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Molecular Design of Multivalent Glycosides Bearing GlcNAc, (GlcNAc)$_2$ and LacNAc - Analysis of Cross-linking Activities with WGA and ECA Lectins

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1. Introduction

The cross-linking properties of a variety of plant and animal lectins with multivalent carbohydrates and glycoproteins have been reviewed (Sacchettini et al., 2001; Brewer 1997; Dessen et al., 1995; Pieters 2004). These studies show that a number of lectins form cross-linked complexes with branched chain oligosaccharides [Bhattacharyya et al., 1988(a); Bhattacharyya et al., 1990], glycopeptides [Bhattacharyya et al., 1987(a); Bhattacharyya et al., 1990], and glycoproteins (Gupta & Brewer, 1994; Gupta et al., 1996). High affinity lectin ligands are of great interest in the cross-linking activities of such carbohydrate-mediated processes. The generation of high affinity ligands, however, is not trivial because the interaction of individual carbohydrate epitopes with lectin is, in many cases, weak and undiscriminating. The binding affinity can be dramatically increased by clustering lectin binding sites and carbohydrate recognition units (Mammen et al., 1998; Roy 2003; Lindhorst 2002; Houseman & Mrksich, 2002; Lee & Lee, 2000). The observation that binding affinity increases exponentially with the number of binding sites has been termed the glycoside cluster effect (Lee & Lee, 1995; Lundquist & Toone, 2002). In general, monovalent carbohydrate-protein interactions often occur with low binding affinities (Kd~10^{-3} M) (Mandal et al., 1994). However, multivalent interactions have several advantages over monomeric ones and are often used by nature to control a wide variety of cellular processes. With this in mind, multivalent carbohydrate analogs for high affinity binding to target lectins have been designed (Maierhofer et al., 2007; Roy 1996; Kiessling & Pohl, 1996; Mammen et al., 1995; Toone 1994; Kitov et al., 2000). It is becoming increasingly clear that multivalency is a powerful design approach to increase the binding strength of synthetic ligands. Considering that strong binding is required for practical application of interference strategies, the synthesis and evaluation of multivalent carbohydrates is a topic of increasing prominence. The multivalency effect can lead to truly large rate enhancement, even for systems of low valency, such as a divalent system (Dam et al., 2005). We are interested in developing an efficient synthetic route to multivalent glycosides, for glycomimetics, as they tend to have enhanced affinity due to their multivalency for specific lectins. Here we describe a simple strategy to design di- and tetravalent glycosides of *N*-acetylglucosamine (GlcNAc), *N,N'*-diacetylchitobiose [(GlcNAc)$_2$] and *N*-acetyllactosamine (LacNAc), which

have an enhanced multivalent effect due to a scaffold structure when bound to wheat germ (*Triticum vulgaris*) agglutinin (WGA) and coral tree (*Erythrina cristagalli*) agglutinin (ECA). Using these di- and tetravalent glycosides, interactions with lectins were analyzed using a hemagglutination inhibition assay, a precipitation assay, double-diffusion test, and surface plasmon resonance analysis.

2. Enzymatic synthesis of spacer-O-linked divalent glycosides and their cross-linking activities with WGA lectin

This section describes a convenient enzymatic synthesis of divalent spacer-O-linked GlcNAc-glycosides carrying alkyl spacers and their binding property with WGA.

2.1 Enzymatic synthesis of divalent spacer-O-linked GlcNAc-glycosides

Amycolatopsis orientalis (IFO 12806[T]) was cultured with colloidal chitin. Increasing the activities of both *N*-acetylhexosaminidase (NAHase) and chitinase was observed in course of culture (Usui et al., 1987). When NAHase activity reached a maximum, the culture was centrifuged, and then the supernatant was desalted and freeze-dried. The dried powder was directly used for transglycosylation without purification. The enzyme catalyzed the synthesis of target spacer-O-linked divalent glycoside **1** together with monovalent 6-hydroxyhexyl β-D-*N*-acetylglucosaminide and 6-hydroxyhexyl β-D-*N,N'*-diacetylchitobioside through *N*-acetylglucosaminyl transfer from (GlcNAc)$_4$ to 1,6-hexanediol (Scheme 1). The target glycoside was separated by successive chromatographies of charcoal-Celite and silica gel columns to obtain in a 10.5% overall yield based on donor added. The synthesis of short length (1,3-propanediol) of spacer-O-linked divalent glycoside **2** was achieved in a similar manner by using chitinolytic enzyme (Scheme 1).
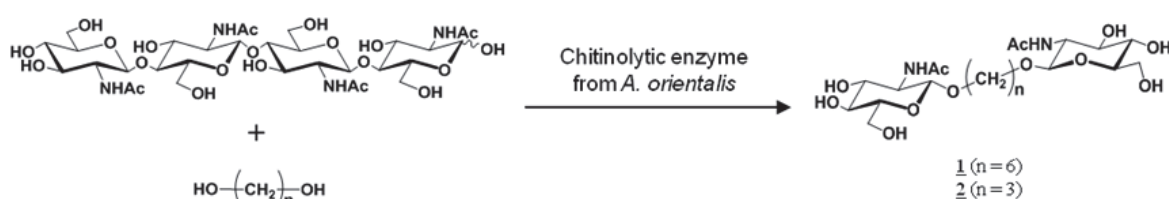
Scheme 1. Enzymatic synthesis of spacer-O-linked divalent GlcNAc-glycosides.

