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Structural Effects of Oligosaccharide-Branched Cyclodextrins on the Dual Recognition toward Lectin and Drug

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Abstract

Bi-galactose-branched cyclodextrins (*Bi*-Gal-CDs) (**1–6**) having different spacer arm lengths between two terminal galactoses were found to have the optimum length for association with PNA lectin. Also, the inclusion interaction of the drug depended on the length of the spacer arms. The dual association of these compounds was quantitatively evaluated by SPR and compared to the other oligosaccharide-branched CDs (Scheme 1). The number and the length of the spacer arm are important for the association both with the lectin and drug for the purpose of targeting drug-delivery systems. The association constants *K* of *bi*-Gal-CD (**2**) with rat liver cells showed a 60 times higher association than with PNA. Direct evidence of the association between PNA and *bi*-Gal-CD (**2**) was characterized by AFM observations. The results obtained strongly suggested a method to find a new design for the targeting drug carrier. In order to increase the association with the cell, a sufficient spacer arm length is necessary for the effective dual recognition of the oligosaccharide-CDs. In order to increase the inclusion of the drug, the CD structure of a multi-saccharide branch is necessary.

Introduction

The interactions between saccharides and protein on the cell surface often involve multivalent binding sites [1], which is known as the glycoside cluster effect [2]. This concept has attracted considerable attention in the investigation of the receptor-binding properties of a variety of multi-antennary synthetic saccharide-modified carriers such as liposomes [3], dendrimers [4], calixarenes [5], and cyclodextrins [6] for the purpose of targeting drug-delivery systems. Our series of investigation have investigated the saccharide-branched CDs [7] which function both as the saccharide recognition to protein and the drug inclusion ability of the CD cavity [8]. The high mannose-type oligosaccharide-branched CDs showed a satisfactory high association constant both with lectin protein and with drug models [7g, 7h]. Also, the multi-antennary branched CDs showed a higher association with both proteins and drugs [7a, 7b].

This paper will describes the effect of the length of the spacer arm between the two terminal galactoses attached to the CD molecules. The synthesis and evaluation of the dual recognition of the *bi*-Gal-CDs (**1–6**) having different spacer arms, i.e., saccharide recognition to lectin protein PNA on the cuvette of an optical biosensor based on SPR, and the inclusion association with the anticancer agent, doxorubicin (DXR), were carried out.

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Materials and methods

*Bi-galactose-branched CDs (**1–6**)*

Lactonolactone was prepared from lactose according to the literature by the oxidation followed by ring closure to form the lactone. It was reacted with 6-amino capronic acid in DMF. Purification was done by chromatography using a Sephadex column and galactosyl- glucono-amide-capronic acid was prepared as the galactose-spacer arm units. This galactosyl- glucono-amide-capronic acid and methyl 6-amionocapronate was connected using the DCC-HONSu in DMF. Next, the methyl ester group was removed. By repeating this procedure, various lengths of spacer arms were prepared as shown in Figure 1. The products were purified using a Sephadex column, and the molecular weight was determined by FAB-MS.

The condensation reaction of the spacer arm units with *6A,6D*-diamino-β-CD by the DCC-HONSu method gave compounds **1–6**. The final products were purified by chromatography using a Sephadex column, and molecular weight was determined by FAB-MS. The products gave a single spot on TLC using n-BuOH: EtOH: H_2O, 5:4:3 by coloring with anisaldehyde. **1**: yield 86%, TLC R_f 0.41, FAB-MS m/z found 1811, calcd. 1811.60 [M-H$^+$]. **2**: yield 42%, TLC R_f 0.46, FAB-MS m/z found 2036, calcd. 2035.89 [M-H$^+$]. **3**: yield 36%, TLC R_f 0.49, FAB-MS m/z found 2265, calcd. 2264.22 [M-H$^+$]. **4**: yield 58%, TLC R_f 0.51, FAB-MS m/z found 2490, calcd. 2490.54 [M-H$^+$]. **5**: yield 21%, TLC R_f 0.53, FAB-MS m/z found 2717, calcd.

2716.85 [M-H$^+$]. **6**: yield 23%, TLC R_f 0.57, FAB-MS m/z found 2943, calcd. 2943.16 [M-H$^+$].

Parenchymal cell of rat liver

Rat cells were purchased from Rockland, USA. Separation and immobilization of the rat liver parenchymal cells were carried out according to the method of Seglen [9]. The rat liver was treated with collagenase and centrifuged. The observed image by optical microscopy shows the same image for the separated and reported cells.

Other materials

PNA lectin (Peanut Lectin, Lyophilized) and DXR (Doxorubicin Hydrochloride) were commercially available (Wako Pure Chemical Industries, Ltd., Japan).

SPR optical biosensor

The molecular interaction of **1–6** was examined using an optical biosensor "Iasys" based on SPR (Affinity Sensors, UK) both with the immobilized PNA and DXR [10, 11]. The SPR assay is a technique for the analysis of the association of a free analyte with the immobilized ligand on a sensor metal that induces a change in the refractive index of the biosensor surface. Changes in the refractive index (which is termed Response, R, in arc sec unit) depend on the interacting mass with the immobilized ligand irrespective of the type of molecules. The information about the association and dissociation kinetics of the associating ligand is in real time and the overall Ka is obtained.

