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Saccharide-branched Cyclodextrins as Targeting Drug Carriers

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Abstract

A synthetic series of *heptakis*-galactose-branched cyclodextrins (termed CDs) having a longer spacer arm using two amino-caproic acids as an enlarging unit were prepared. Starting with *heptakis*-amino-β-CD or *heptakis*-amino-caproic-amide-β-CD, treated with galactosyl-glucono-amide-caproic acid, the new compounds *heptakis* (Gal-cap1)-CD (**4**) or *heptakis* (Gal-cap2)-CD (**5**) were obtained. The longer galactose spacer arm extremely favors the PNA association. The effect of branch length on K with PNA was enhanced up to 138-fold **3** as well as with DXR enhanced up to 81-fold. *Hexakis* (Gal-cap2)-CD (6) was prepared and the association constants with rat liver cells were observed to be 2.5×10^{10} M^{-1}. A multi-high mannose type oligosaccharide branched CD (**7**) showed a large association constant with DXR up to 1.1×10^{9} M^{-1}. The two-dimensional map for the association constants of newly synthesized oligosaccharide-branched CDs toward lectin or liver cells *versus* the association constants toward a drug (doxorubicin) suggested a method of finding a better targeting drug carrier. The structural effect of the oligosaccharide-CDs showed that the number and length of the branch were dominant factors in designing for enhanced dual recognition.

Introduction

The oligosaccharide-branched CD can be associated with lectin on the specific cell surface through a hydrogen bond between the saccharide and protein. Another associating point for the saccharide-modified CD is the inclusion of a drug in the CD hydrophobic cavity. The SPR assay is a technique for the analysis of the association of a free analyte with an immobilized ligand on a sensor metal that induces a change in the refractive index of the biosensor surface. Changes in the refractive index depend on the interacting mass with the immobilized ligand irrespective of the type of molecules. Information on the association and dissociation kinetics of the associating ligand in real time and the overall K_a was obtained. The immobilization of lectin protein, a liver cell or an anticancer agent, doxorubicin (DXR), on the sensor cuvette was carried out by the reaction of a reactive linker with the sensor surface having an amino group [1].

The first series of the compounds was designed as *bis*-galactose-branched CDs having various spacer arm lengths, *6A*, *6D-bis*-galactosyl-branched β-CDs (**1**) [2]. The association constants K_a between **1** and the immobilized PNA became larger along with the length of the spacer arm, and the maximum value of K_a was found to be around 8 nm in the spacer arm length. The length of two binding sites of PNA was estimated by X-ray analysis to be about 6.5 nm. With a shorter length of the spacer arm, compound **1** ($n = 0$–3) can be considered to associate only at a single binding site of PNA. A longer length of the arms ($n = 4$–5) is sufficient to bind at two sites simultaneously. An inclusion interaction with immobilized DXR was also observed. The value of K_a increases gradually along with the length of the spacer arms. We presume this is due to the enhancement of the hydrophobic cavity formed by the arm spacers.

The second series of the synthetic design was examined for the structural effect of the number and the length of the arm spacers between the *heptakis*-galactose-branched CD (**3**) on the dual association with protein and the drug. The K_a value of **3** with the PNA lectin showed a 16-fold larger value than the *mono*-galactose-branched CD (**2**) [3].

However, in this case, when we consider the increase in the local concentration of the 7-fold galactose branch, the net increase in the association constant was 2-fold. This might be a glyco-cluster effect. On the other hand, the association constant of the *heptakis*-galactose-branched

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CD (**3**) with DXR showed a 21-fold tighter inclusion than the *mono*-galactose-branched CD (**2**). To our surprise, in the latter case, the driving force for the larger K comes only from the large decrease in k_d. That means that this compound includes DXR ordinarily, but it did not like to release the drug. This scheme explains the reason for the decreased dissociation rate constant, k_d. The wide-open saccharide antenna of the *heptakis*-galactose-branched CD (**3**) in aqueous solution may close on complex formation with DXR. This phenomenon is known as a kind of 'induced-fit' in enzymatic catalysis [4] involving changing the conformation to achieve advantageous behavior in catalysis. The computer-assisted molecular modeling using MM2 calculation supported this idea. I would like to suggest the 'Sea anemone effect' in this case. This kind of compound can be developed for various drug carriers showing a high association constant with drugs as well as with specific cells or viruses.

New synthetic series of *heptakis*-galactose-branched CDs having longer spacer arms [3]

The new series of *heptakis*-galactose-branched cyclodextrins (termed CDs) having longer spacer arms using one or two amino-caproic acids as an enlarging unit was prepared. Starting with *heptakis*-amino-β-CD or *heptakis*-amino-caproic-amide-β-CD, treated with galactosyl-glucono-amide-caproic acid, the new compounds *heptakis* (Gal-cap1)-CD (**4**) and *heptakis* (Gal-cap2)-CD (**5**) were obtained. After the purification with an ion exchanger and a GPC column, the yield was more than 30% as is shown in Figure 1.