2.2 Cross-linking property of spacer-O-linked divalent glycosides as divalent ligand

Interaction of the resulting spacer-O-linked divalent glycosides carrying GlcNAc with WGA was analyzed by two different methods: precipitation and biosensor analysis. The avility of the syhthetic divalent glycosides (**1** and **2**) to bind to WGA was first analyzed by precipitation analysis. WGA (128 μM) and divalent glycoside (0.025-12.8 mM) were mixed on a 96-well microplate. When each divalent GlcNAc glycoside was added to the WGA solution under appropriate conditions, glycoside **1** with the long spacer (C-6) formed a precipitate within a few minutes, whereas glycoside **2** with the short spacer (C-3) did not little. However, the presence of chitin oligosaccharide [(GlcNAc)$_2$ and (GlcNAc)$_3$] prevented the formation of precipitates with **1**. The precipitates with glycoside **1** dissolved upon addition of the di- and trisaccharide (data not shown). These results indicate that the divalent GlcNAc glycoside bind specifically to WGA. Monovalent glycosides carrying GlcNAc and (GlcNAc)$_2$ on one side did not form a precipitate. Precipitin curves for WGA in

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the presence of the divalent glycosides **1** and **2** were prepared by measuring the lectin concentration in the supernatant (Fig. 1). Spacer-O-linked divalent glycoside **2** with the short spacer did little form precipitate. By contrast, the concentration of **1** at the equivalence point was in the region of 0.1-2.5 mM, although the value was not accurately determined under the present conditions. Precipitation analysis showed that spacer-O-linked divalent GlcNAc glycoside specifically binds to WGA. Furthermore, the length of spacer group between the two GlcNAc residues appears to affect the ability to precipitate WGA. From these results, the length of spacer might be important in allowing the molecule to adopt a favorable orientation during interaction with the GlcNAc binding site on WGA. Some reserchers reported that the binding and cross-linking properties of lectin are sensitive to the degree of flexibility and spacing between the carbohydrate epitopes of the analogs (Krishnamurthy et al., 2007), which are entirely consistent with the present results.
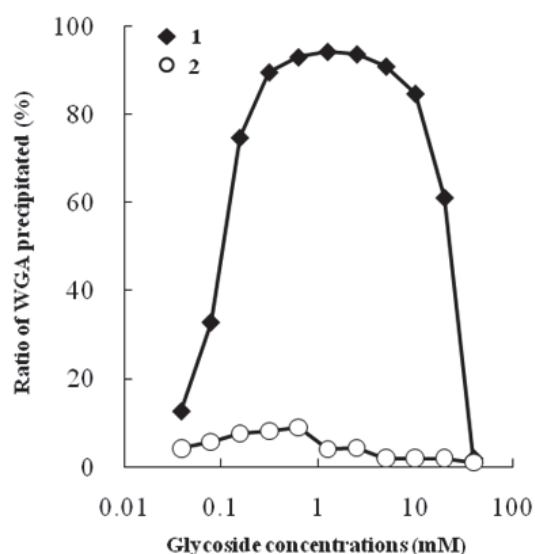

Fig. 1. Precipitin curves for the precipitation of WGA with spacer-O-linked divalent glycosides. The percentage of precipitated WGA was calculated by subtracting the amount of WGA in the supernatant from the total amount of WGA.

We have already reported that by surface plasmon resonance (SPR) analysis, spacer-linked divalent glycoside promotes, rather than inhibits, binding of WGA to surface-bound asialofetuin on a sensor chip (Misawa et al., 2008). This result is dramatically different from that of the corresponding monovalent glycoside, which inhibits the binding of the lectin. In the part, a SPR binding assay was used to monitor the cross-linking effect of synthetic divalent glycosides on the interaction of soluble WGA with a surface-bound WGA, which was directly immobilized onto the surface of a sensor chip using the amine coupling method. Cross-linking of the spacer-O-linked divalent glycosides with WGA in solution was minotored by coinjecting an equilibrium mixture of a fixed amount of WGA with a variable amount of glycoside onto a surface-bound WGA. The surface was regenerated at the end of each cycle using 50 mM H_3PO_4. Based on the sensorgrams, we plotted the RUs (response units) at 190 s, corrsponding to the cross-linking maximum responses for the divalent glycosides to the surface-bound WGA, against glycoside concentration (Fig. 2). As anticipated, injection of a solution of WGA in the absence of divalent glycoside (*i.e.*, negative control) resulted in no change of RU. However, as the concentration of divalent glycoside (**1**