The immobilization of PNA lectin on the sensor cuvette was carried out by the reaction of a reactive linker molecule with the cuvette surface having an aminobutyl group in neutral aqueous buffer solution. A spacer arm (BS3) was connected with an aminosilane group on the surface of the sensor metal. The amino group of PNA and DXR was reacted with the succinic acid amide ester group of BS3. Lectin protein, PNA, in an acetate buffer of pH 5.3 was added to the cuvette. Under this condition, PNA was supposed to have dimeric subunits. With the increase in response, we know that 3.5 ng/mm^2 of PNA was immobilized based on the sensitivity factor of 600 arc sec/ng. This means that the density of the immobilized PNA on the sensor surface is almost entirely covered when considering the volume of PNA.

If a drug molecule has a functional group to connect on the sensor surface, we can also observe the inclusion phenomenon with the CD cavity. The immobilization of the anticancer agent, DXR, on the cuvette surface was connected. The amount of immobilized DXR was 0.67 ng/mm^2. The immobilization of PNA was 8.7% and the immobilization of DXR was 83% of the aminosilane based on the assumption that the density of the aminosilane was one per 1 nm^2 area.

The association constant (K_a) was obtained by measuring the change in the response (R) according to the usual procedure as previously described [7].

The determination of Ka was carried out as follows: Based on the SPR principle, the concentration of the associated complex is proportional to the response R. The following relationship was derived: $dR/dt = k_a[\text{analyte}]R_{\max} - k_{\text{on}}R$, where $R_{\max}$ is the response when the entire ligand forms the complex, and $k_{\text{on}} = k_a[\text{analyte}] + k_d$. Plotting R *vs.* dR/dt, the slope gave $-k_{\text{on}}$, and plotting [analyte] *vs.* k_{on}, the slope gave k_a, and the intercept gave k_d. The computational results were derived using the software FASTfit equipped in the IAsys. The association constant was calculated by the relationship, $K_a = k_a/k_d$.

To obtain the kinetic plots, several time-response curves are determined by changing the analyte concentration. The association constants were obtained from the linear plots between the on-rate-constant, k_{on}, and the concentration of the oligosaccharide-CD. k_{on}, was obtained from the slope of the plots between dR/dt *vs.* R from the saturation curves.

The interaction curves were measured at the concentration of the *bi*-Gal-CDs (**1–6**) $10^{-3} \sim 10^{-2}$ M in *p*H 5.3 × 10 mM acetate buffer with [$MgCl_2$] = 1.00 mM, [$CaCl_2$] = 1.00 mM, [NaCl] = 100 mM at 25.0 °C according to our previous study [7c]. The association constants K_a (PNA) between the immobilized PNA and free analyte (**1–6**) were derived.

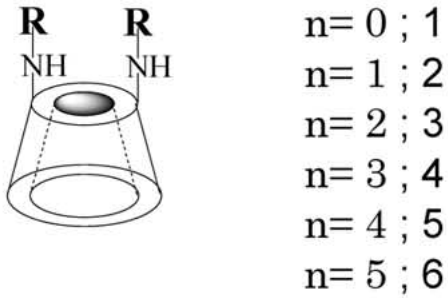
Atomic force microscopy

A separable cuvette, the 'Screw-in-Cuvette' (ThermoLabsystems, UK) was used for the SPR/AFM measurement [12]. After the usual procedure of the SPR optical biosensor assay, the block part of the removable cuvette was used for the AFM measurement. The AFM images and the section analysis were observed using a Shimazu SPM-9500J2 apparatus in the contact mode in air at a resonance frequency of 300 kHz at 25 °C. The samples were imaged with silicon nitride cantilevers with a nominal spring constant of 42 N/m. The images were captured at a scan angle of 0° and at a scan rate of 1 Hz.

Results and discussion

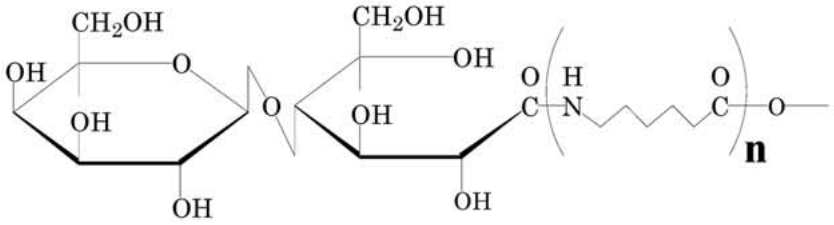
*Synthesis of bi-galactose-branched CDs (**1–6**)*

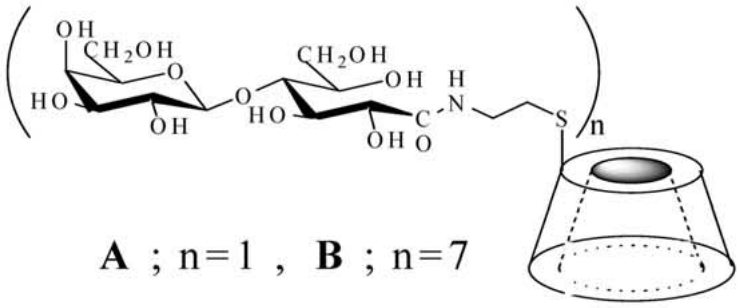
Lactonolactone was reacted with 6-aminocaproic acid in DMF. This galactosyl-glucono-amide-caproic acid was connected with the 6-aminocaproic acid methyl ester using DCC and *N*-hydroxysuccinic imide (HONSu) in DMF, consequently, the methyl group was removed. Repeating this procedure, after purification by chromatography using the Sephadex G-15 column, six different length spacer arms were prepared. The condensation reaction of the spacer units with *6A,6D*-diamino-β-CD by the DCC-HONSu method gave the products **1–6**. The products were identified by TLC and FAB-MS.