The MALDI TOF-MS spectrum showed the same value between Calcd. and Found. Also the analytical HPLC showed a single peak. This supported the preparation of the compound (Figures 2 and 3).

The relation between the association constant K_a for PNA lectin and the number of the galactose branches is plotted in this graph changing the number to 1, 2 and 7. The relative ratio of K_a increased up to 41-fold for the *heptakis* branches. Increase in the galactose branches on the CD favors the PNA association. This can be thought to be a glyco-cluster effect [5].

The relation of K for PNA depending on the arm spacer length is shown in Figures 4 and 5. The K_a ratio increased up to 138-fold depending on the arm length from 0.91 to 1.79 nm. The longer galactose spacer arm extremely favors for the PNA association.

On the other hand, the relation between K_a for the drug DXR and the number of the galactose branches is plotted in Figure 6. The ratio of K_a increased up to 40-fold corresponding to the heptakis branches. This can be thought to be due to enhancement of the hydrophobic effect and also to the Sea anemone synergic effect.

The relation of K for DXR with the arm spacer length is shown here. The length of a branch influenced K corresponding to the ratio up to 81-fold. A synergic effect with hydrophobic enforcement and the 'Sea anemone effect' may be involved.

Table 1 shows a summary of the obtained results. The association with lectin increased with an increase in the number and length of the branches. This comes from a 'Glyco-cluster effect'. Association with the drug increased with an increase in the number and length because of the conformational change. We would like

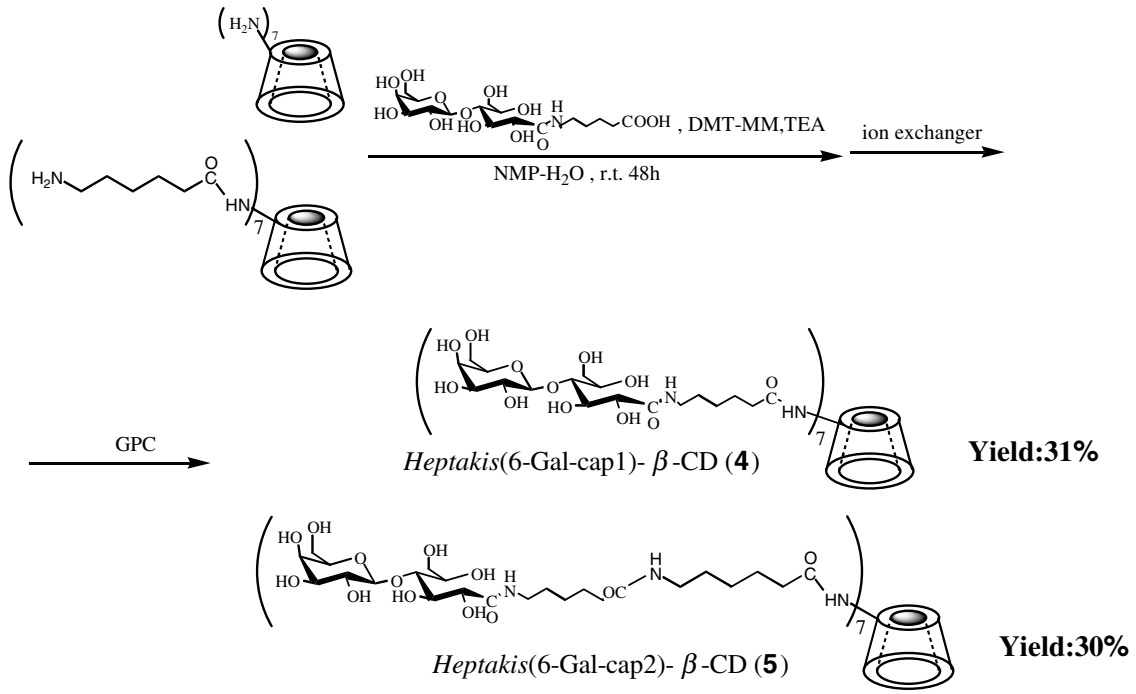


Figure 1. Syntheses of *heptakis* (Gal-cap1)-CD (**4**) and *heptakis* (Gal-cap2)-CD (**5**) having longer spacer arms.