and **2**) increased (*i.e.*, from 0.001 mM to 0.1 mM) there was an increse in RU (Fig. 2). GlcNAc, (GlcNAc)$_2$ and monovalent glycoside carrying GlcNAc were also injected as a control and then ploted (Fig. 2). They were not seen on a top of the peak as seen in **1** and **2**, although they seemed to show a silght increase of RU. It suggests that these control samples cannot cross-link between surface- and free-WGA. The maximal RU of glycoside **1**, was 5 times greater than that of **2**. Our data suggests that coinjection of divalent glycoside and tetravalent WGA as analyse results in simultaneous cross-linking. The cross-linking complexes presumably also bind to the surface-bound WGA through unbound divalent glycoside (Fig. 3). Burke *at al.* used SPR to show the trivalent mannose macrocycle, which is more potent than the corresponding monovalent derivative, functions by cross-linking Con A in solution (Burke et al., 2000). This is consistent with our results.
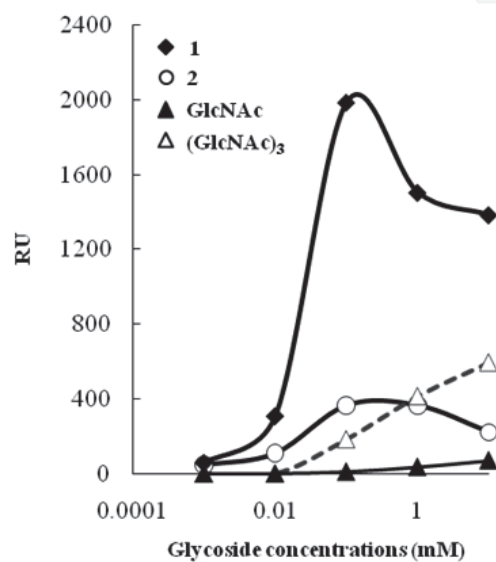

Fig. 2. Cross-linking formation of WGA with spacer-O-linked divalent glycosides on the sensor chip. WGA corresponding to 5700 response units was immobilized onto the sensor chip.
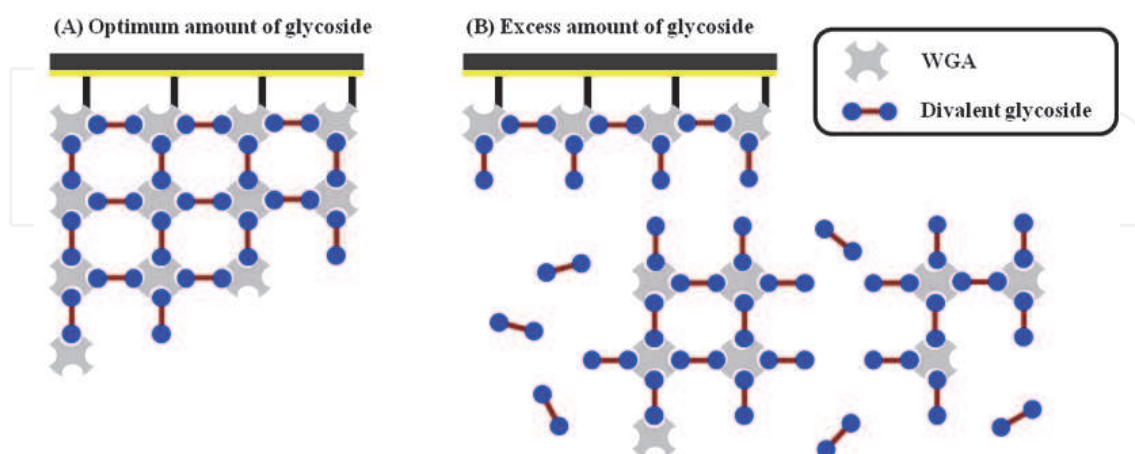

Fig. 3. Diagrams of cross-linking formation and collapse of cross-linking complex on the chip. (A) The formation of complex under optimum amount of spacer-O-linked divalent glycosides. (B) The collapse of complex under excess amount of spacer-O-linked divalent glycosides.

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3. Synthesis of spacer-N-linked divalent glycosides and their cross-linking activities with WGA lectin

We showed an enzymatic method for preparing spacer-O-linked divalent glycosides bearing GlcNAc by chitinolytic enzyme-mediated transglycosylation (Scheme 1) [Misawa et al., 2008(a); Misawa et al., 2009]. The enzyme catalyzes the direct transfer of GlcNAc residue to a primary diol acceptor. The divalent glycoside was shown to bind, cross-link and precipitate WGA. These results encouraged us to prepare spacer-N-linked divalent GlcNAc, (GlcNAc)₂ and LacNAc-glycosides as ligands that possess cross-linking activity with target lectins [Misawa et al., 2008(b)].