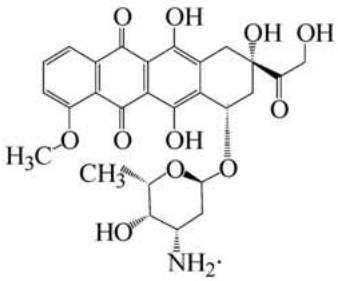
n= 0 ; 1
n= 1 ; 2
n= 2 ; 3
n= 3 ; 4
n= 4 ; 5
n= 5 ; 6

R =

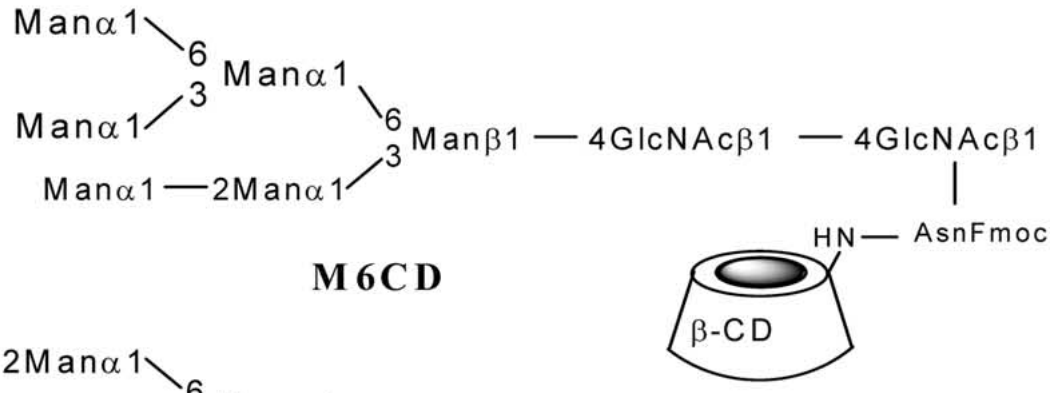




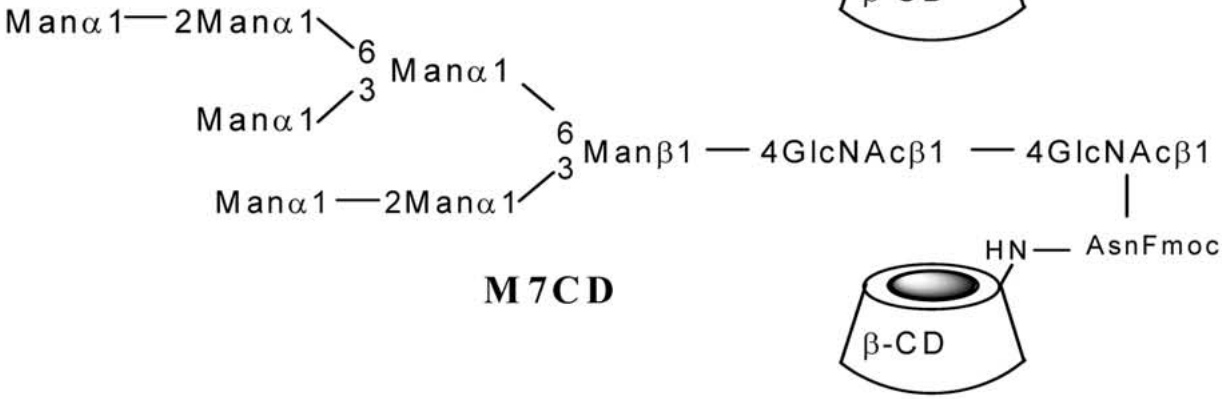
A ; n=1 , **B** ; n=7



DXR



M6CD



M7CD

Scheme 1. The structures of *6A,6D-bi*-antennary galactose-branched β-cyclodextrins (**1–6**), galactose-branched CDs (**A**, **B**), natural high mannose type oligosaccharide-branched CDs (**M6CD**, **M7CD**) and doxorubicin (**DXR**).