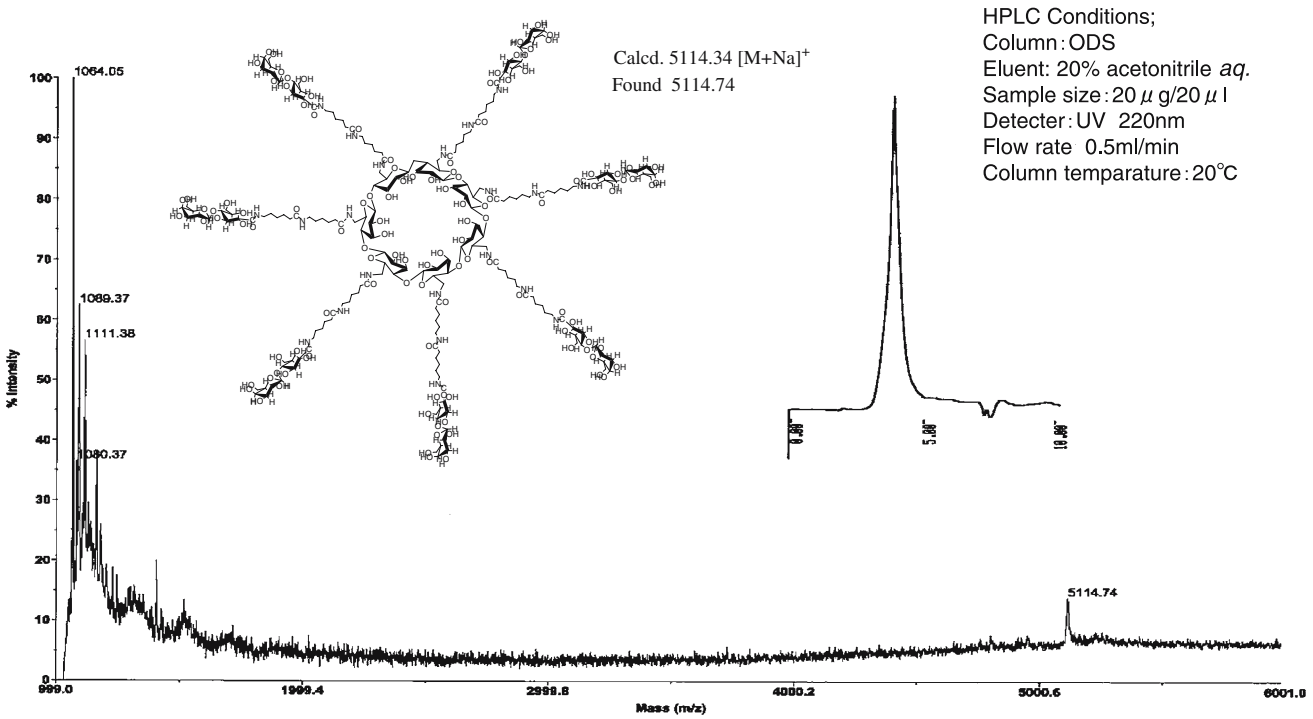


Figure 2. MALDI TOF-MS spectrum and HPLC of *heptakis* (Gal-cap1)-CD (4).

