by hydrogen bonding.

Turbidity measurements also supported that the interaction between each glycopolymer is dependent on the kind of monosaccharide (Fig.4).[25] The turbidity was recorded using glycopolymers (**pMan$_{200}$**, **pGal$_{200}$**, and **pGlc$_{200}$**). While **pMan$_{200}$**

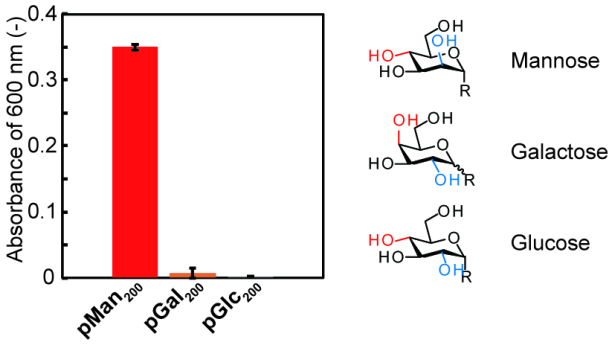


Figure 4 Turbidity measurement of pMan$_{200}$, pGal$_{200}$ and pGlc$_{200}$ and chemical structure of monosaccharides. he turbidity of each glycopolymer were measured after dissolution in HEPES buffer at 1 g L^{-1} and standing at 15 ˚C for 48 h.

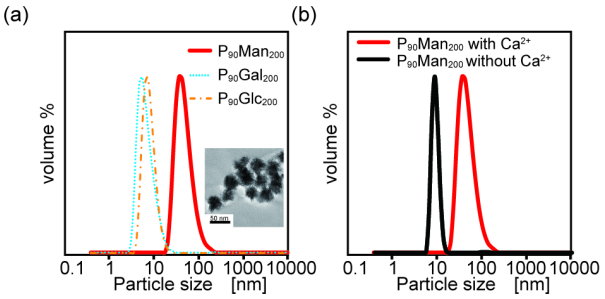


Figure 5 (a) DLS measurement of P$_{90}$Man$_{200}$, P$_{90}$Gal$_{200}$ and P$_{90}$Glc$_{200}$ and TEM image of P$_{90}$Man$_{200}$ (inlet). (b) DLS measurement of P$_{90}$Man$_{200}$ under with (red) and without (black) Ca^{2+}.

showed significant turbidity, while **pGal$_{200}$** and **pGlc$_{200}$** did not. The increase in the turbidity was caused by the interaction between the glycopolymers in aqueous solution and agreed with the QCM results, which indicated that a difference in the stereochemical structure of the saccharide affected the interaction.

These results indicate that the interaction between glycopolymers depends on the saccharide structure, which has similarity to the self-assembly capability of PEG-terminated glycopolymers. Only **P$_{90}$Man$_{200}$** showed a significant scattering, with its hydrodynamic diameter and polydispersity index (PDI) of 50±3 nm and 0.17, respectively. In addition, TEM observation of **P$_{90}$Man$_{200}$** also confirmed that it had a spherical structure with a 30±5 nm diameter (Fig.5 (a) inlet). These results indicated that among these three PEG-terminated glycopolymers, **P$_{90}$Man$_{200}$** can self-assemble in aqueous solution. Although all the monosaccharides (mannose, galactose, and glucose) have the same chemical formula, their self-assembly capability differs depending on their structure. In addition, the DLS measurements in the absence of calcium ions did not show any significant scattering in the case of **P$_{90}$Man$_{200}$**, which indicates that calcium ions are involved in the interactions between the glycopolymers and agrees with the results obtained by the QCM measurement (Fig. 5(b)).

Interestingly, the strong CCI was observed with mannose cluster but not with galactose and glucose clusters. There is a possibility that hydrophobicity of the polymer backbone and the triazole contribute the interaction. However, in the case of polymers with glucose and galactose as the side chain, the interaction between the glycopolymers was significantly decreased, which shows that the interaction is caused by the mannose moiety. In our previous studies, the self-assembly of glycopolymers with mannose was mediated by hydrogen bonds and calcium coordination bonds, and not by hydrophobic interactions.[22] At the same time, it is clear that the dense α–mannose along polymer backbone induced intermolecular interactions.

Considering the previous results, the apparent binding constants (K_a) between mannose clusters in this study are weaker than those between carbohydrate and lectin, but are comparable in strength.[26–29] The CCI has been observed only qualitatively in most studies to date, and there are few quantitative results, the reported CCI is still limited to the complex oligosaccharides. Abeyratne-Perera *et al.* also reported the formation of strong hydrogen bonds between mannobiose using AFM. They reported a quantitative mannobiose cluster force and it is comparable to the force of the CCI between specific combinations of oligosaccharides such as Le^x, which is different quantitative data to our binding constants.[30] The galactose interaction was comparable to the mannose interaction in the presence of calcium, which was similar to the CCI of lactose. The mannose interaction was remarkably strong even in the absence of calcium ion, and the contribution of hydrogen bonding was considered to be significant.

4. Conclusion

We evaluated the stereochemical dependence on the interaction between each glycopolymer moiety by QCM. The interaction between glycopolymers depended on the stereochemistry of the monosaccharide. The binding constants between glycopolymers carrying mannose was comparable to the carbohydrate-lectin recognition. The interaction was strongly affected by calcium ions. Comparing the self-assembly capability of the three kinds of PEG-terminated glycopolymers, only the mannose carrying polymer could self-assemble in an aqueous solution. Mannose is abundant on the surface of viruses and pathogens, and the human body has many mannose receptors. In addition, the role of mannose has become very important because high mannose type *N*-glycan plays a major role in protein quality control and transport. This mannose-mannose interaction could be an important interaction in biological systems. Mannose-mannose interaction has not been found before, and the bioinpired polymer made a significant contribution to the measurement.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

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