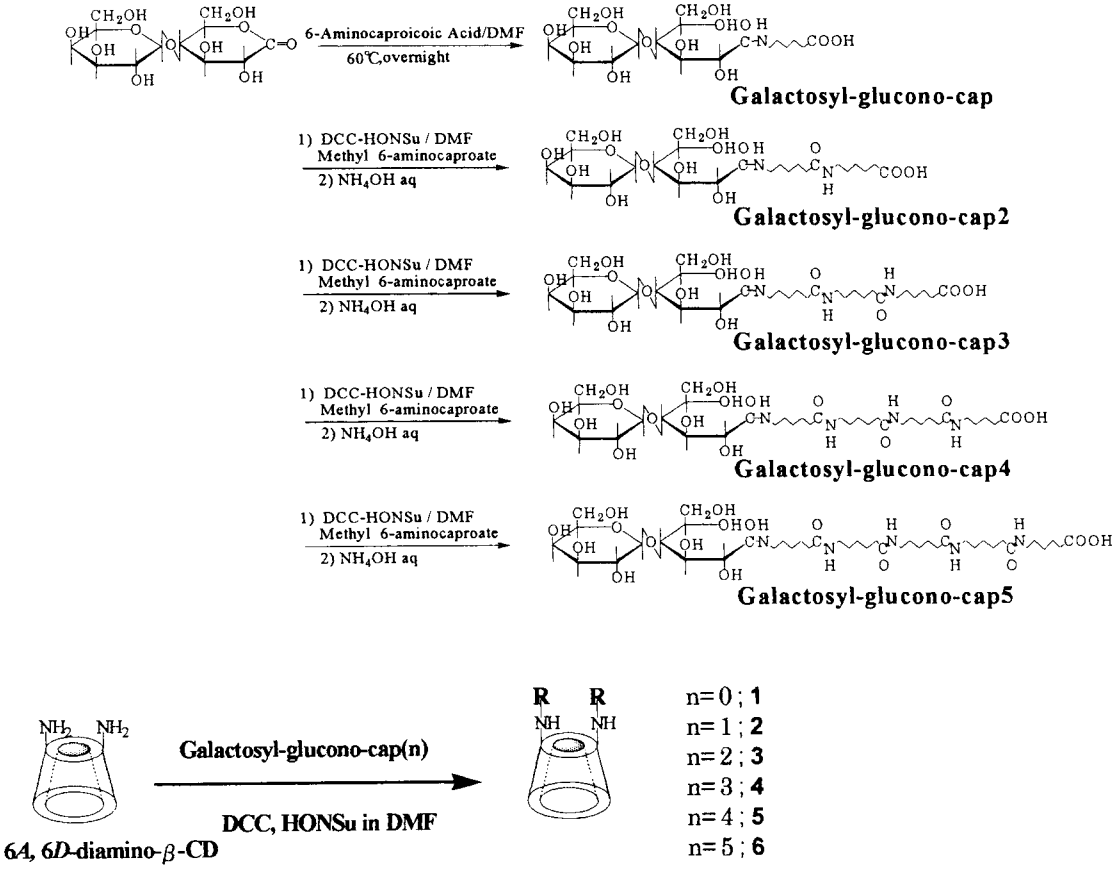


Figure 1. Preparation of *6A,6D-bi*-antennary galactose-branched β-cyclodextrins (**1–6**).

Evaluation of association between bi-galactose-branched CDs and PNA by SPR biosensor

The relation between these association constants, K_a (*vs.* PNA), and the length of the spacer between the galactoses is shown in Figure 2 from the kinetic results in Table 1. Molecular modeling using MM2 suggested that the estimated lengths between the two terminal galactoses of **1–6** are 1.6, 3.2, 4.7, 6.2, 7.7, and 9.2 nm, respectively, as schematically shown in Figure 3. The K_a (*vs.* PNA) increased along with the length of the spacer arm, and the maximum K_a (*vs.* PNA) value was observed at the 7.7 nm spacer length. The smaller K_a values of compounds **1–4** are thought to be due to the effect of an insufficient arm length to reach the binding site of a PNA subunit. For compounds **5** and **6**, the length of the arms is sufficient and the length between the two terminal galactoses is estimated to be adequate to simultaneously associate at the two binding sites of two dimeric PNA subunits. An X-ray crystal structure analysis has already been done with PNA [14] in comparison with legume lectins. The binding site structure between PNA and Galβ1-3GalNAc was described by Vijayan *et. al.* [14a]. The subunit conformation in the structure is similar to the other legume lectins. Kissling's group suggested that the length of the dimeric subunits of concanavalin A is approximately 6.5 nm [11d]. This may also be true in PNA because the homogeneity of the structure of the legume lectins has been discussed by Vijiyan's group [14a]. Our findings of the maximum K_a at 7.7 nm between the two terminal galactoses coincided with the results of the 6.5 nm length between the two binding sites.

Table 1. Kinetic parameters of *bi*-galactose-branched CDs in the association with immobilized PNA

Galactose branched CD	$K_a \times 10^3/M^{-1}$	$k_{ass} \times 10^1/M^{-1}\,s^{-1}$	$k_{diss} \times 10^{-3}/s^{-1}$
1	6.4±0.9	0.3±0.7	0.5±0.0
2	21±0.1	1.5±0.2	0.7±0.0
3	960±0.5	450±1.2	4.7±0.0
4	2500±5.9	430±0.5	1.7±0.0
5	4600±6.0	730±0.5	1.6±0.0
6	4400±50	180±2.3	0.4±0.0
A	8.1±0.2	2.1±0.2	2.6±0.2
B	130±0.2	14±0.1	1.1±0.8

Evaluation of association between bi-galactose-branched CDs and rat liver cell by SPR biosensor

In the study by Ashwell [13a], the structure of the receptor protein on the liver cell surface was known as the six subunits and recognizing sites exist in each subunit. The asialoglycoprotein-binding protein, ASGP-BP, has three kinds of subunits, RHL-1, RHL-2 and RHL-3 [13b, 13c].