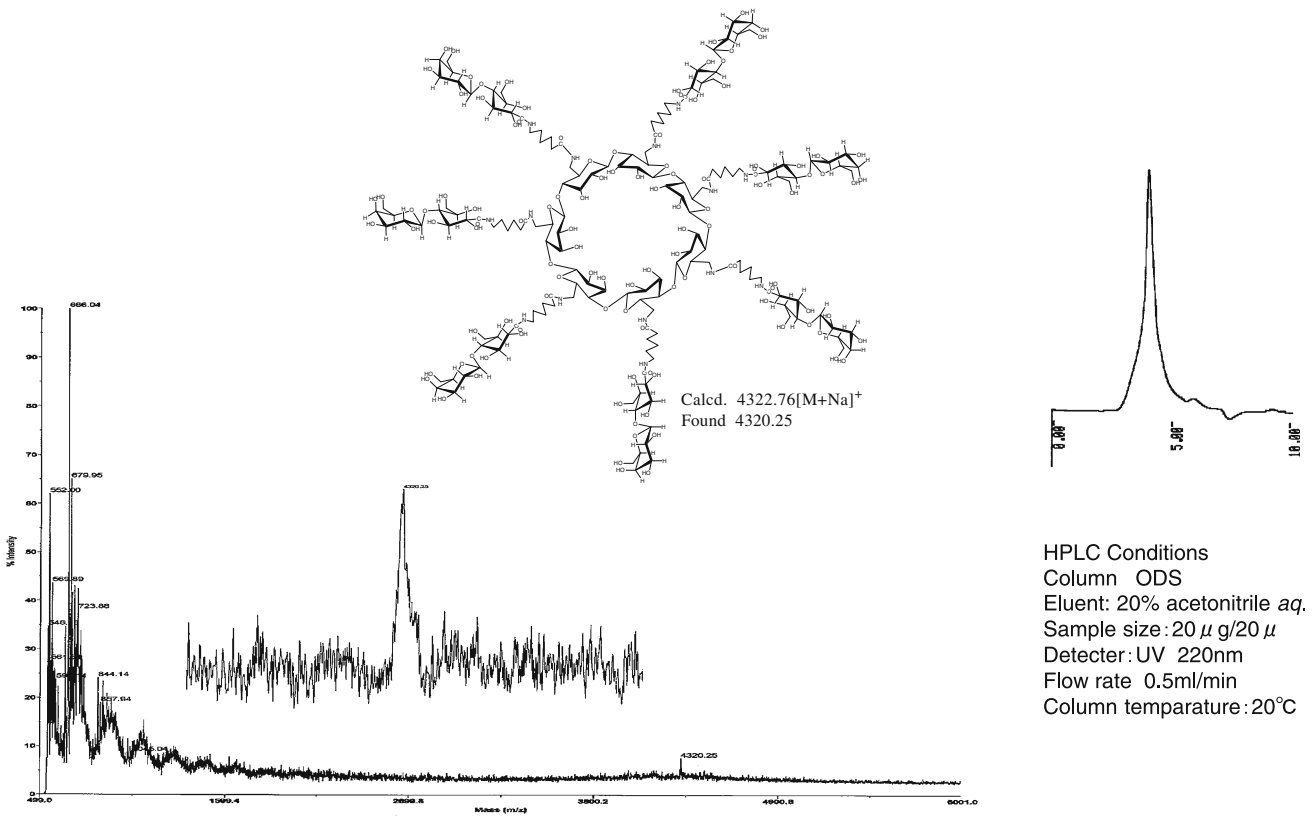
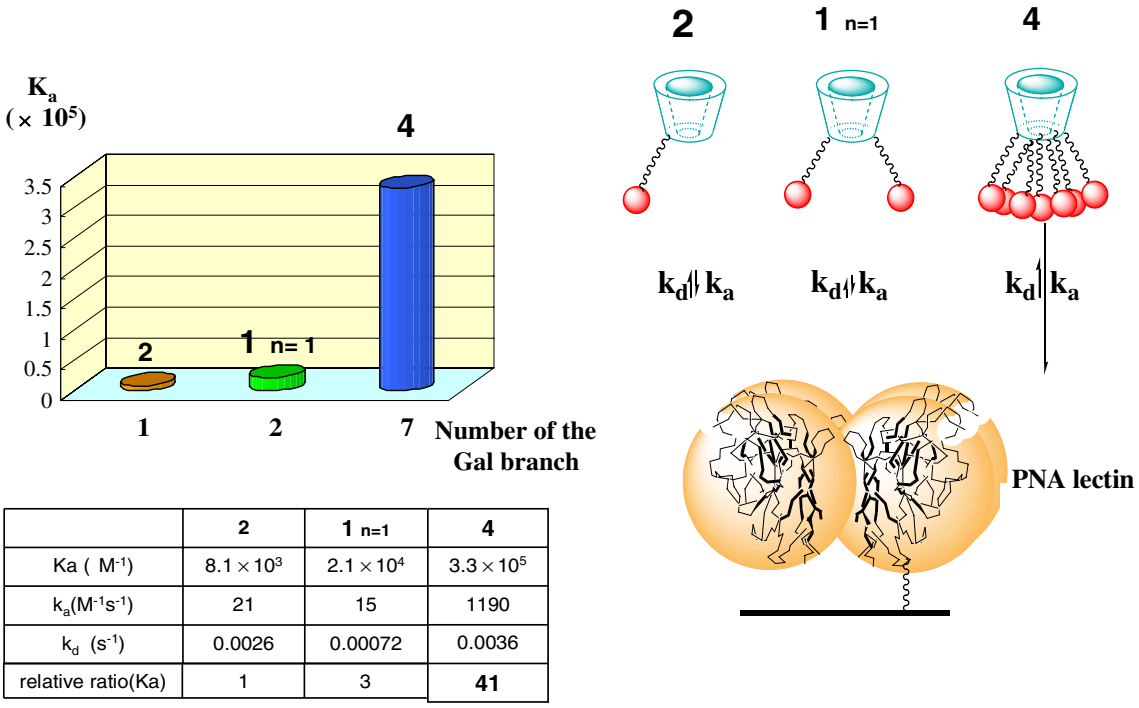


Figure 3. MALDI TOF-MS spectrum and HPLC of *heptakis* (Gal-cap2)-CD (5).

to suggest a 'Sea Anemone effect' in these phenomena. Also there may be an effect due to enforcement of the hydrophobic interaction. A synergy effect of the Glyco-cluster effect and the Sea anemone effect and other effects may function effectively in these nanostructures.

The galactose-branched CDs association with rat liver cells in place of PNA was examined. Isolation of rat



	2	1 n=1	4
Ka (M^{-1})	8.1×10^3	2.1×10^4	3.3×10^5
$k_a(M^{-1}s^{-1})$	21	15	1190
k_d (s^{-1})	0.0026	0.00072	0.0036
relative ratio(Ka)	1	3	**41**

Figure 4. Relation between K_a(lectin PNA) and the number of galactose branches.