3.1 Synthesis of spacer-N-linked divalent glycosides

As represented in Scheme 2, an amino function was introduced into the anomer position of GlcNAc, (GlcNAc)₂, and LacNAc with ammonia according to the method of Hiratake and co-workers (Kato et al., 2005). The resulting N-β-mono- and diglycosylamines were then condensed with one of the three dicarboxylic acids (n = 3, 4 or 5) with HBTU and diisopropyl ethanolamine (DIEA) in DMSO. The N-glycosylation proceeded stereoselectively within a few hours to give only the β-glycoside without the need for any protection and deprotection step. The yields of **3-8** were 50-60%, based on the amount of dicarboxylic acid added. The target compounds were purified by chromatography on a single silica gel column and their structures were determined by ^{1}H and ^{13}C NMR absorptions on the basis of two dimensional C-H COSY techniques. The compounds were soluble in water and DMSO. This method affords an easy and efficient synthesis of spacer-N-linked divalent glycosides bearing GlcNAc, (GlcNAc)₂, and LacNAc from the corresponding glycosylamine.
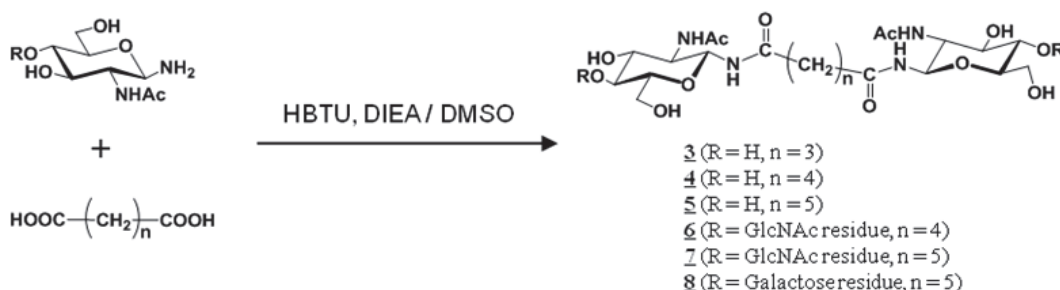
Scheme 2. Synthesis of spacer-N-linked divalent glycosides.

3.2 Interaction of spacer-N-linked divalent glycoside with WGA
3.2.1 Precipitation analysis

The interaction of the resulting spacer-N-linked divalent glycosides carrying GlcNAc or (GlcNAc)₂ with WGA was analyzed by two different methods: precipitation and biosensor analyses. Spacer-O-linked divalent glycoside **1**, which has the ability of precipitating WGA as a divalent ligand, was used as a control. The ability of the synthetic spacer-N-linked divalent glycosides (**3-7**) to bind to WGA was first analyzed by precipitation analysis. WGA (256 μM) and divalent glycoside (0.025-12.8 mM) were mixed on a 96-well microplate. When each divalent GlcNAc- and (GlcNAc)₂-glycoside was added to the WGA solution under appropriate conditions, **3-7** formed a precipitate within a few mnites. However, the presence of chitooligosaccharide [(GlcNAc)₂ and (GlcNAc)₃] prevented the formation of precipitates with **3-7**. Furthermore, the precipitates formed with **3-7** dissolved upon the addition of the

oligomer. These results indicate that the divalent glycosides **3-5** were prepared by measuring the lectin concentration in the supernatant (Fig. 4A).
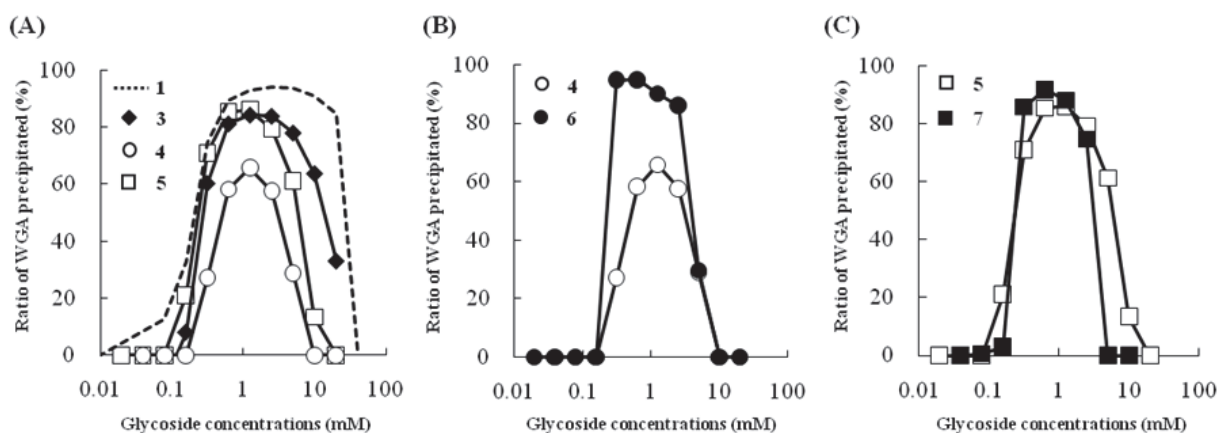

Fig. 4. Precipitin curves for the precipitation of WGA with spacer-N-linked divalent glycosides. The percentage of precipitated WGA was calculated by subtracting the amount of WGA in the supernatant from the total amount of WGA. (A) Spacer-N-linked divalent GlcNAc-glycosides **3-5** precipitated WGA. The curve corresponding to spacer-O-linked divalent glycoside **1** is shown by a dashed line for comparison. (B) Spacer-N-linked divalent GlcNAc- and (GlcNAc)$_2$-glycosides bearing -(CH$_2$)$_4$- on the spacer precipitated WGA. (C) Spacer-N-linked divalent GlcNAc- and (GlcNAc)$_2$-glycosides bearing -(CH$_2$)$_5$- on the spacer precipitated WGA.