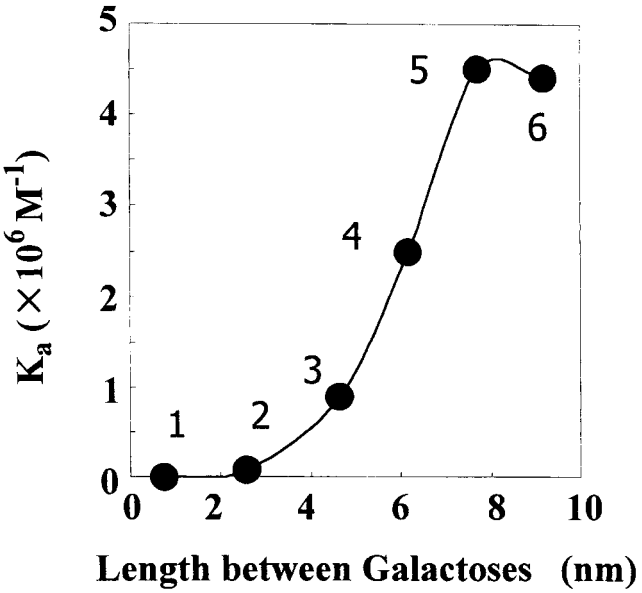


Figure 2. The dependency of the association constant K_a (PNA) on the length between the terminal *bi*-galactoses.

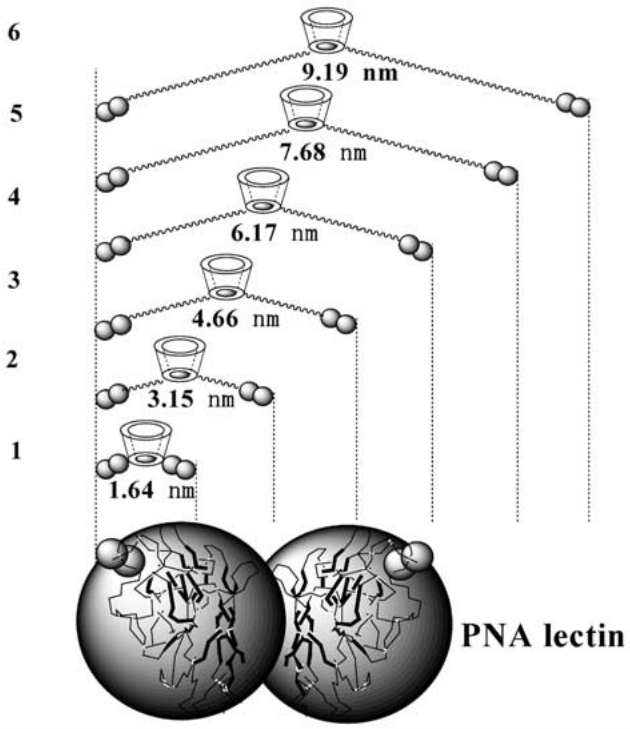


Figure 3. Comparison of the sizes between *bi*-galactose branched CDs and the PNA lectin.

Kinetic plots for the immobilized liver cells and A,D-biantenary galactosyl-glucono-amide-caproic-amide-CD was observed by changing the concentration of the galactose-CD. The obtained result was a fairly good straight line and provided the association constant on the order 10^{-6} M^{-1}. This value shows an extraordinary higher binding of the receptor on the liver cell than the PNA lectin. The association constants K of *bi*-Gal-CD (**2**) with the liver cells and with PNA are compared in Table 2. The liver cell receptor showed a 60 times higher association constant than

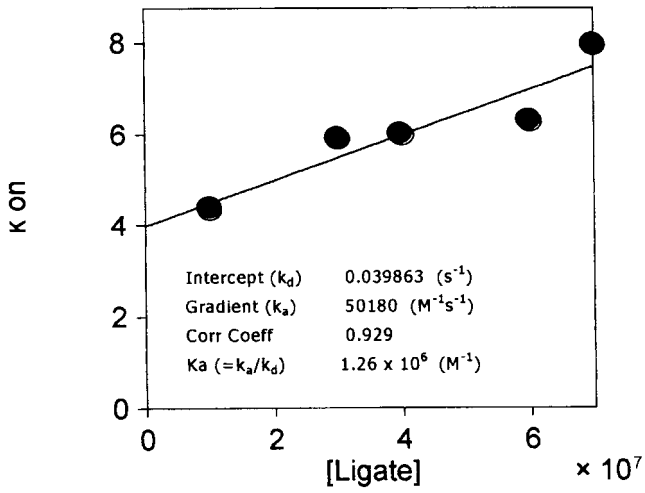


Figure 4. Kinetic plots for immobilized liver cell and *bi*-galactose branched CD (**2**).

Table 2. Kinetic parameters of galactose-branched CDs in the association with immobilized DXR

Galactose branched CD	$K_a \times 10^3/M^{-1}$	$k_{ass} \times 10^1/M^{-1}\ s^{-1}$	$k_{diss} \times 10^{-3}/s^{-1}$
1	17±0.3	0.2±0.0	0.1±0.0
2	23±0.5	0.4±0.1	0.2±0.0
3	37±0.4	3.9±0.8	1.0±0.3
4	43±1.5	1.1±0.2	0.2±0.0
5	–	–	–
6	51±2.5	4.1±0.3	0.8±0.1
A	3.1±0.2	12±0.5	41±3.0
B	62±1.0	11±1.0	1.9±4.0

PNA. The association rate of Gal-CD to the liver cell was 3300 times faster than the PNA lectin. The dissociation rate of the Gal-CD from the cell is faster than PNA, but the extent is moderate compared with the association rate.