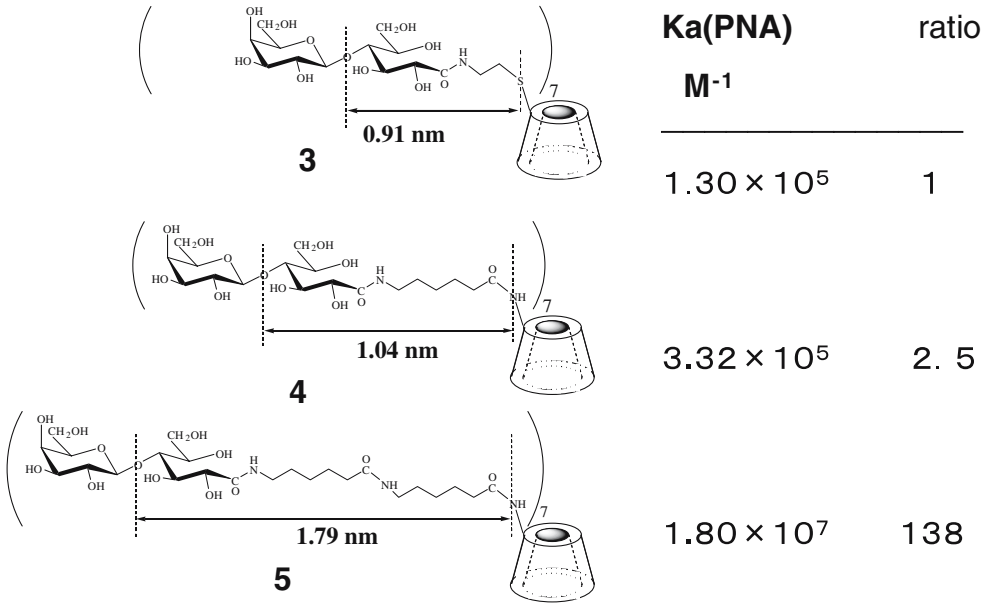
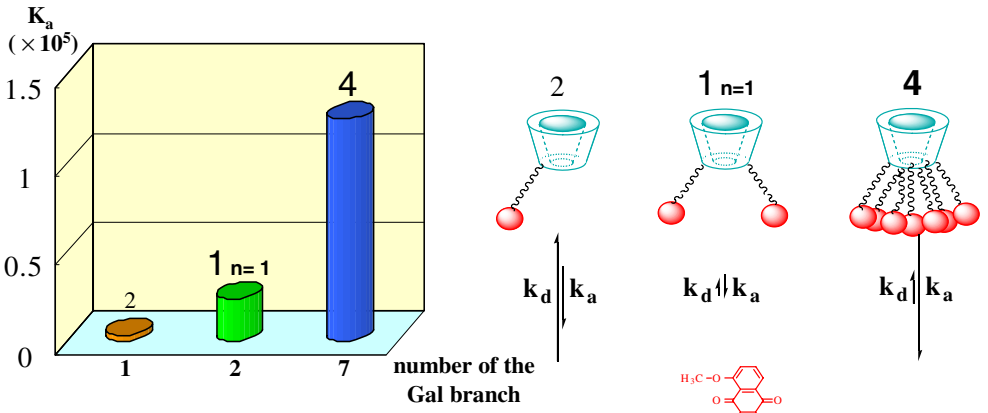


Figure 5. Relation of K_a(lectin PNA) of *heptakis*-Gal-CDs to the arm length.

Table 1. Summary of effects of the number and length of the arm spacer of branched CDs (**4** and **5**) on the association constant K_a

Association	Number of the branch mono- → bis- → hepta-	Length of the arm spacer 0.91 nm → 1.79 nm	Presumed effect
With lectin PNA	Increase 1 → 3 → 41	Increase 1 → 2,5 → 138	'Glyco-cluster'
With drug DXR	Increase 1 → 8 → 40	Increase 1 → 5.2 → 81	'Sea anemone' and Hydrophobic enforcement



	2	1 n=1	4
Ka (M^{-1})	3.1×10^3	2.4×10^4	1.25×10^5
$k_a(M^{-1}s^{-1})$	124	42	264
k_d (s^{-1})	0.041	0.0002	0.0021
relative ratio(Ka)	1	8	**40**

Figure 6. Relation between K_a(drug DXR) and the number of the saccharide branches.