The ability to form a precipitate was compared with that of spacer-O-linked divalent glycoside **1**. The concentration of **3**, **4** or **5** at the equivalence point (region of maximum precipitation) of the precipitin curve for 128 μM of WGA was about 1 mM. By constract, the concentration of **1** at the equivalence point was in the region of 0.1-2.5 mM, although the value was not accurately determined under the present conditions. Figure 4B compares the precipitin curves of **4** and **6** bearing GlcNAc or (GlcNAc)$_2$, respectively, with the same spacer group. These results clearly show that the addition of GlcNAc to **4** increases the amount of precipitate obtained in the assay. On the other hand, the addition of GlcNAc to **5** did not substantially increase the amount of precipitate generated in the assay (Fig. 4C). Precipitation analysis showed that spacer-N-linked divalent GlcNAc glycosides specifically bind to WGA in an analogy with spacer-O-linked divalent GlcNAc glysoides. However, the effect of the spacer-N-linked divalent GlcNAc glycosides on the interaction with the lectin was somewhat weaker than that of sapcer-O-linked divalent GlcNAc glycoside **1**. Our results demonstrate that divalent analogs with flexible spacer groups (O-linked) between the two GlcNAc residues possess higher affinity for the lectin compared to those bearing an inflexible spacer group (N-linked) through an NHCO linkage. Furthermore, the length of the spacer group between the two GlcNAc residues appears to affect the ability to precipitate WGA. The maximum amount of precipitate generated with divalent glycosides increased 30-33% when the spacer unit contained an odd number of methylene groups (**3** and **5**) compared to a corresponding glycoside with an even number of methylene groups in the spacer unit (**4**). From these results, the length of the spacer might be important in allowing the molecule to adopt a favorable orientation during interaction with the GlcNAc binding site. It has been reported that the binding and cross-linking properties of lectins are sensitive to the degree of

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flexibility and spacing between the carbohydrate epitopes of the analogs (Dam et al., 2005; Krishnamurthy et al., 2007), which is entirely consistent with the present results.

3.2.2 SPR analysis

We have already reported that by SPR analysis, spacer-O-linked divalent glycoside **1** promotes, rather than inhibits, binding of WGA to surface- bound asialofetuin on a sensor chip [Misawa et al., 2008(a); Misawa et al., 2009]. Thus, we plotted the RUs at 190 s, which corresponds to the cross-linking maximum responses for the divalent glycosides to the surface-bound WGA, against glycoside concentration (as shown in Fig. 5), although some of the sensorgrams did not reach equilibrium. Our main purpose was to prove its ability to act as a divalent ligand through SPR analysis. In this case, the amount corresponded to 5700 RU of WGA (Figs. 5A and 5B) immobilized onto the surface, while 8700 RU in Fig. 5C. The cross-linking activity with the GlcNAc divalent glycosides increased in the order of **1 > 3 = 5 > 4** (Fig. 5A). The maximal RU of compound **6**, bearing (GlcNAc)$_2$, was 30% greater than that of **4**, bearing a single GlcNAc with the same spacer unit (Fig. 5B). In contrast, the maximal RU of compound **7** bearing (GlcNAc)$_2$ was 30% smaller than that of **5** bearing GlcNAc (Fig. 5C). The binding activity corresponds to the results from the precipitation analysis. Our data suggest that co-injection of divalent glycoside and tetravalent WGA as analyte results in simultaneous cross-linking. This result shows that the length of the spacer group between two terminal residues greatly influences the cross-linking activity.
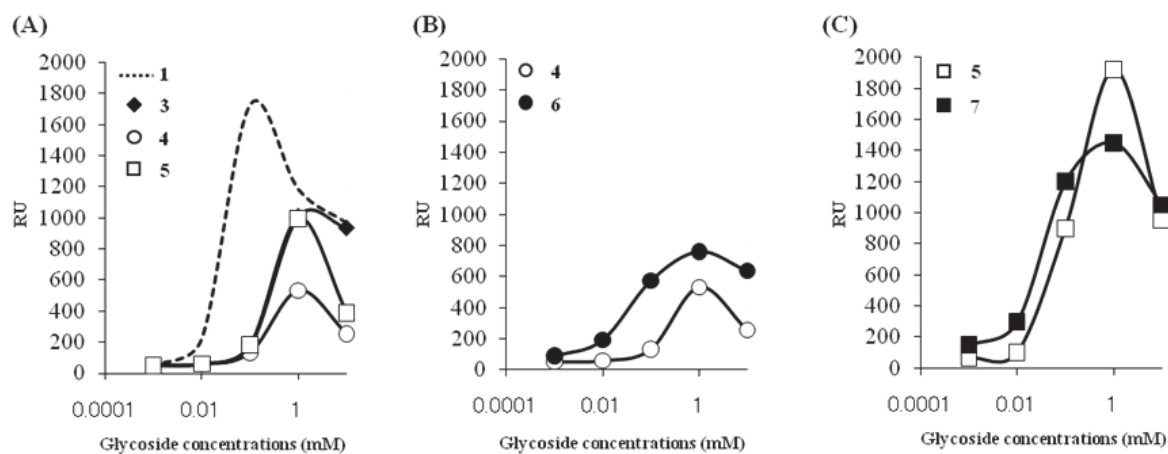