Atomic force microscopy of the associated PNA with bi-Gal-CDs

Direct evidence of the association between the lectin and Gal-CD was characterized by Atomic Force Microscopy, AFM. We can observe the immobilized lectin on the aminosilane cuvette surface. Also, we expected to have direct evidence of the interaction between the saccharide-CD on the lectin protein. This could be the direct evidence for the SPR observations.

The analysis of the section images supported the PNA immobilization. The heights of the white spots were measured to be around 4 and 8 nm. From the X-ray analysis, the crystallized PNA lectin was reported to be a cubic structure subunit having a 4 nm length. The immobilization condition was at a pH of 5.3 where the conformation of the dimeric subunit of PNA lectin was presumed. The PNA size in the AFM image was nearly the same as the known size. The AFM has a characteristic that the horizontal diameter of the

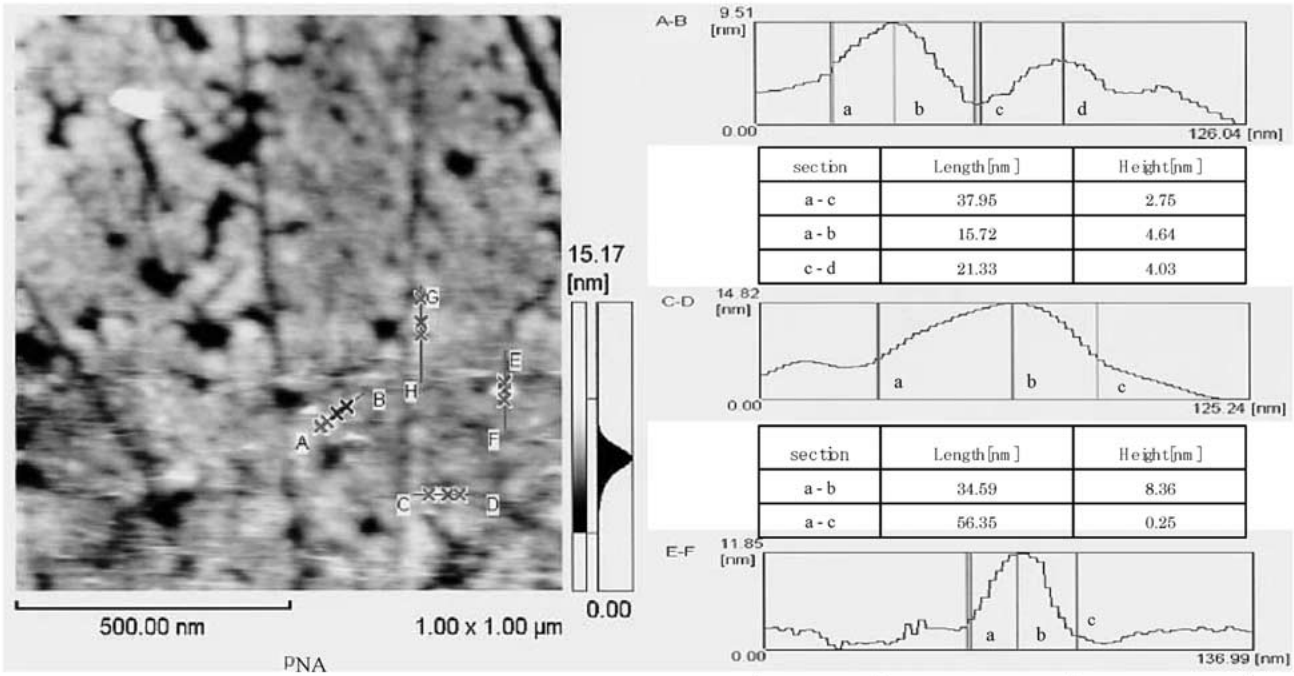


Figure 5. AFM image analysis of the immobilized PNA on the SPR cuvette.