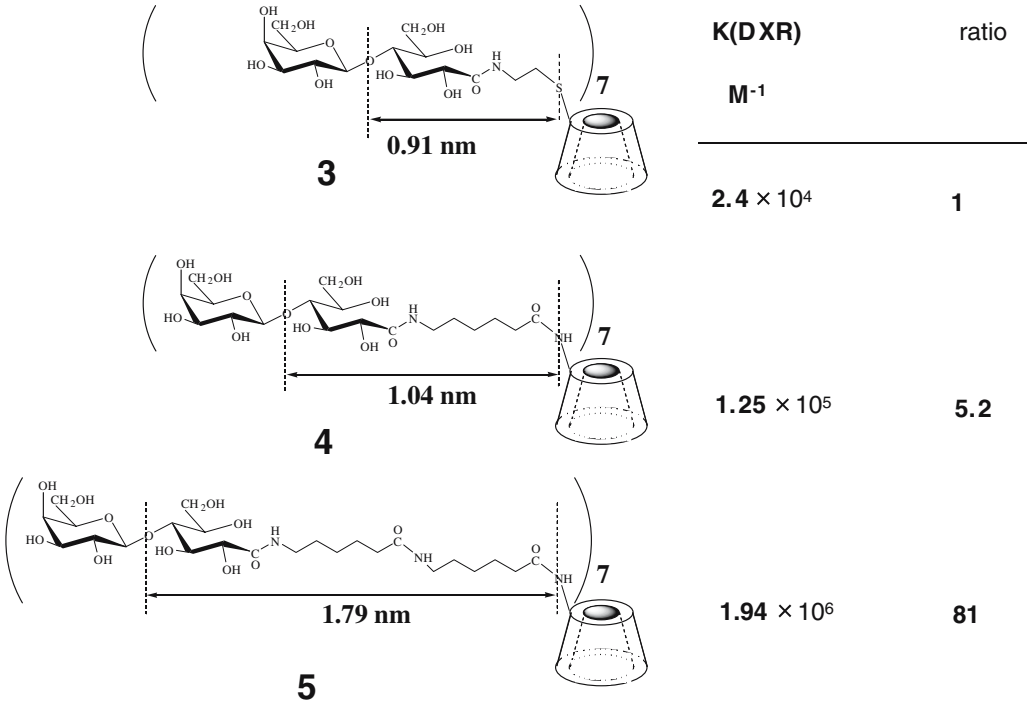


Figure 7. Relation of K_a(drug DXR) of saccharide-CDs to the arm length.

liver parenchymal cells was carried out according to the method of Seglen [6] and also Tanaka and Ichikawa [7]. A rat liver was cut, treated with collagenase and centrifuged. This shows the observed cell image by optical microscopy compared with the reported cell image. Immobilization of the rat liver cell on the SPR optical biosensor cuvette surface was carried out. We can monitor by the SPR sensing curve for the mass change on the cuvette surface. On an aminosilane type cuvette, using a reactive arm spacer, the rat liver cell was immobilized as shown in Figures 6–8.

The association parameters of the rat liver cell *versus* Gal-CD are summarized in Tables 2 and 3. The results using *hexakis*-galactose-branched CD with the rat liver cell shows 93,000-fold stronger interaction than lactose. The rate constants go up to 2.5×10^{10} M^{-1}. This is an extraordinarily large number. This assures the association of the galactose-CD with the rat liver cell. Other