Fig. 5. Cross-linking formation of WGA with spacer-N-linked divalent glycosides on the sensor chip. In case of (A) and (B), WGA corresponding to 5700 response units was immobilized onto the sensor chip. In case of (C), WGA corresponding to 8700 response units was immobilized onto the sensor chip. (A) Spacer-N- and O-linked divalent GlcNAc-glycosides **1** and **3-5**. (B) Spacer-N-linked divalent GlcNAc- and (GlcNAc)$_2$-glycosides bearing -(CH$_2$)$_4$- on the spacer. (C) Spacer-N-linked divalent GlcNAc- and (GlcNAc)$_2$-glycosides bearing -(CH$_2$)$_5$- on the spacer.
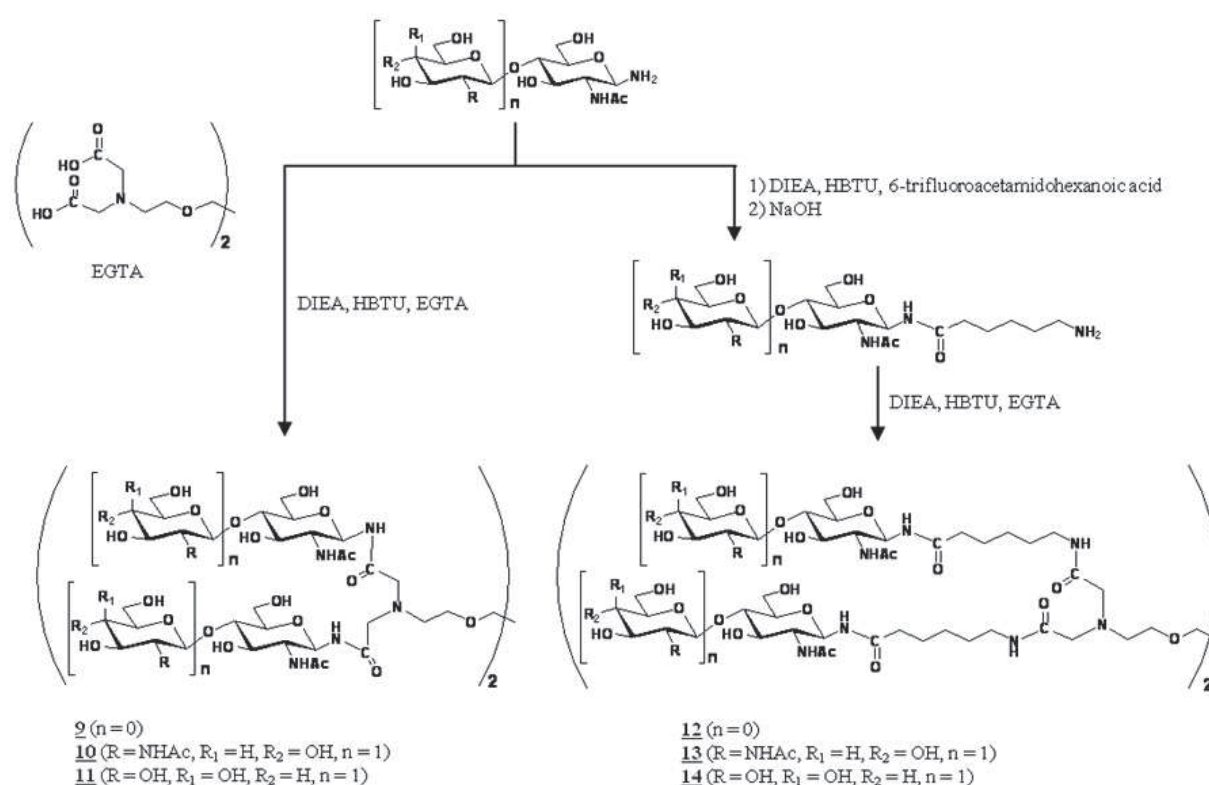
4. Molecular design of spacer-N-linked tetravalent glycosides and their cross-linking activities with WGA and ECA lectins

We showed a synthetic method for generating spacer-O/N-linked divalent glycosides bearing GlcNAc, (GlcNAc)$_2$ and LacNAc with different spacer groups. The divalent

glycosides were shown to be capable of precipitating WGA and jack bean (*Canavalia ensiformis*) agglutinin (Con A) as divalent ligands. They were even able to achieve divalency in systems of low valency. These results encouraged us to prepare multivalent carbohydrate analogs for high affinity binding to target lectins (Masaka et al., 2010).