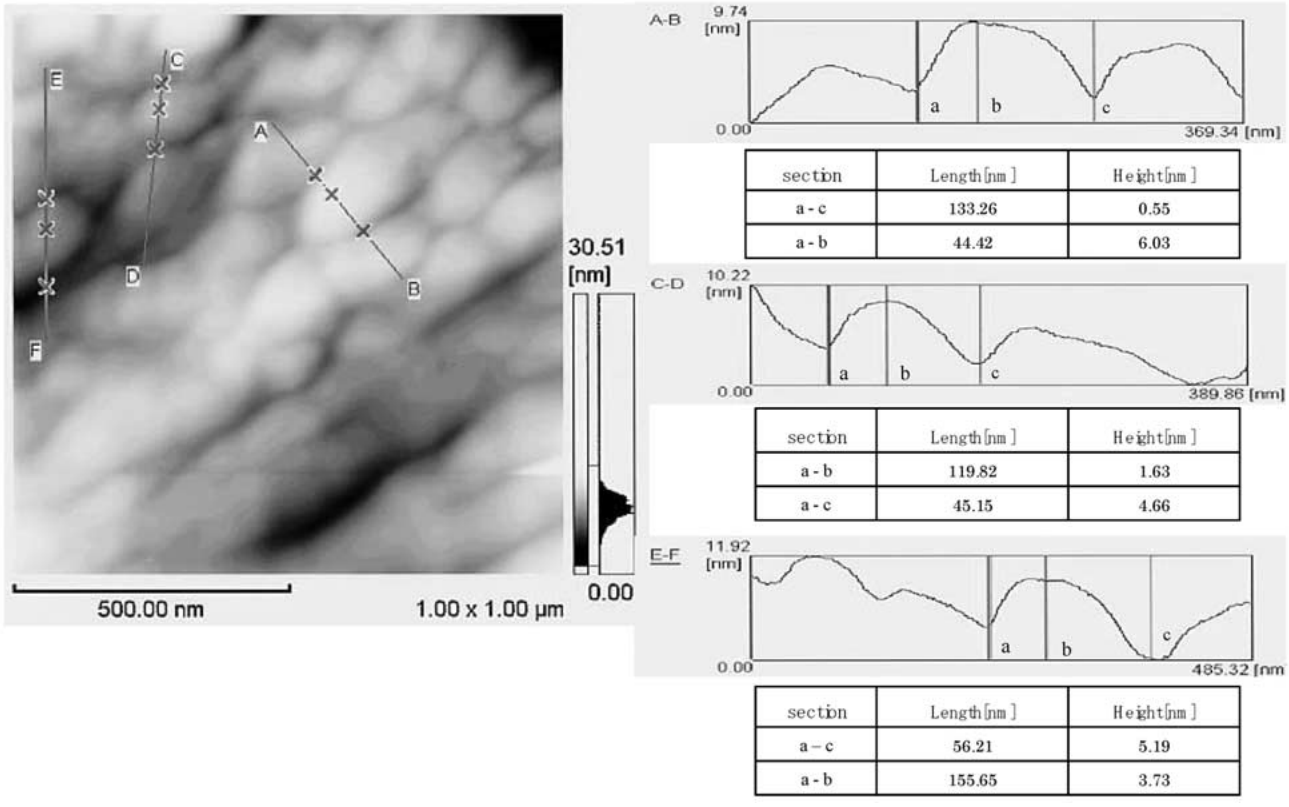


Figure 6. AFM image analysis of the PNA associated with *bi*-Gal-CD (**2**).

Table 3. Kinetic parameters of *bi*-Gal-CDs (**2**) in the association with immobilized liver cell

Immobilized ligand	K_a/M^{-1}	$k_a/M^{-1}\ s^{-1}$	k_d/s^{-1}
Liver cell	1.3×10^6	5.0×10^4	3.9×10^{-2}
PNA	2.2×10^4	1.5×10	7.1×10^{-4}
Cell/PNA	60	3300	55

spots looks enlarged because of the resolution limit of the AFM. The top of the cantilever has a size greater than several times that of the proteins and the horizontal sizes are apt to be exaggerated from the real size.

After the addition of the *bi*-antennary Gal-CD on the immobilized PNA, the AFM image changed. The section analysis showed some deformed curves in the asymmetric peaks. There was also a shoulder in the peaks. This may be caused by the associated Gal-CD on the protein. The size of the β-CD is a 0.8 nm depth and 1.5 nm diameter. This size will deform the PNA structures as seen in the section analysis curves.

The association of *bi*-Gal-CD on the AFM image was also ascertained after the regenerating treatment of PNA with urea solution. The AFM image recovered its symmetric peaks during the analysis of the curves.

Evaluation of association between bi-galactose-branched CDs and DXR by SPR biosensor

The inclusion association constants of **1–4**, **6** were also evaluated. An inclusion interaction concerning the *bi*-galactose-branched CDs (**1–4**, **6**) with immobilized DXR on a cuvette was measured. The relations between K_a (*vs.* DXR) and the distance between the two galactoses are shown in Figure 7. These data were greater than the *mono*-galactose-modified CD and smaller than the compound *hepta*-galactose-branched CD [7a]. The K_a (*vs.* DXR) value gradually increases along with the spacer length. K_a (*vs.* DXR) reflects the number of branches. If the modification of the multi-branched galactose unit on a CD with long arms is carried out, a significantly higher association constant can be presumably obtained in the inclusion interaction.

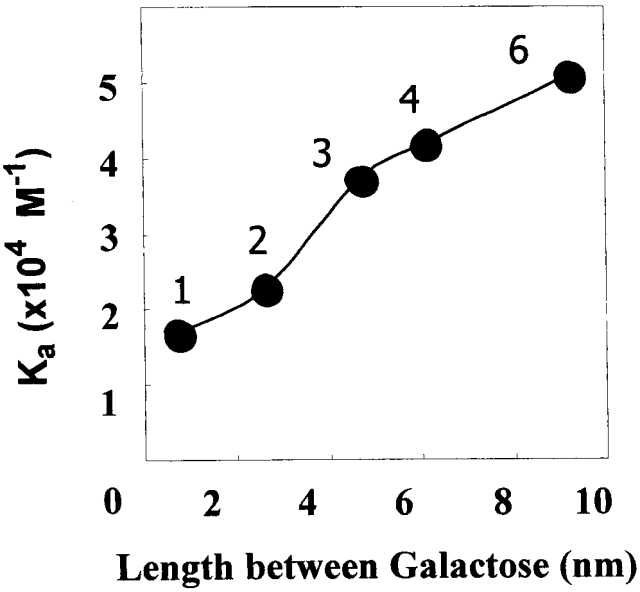


Figure 7. The dependency of the association constant K_a (DXR) on the length between the terminal *bi*-galactoses.