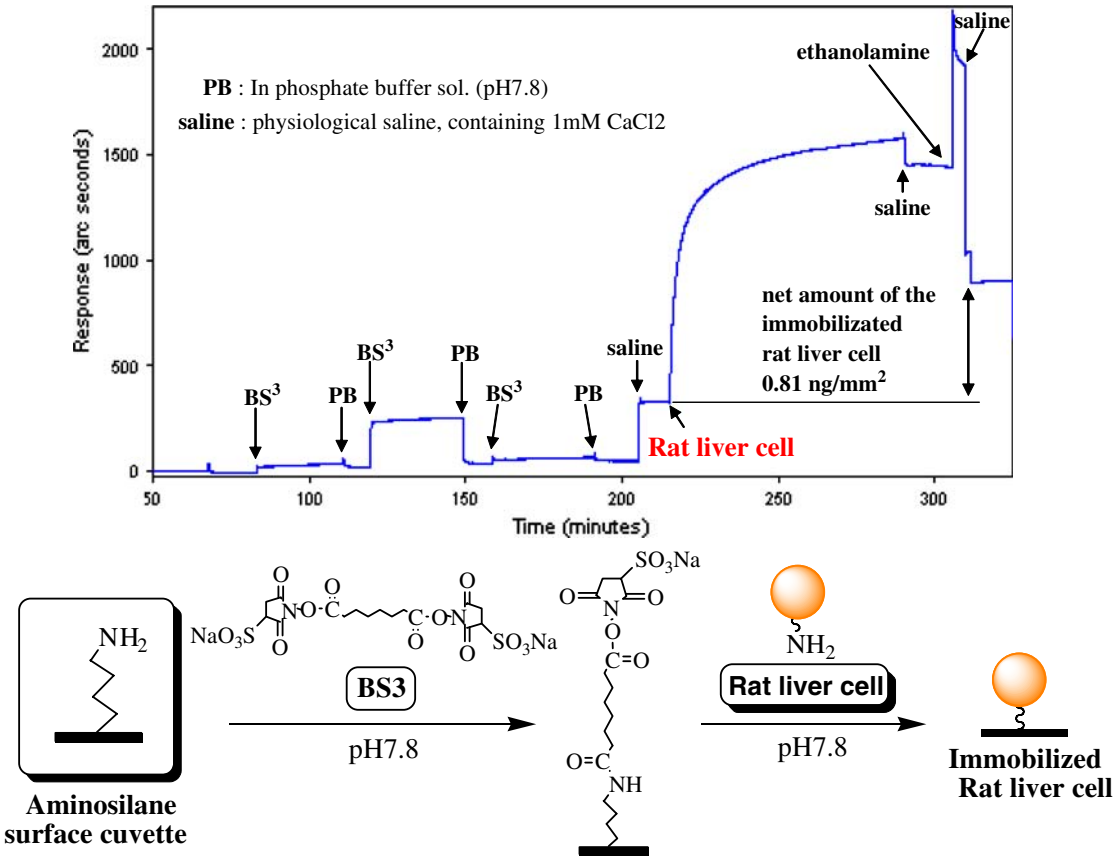


Figure 8. Immobilization of rat liver cell on the SPR biosensor cuvette surface.

Table 2. Association parameters of *hexakis*-galactose-branched CD (**6**) *versus* rat liver cell

Gal-CD	Immobilized Ligand	k_a ($\times 10^2$ M^{-1} s^{-1})	k_d ($\times 10^2$ s^{-1})	K_a/ ($\times 10^2$ M^{-1})	Ratio
1	Rat liver cell	8.0	0.32	2.7	1
2	Rat liver cell	502	3.99	12.6	4.67
2	PNA	0.15	0.07	0.22	0.08
3	PNA	89.3	0.05	180	66.7
4	Rat liver cell	22,200,000	8.87	251,000	93,000

Table 3. Association behavior of *multiple* natural high mannose-branched CD with an immobilized guest

Natural high mannose-CDs	Immobilized guest	Association constant K_a M^{-1}	Association rate constant k_a M^{-1} s^{-1}	Dissociation rate constant k_d s^{-1}
Mono-M6CD $n=1$	Cholic acid	1.3×10^7	3.3×10^4	2.6×10^{-3}
Multi-M6CD $n=4$, 5 (mixt.)	DXR	1.1×10^9	5.7×10^7	5.0×10^{-2}
Multi/Mono ratio		100	1500	15

important results of the *bis*-branched CD with rat liver shows a 60 times stronger interaction than PNA lectin. Therefore, it can be said that PNA is a more moderate receptor than the receptor in the liver cell.

Previously, we prepared naturally originated high mannose-branched CD (termed Mono-M6CD) [8] in Figure 9. We were challenged to attach several such natural branches (M6) on *heptakis* aminoethylythio-CD. After the treatment to form an amide linkage and purification using GPC chromatography methods, we obtained the mixed product having four and five high mannose-branches (termed Multi-M6CD) in the preliminary results.

This *multiple*-M6CD showed an extremely large association constant with DXR, 10^9 M^{-1}. That is a 100-fold increase compared to *mono*-M6CD.

A two-dimensional map for the association constant both with lectin and a drug was suggested as a summary of our results. The map suggested a method of finding new targeting drug carriers with better CD-based conjugates. In this map, the *x*-axis is the log K_a for the drug and the *y*-axis is the log K_a