4.1 Synthesis of tetravalent glycosides

We designed two types of nonspacer- and spacer-N-linked tetravalent glycosides to increase the binding strength with specific lectins. Two types of N-linked tetravalent glycosides bearing GlcNAc, (GlcNAc)$_2$ and LacNAc were designed and prepared using ethylene glycol bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetate (EGTA) as a precursor, which is widely used as a calcium-specific chelator, Scheme 3.

9 (n = 0)
10 (R = NHAc, R$_1$ = H, R$_2$ = OH, n = 1)
11 (R = OH, R$_1$ = OH, R$_2$ = H, n = 1)

12 (n = 0)
13 (R = NHAc, R$_1$ = H, R$_2$ = OH, n = 1)
14 (R = OH, R$_1$ = OH, R$_2$ = H, n = 1)

Scheme 3. Synthesis of nonspacer- and spacer-N-linked tetravalent glycosides.

Nonspacer-N-linked tetravalent glycosides were firstly prepared as follows. The *N*-β-mono- and diglycosylamines bearing GlcNAc, (GlcNAc)$_2$ and LacNAc were directly condensed with tetra-acetate EGTA in DMSO containing HBTU and DIEA. The tetravalent glycosides **9**, **10** and **11** were purified by chromatography on a Bio-Gel P-2 column and a charcoal-Celite column, producing yields of 55, 26 and 23%, respectively, based on the amount of EGTA added (Scheme 3). For an alternative design, we made a spacer-N-linked tetravalent glycoside by insertion of a spacer group between the sugar and EGTA. Thus, the corresponding *N*-β-glycosylamines were first condensed with the carboxyl group of 6-trifluoroacetamidohexanoic acid as in Scheme 3. The resulting spacer-N-linked glycosides were then deacylated to the corresponding amino group by alkali treatment, and the amino group was reacted with EGTA, as described above. Targets **12**, **13** and **14** were purified by chromatography on a Bio-Gel P-2 column and an ODS column producing high yields of 71,

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74 and 67%, respectively, based on the amount of EGTA added. The tetravalent glycosides were elucidated by ^{1}H and ^{13}C NMR analyses, as described previously [Misawa et al., 2008(b); Ogata et al., 2009]. Structures of tetravalent glycosides bearing (GlcNAc)₂ were used as a reference in the analysis of ^{1}H NMR spectra. In the ^{1}H NMR spectra of **10** and **13**, two types of glycosidic proton signals were clearly observed in the lower field with larger coupling constants (**10**, δ 5.11, $J_{1,2}$ 9.8 Hz, H-1 and δ 4.63, $J_{1',2'}$ 8.6 Hz, H-1'; **13**, δ 5.06, $J_{1,2}$ 9.0 Hz, H-1 and δ 4.62, $J_{1',2'}$ 8.5 Hz, H-1'). In ^{13}C NMR spectra, two types of glycosidic signals were also characterized by the lower field (**10**, δ 104.2, C-1'β and δ 81.0, C-1β; **13**, δ 104.2, C-1'β and δ 81.1, C-1β). The spectra showed only the separated and overlapping resonances corresponding to respective sugar moieties. This simplicity suggests that these four sugar moieties can be superposed upon each other. ESI-MS analysis of tetra-headed glycosides **9**, **10**, **11**, **12**, **13** and **14** showed molecular ions at *m/z* 1211.5, 2023.8, 1859.7, 1663.8, 2477.4 and 2312.3, respectively, arising from the [M + Na]⁺ ions. These results indicate that the resulting tetravalent glycosides consist exclusively of stereoregular sugars with tetravalent units. These compounds were soluble in water and the solubilities were 5 ~ 10%. Our synthetic methods are easy and efficient in the synthesis of tetravalent glycosides with stereoregular sugars through tetravalent carboxyl groups.

4.2 Interaction of tetravalent glycosides with specific lectins
The resulting tetravalent glycosides were used in the analysis of WGA and ECA: Interactions were measured using four methods: hemagglutination inhibition assay, precipitation assay, double-diffusion test, and biosensor analysis.

4.2.1 Hemagglutination inhibition assay
The interaction abilities of a series of divalent N^1,N^7-di-(2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl)-pimeamide (**7**) and tetravalent glycosides related to GlcNAc were compared with those of (GlcNAc)₂ and (GlcNAc)₃, which were used as control samples and are potent inhibitors of the hemagglutination of WGA (Table 1). In the hemagglutination inhibition assay, a multivalency effect was observed for **10**, **12** and **13**, but not for **9** bearing GlcNAc, when compared with (GlcNAc)₂, (GlcNAc)₃ and divalent **7**. Compounds **10**, **12** and **13** acted as inhibitors and their activities were 2 ~ 1042 fold higher than the reducing sugars (GlcNAc)₂ and (GlcNAc)₃ and 1 ~ 260 fold higher than divalent **7**. Compound **13** was the most effective inhibitor at a low concentration among the tetravalent glycosides: IC₅₀ was 0.18 μM. Inhibition by flexible tetravalent **13**, bearing tandem GlcNAc, was much higher than the analog **12**, bearing a single GlcNAc and rigid nonspaced **10**, bearing tandem GlcNAc. The inhibition activity of the tetravalent glycosides increased in the order of **13** > **10** > **12** > **9**. It is worth noting that **13**, despite its low molecular weight, increases binding by a factor of 4 ~ 8 when compared with ovalbumin and asialofetuin.