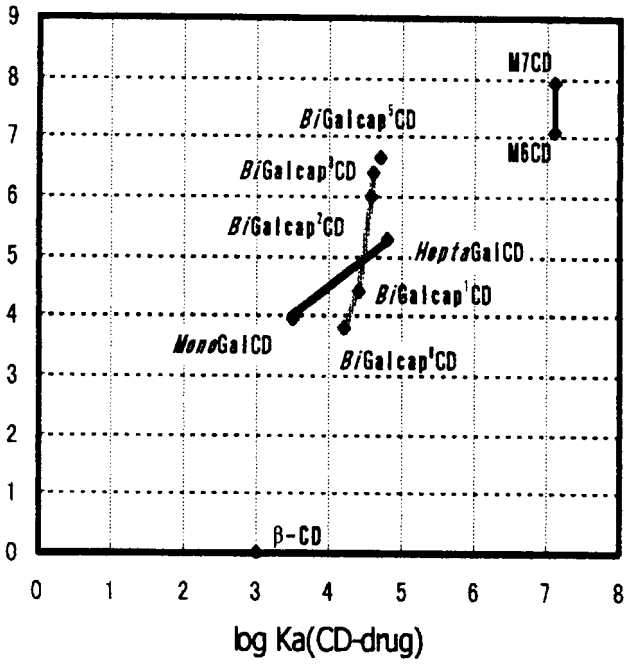


Figure 8. Two-dimensional map for dual recognition.

Two-dimensional map for dual recognition

In order to clearly discuss the dual association behavior of the oligosaccharide-branched CDs as a carrier for the targeting drug-delivery system, a two-dimensional map for the dual interaction of the oligosaccharide-branched CDs was suggested in Figure 8. In the map, the x-axis is log K_a of the inclusion with a drug and the y-axis is log K_a of the association with a lectin protein. The usual saccharide-branched CD exists in the area around $3 \sim 5$ for the inclusion constant and around 3–5 for the saccharide recognition. β- CD itself exists at this low point. The series with the longer arm of the *bi*-Gal-CDs (**1** $\sim$ **4** and **6**) made the positions on the vertical line from **1** ($x = 4.2$, $y = 3.8$) to **6** ($x = 4.7$, $y = 6.6$). The *mono*-antennary galactose-CD horizontally goes up to the right and increases in this map when changed to the *hepta*-antennary galactose-CD [7a]. However, natural high-mannose-CDs (**M6CD** and **M7CD**) existed on the vertical line in an advantageous position ($x = 7.1$, $y = 7.1$ and $x = 7.1$, $y = 7.9$) of the extensive association with both the protein and drug [7h]. This map strongly suggests that the number and length of the spacer arm are important for the molecular design at an efficient carrier for targeting a drug delivery system.

When we refer to these natural oligosaccharide-CDs of the high mannose type, we wonder if the designed oligosaccharide-modified CDs can be replaced by these natural oligosaccharide-CDs. There are large differences in the

association constant of one-tenth in the CD-lectin interaction and more than one-hundredth in the CD-drug association. The huge saccharide unit of eight residues and one asparagine unit with the Fmoc protecting group may form a very advantageous structure including a site for the cholic acid. Also, three mannose residues with sufficient spacer arm length from the CD may form a tight association with the Concanavalin A lectin.

The obtained results strongly suggested a method to find a new design for the targeting drug carrier. In order to increase the association with the cell, a sufficient spacer arm length is necessary for the effective dual recognition of the oligosaccharide-CDs. Also in order to increase the inclusion for the drug, the CD structure of the multi-saccharide branch is necessary.

Conclusions

The following conclusions can be summarized: (1) the *Bi*-galactose-branched CDs (**1–6**) having different spacer arm lengths were synthesized, (2) Kinetic evaluation by the SPR assay of the newly synthesized oligosaccharide-branched CDs provided us clear parameters for the dual association. The dual interaction behavior was quantitatively compared among the other oligosaccharide-branched CDs, and (3) The structural effects on the dual association were found to depend on the number of branches, the length of the spacer arms, and the size and structure of the saccharides.

Scope

Our research can be regarded as possibly finding a new targeting drug carrier. I would like to add the subjects to be investigated for the application of the oligosaccharide-CDs for targeting DDS as follows.

(1) Is the larger K (lectin) and K (drug) better for the practical targeting carrier?

(2) How to design the oligosaccharide structure for the arbitrary cell or virus? The mannosyl group will associate with the receptors on the Kuffer cell in the liver or on a macrophage. Also, the galactosyl unit can target the liver parenchymal cell. In order to develop this research, we have to observe the interaction with liver cells, bacteria, and viruses.

(3) Finding drugs to carry is important for wide applications.

(4) Finding of the cell or virus to target. Another key for us is to recognize the lock and key in the structural relation between a protein and oligosaccharide. This combination will provide us a new strategy to design the effective targeting DDS such as to target cancer or AIDS. Since the study on the various syntheses of the saccharide-branched CDs are still under way in our laboratory, the expected possibility should be proven in the future.

(5) Experiments *in vitro* and *in vivo*. It will then be necessary to submit practical tests *in vivo*, followed by clinical tests.

(6) Convenient synthesis of the oligosaccharide-CDs on an industrial scale should follow.

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