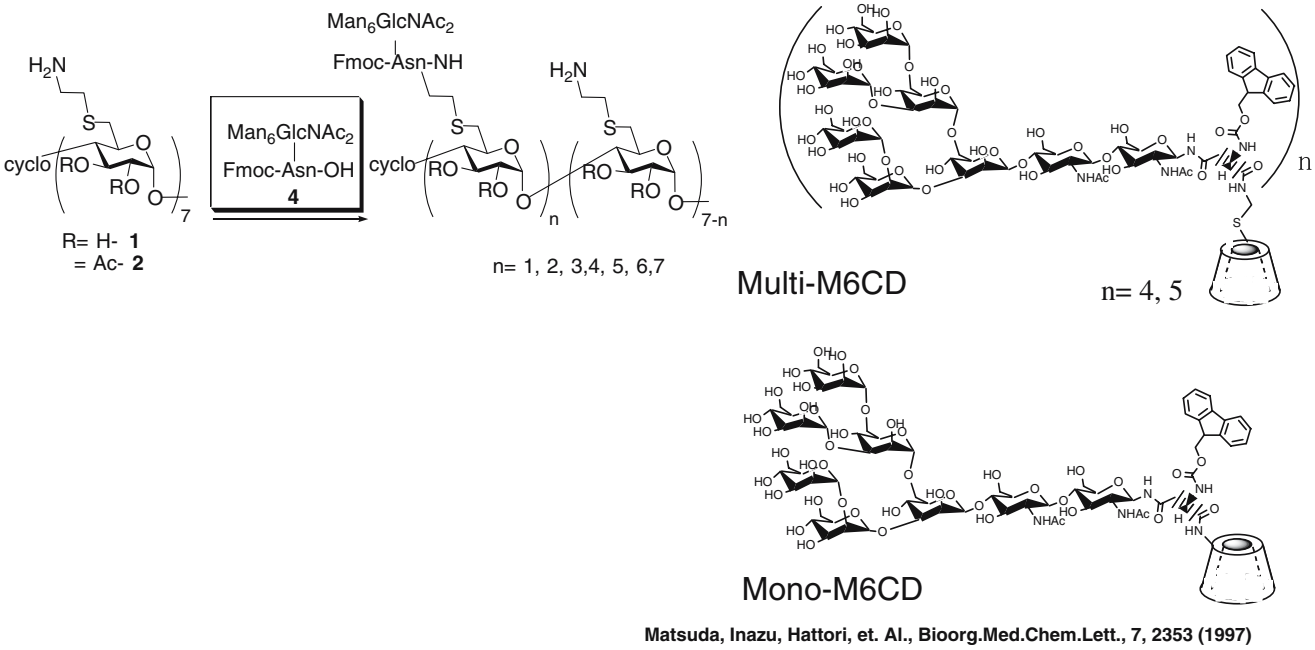


Figure 9. Association between *multiple*-natural high mannose-branched CD (**7**) and a drug.

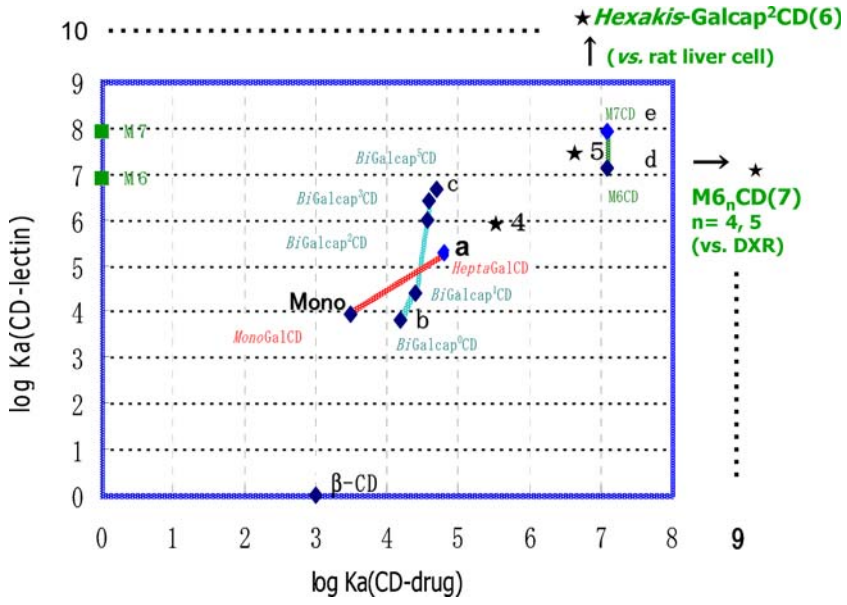


Figure 10. The way to find the better drug carriers in the two-dimensional map for dual association.

for lectin. Here, we summarize the results as in Figure 10.

The first series of the line in Figure 10 from *b* to *c* of increasing arm length of the *bi*-antennary galactose-branched CDs (1; $n = 0$–5) moves the position upward depending on the length of the arms. In the second series of the line in Figure 10 from Mono to *a*, the *mono*-galactose-branched CD (**2**) goes up to the right and increases in this map when changed to the *heptakis* galactose-branched CD (**3**). The CDs with the *heptakis* longer spacer units of **4** and **5** showed extraordinary enhancement of the K_a for both PNA and DXR. However, the third series of line in Figure 10 from *d* to *e* of natural high mannose-branched CDs (M6CD and M7CD) showed an advantageous position of a higher association constant both with the lectin and the drug. In addition, we added two new preliminary data items extrapolated out of this map. One is the *hexakis*-galactose-CD (**6**) having a longer arm with rat liver cells, showing a high K_a with two more digits.

Another one is the *multiple*-natural high mannose-branched CD with 4- and 5-substitution (**7**) which shows an extraordinarily higher K_a with two more digits. This newly synthesized *multiple* saccharide-CD (**7**) showed excellent dual association with rat liver cells and the DXR extrapolated out of the map.

These results allowed us to devise a new design of the CD structure with a sufficient number of saccharide branches with sufficient spacer arm length that may be necessary for the enhanced dual association of the saccharide-CDs to develop new targeting drug carriers.

Conclusions

A conclusive summary of this short review is as follows:

(1) Dual recognition of the designed saccharide-CDs was evaluated by SPR.
(2) The number and the length of the branches were dominant factors in designing for enhanced dual recognition.
(3) A two-dimensional map for the dual association with lectin and a drug was useful in finding new targeting drug carriers.
(4) These newly synthesized compounds can be adopted and developed for the targeting drug delivery carrier. We are progressing in synthesizing various saccharide-branched CDs for the targeting drug carriers.As I mentioned concerning on the galactose and natural high mannose-branched CDs, we tried sialic acid, fucose, mannose, and natural sialic acid-branched CDs. All are targeted to a specific site of a cell or virus. Also we are trying to determine the specific saccharide structure for targeting cancer cells.

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