In contrast, multivalency effects were not much detected in ECA (Table 2). The minimal inhibitory concentrations of the lectin mediated hemagglutination of ECA were: 47 μM for divalent LacNAc-glycoside (**8**), 12 μM for **11** and 5.9 μM for **14**. The relative binding affinity of **14** was one hundred and thirty times less than the binding affinity of asialofetuin. We suggest that ECA has a small multivalency enhancement ability, when comparing with WGA. This would be due to a difference of the binding mode on sugar sequence. ECA acts in an exo-manner on LacNAc sequence, while WGA both in endo and exo-manner on

GlcNAc sequence (Goldstein & Poretz, 1986). We have already reported the ECA is much less subject to the cluster effect by glycopolymer than WGA (Zeng et al., 2000).

Inhibitors	Sugar moiety	Valency	IC$_{50}$[a] (µM)	IC$_{50}$[b] (µM)
(GlcNAc)$_2$	-	Mono	750	750 (1)[d]
(GlcNAc)$_3$	-	Mono	375	375 (2)
7	(GlcNAc)$_2$	Di	93.8	188 (4)
9	GlcNAc	Tetra	>4000	-
10	(GlcNAc)$_2$	Tetra	5.86	23.4 (32)
11	LacNAc	Tetra	>4000	-
12	GlcNAc	Tetra	46.9	188 (4)
13	(GlcNAc)$_2$	Tetra	0.18	0.72 (1042)
14	LacNAc	Tetra	ND[c]	-
Ovalbumin	-	-	1.46	-
Asialofetuin	-	-	0.73	-

[a] Minimum concentrations required for complete inhibition of hemagglutination.
[b] Concentration of sugar units.
[c] ND: not determined.
[d] Relative inhibitory potency. (All data normalized to those of (GlcNAc)$_2$. Higher values indicate greater inhibitory potency.)

Table 1. Inhibition of WGA-mediated hemagglutination by di- and tetravalent glycosides.

Inhibitors	Sugar moiety	Valency	IC$_{50}$[a] (µM)	IC$_{50}$[b] (µM)
LacNAc	-	Mono	94	94 (1)[c]
8	LacNAc	Di	47	94 (1)
11	LacNAc	Tetra	12	48 (2)
14	LacNAc	Tetra	5.9	24 (4)
Asialofetuin	-	-	0.092	-
Fetuin	-	-	0.37	-

[a] Minimum concentrations required for complete inhibition of hemagglutination.
[b] Concentration of LacNAc units.
[c] Relative inhibitory potency. (All data normalized to those of LacNAc. Higher values indicate greater inhibitory potency.)

Table 2. Inhibition of ECA-mediated hemagglutination by di- and tetravalent glycosides.

4.2.2 Precipitation analysis

In the quantitative precipitation assay, tetravalent glycosides were shown to be capable of precipitating WGA/ECA as tetravalent ligands. The result is consistent with the sugar specificities of the corresponding lectins reported for naturally occurring glycoproteins, glycolipids and oligosaccharides. The precipitin profiles (Figs. 6 and 7) are similar to antigen-antibody (Kabat 1976), lectin-polysaccharide (Usui et al., 1981), and lectin-glycopeptide [Bhattacharyya et al., 1988(a)] and complex-type oligosaccharide-Con A precipitin curves [Bhattacharyya et al., 1987(b)] which suggests similar multivalent interactions between the tetravalent glycosides and WGA/ECA, despite the low molecular weights as ligands. The precipitin profile between flexible **12/13** with spacer and WGA was shown to proceed stoichiometrically, compared with rigid **9** and **10** with no spacer.

Compounds **12** and **13** behaved tetravalently in WGA binding. Similar precipitation experiments with spacer-linked **14** showed that it is divalent for ECA binding. No precipitates were observed between divalent glycoside and ECA. The results suggested the spacer-linked glycosides effectively bind and promote cross-linking of WGA in solution, rather than inhibiting the binding of lectin. Precipitation of WGA with **10**, **12** and **13** occurs at room temperature, whereas precipitation of ECA with **11** and **14**, which bind more weakly, requires a lower temperature (4°C). In general, the precipitation data for ligand with lectin reflects the relative affinities of carbohydrate for the proteins. The binding activities of lectins, including WGA and ECA, produced cross-linked complexes with the tetravalent glycosides. The results were supported by the double diffusion test. However, ECA has a much smaller multivalency enhancement than WGA. The present results are easily predictable by previous our reports. Because we have already reported that artificial glycopolymers bearing (GlcNAc)$_2$ unit shows a strong interaction with WGA, because of a cluster effect of multivalent oligosaccharide chains in the polymer, whereas ECA is much less subject to cluster effect by glycopolymer bearing LacNAc unit (Zeng et al., 1998).
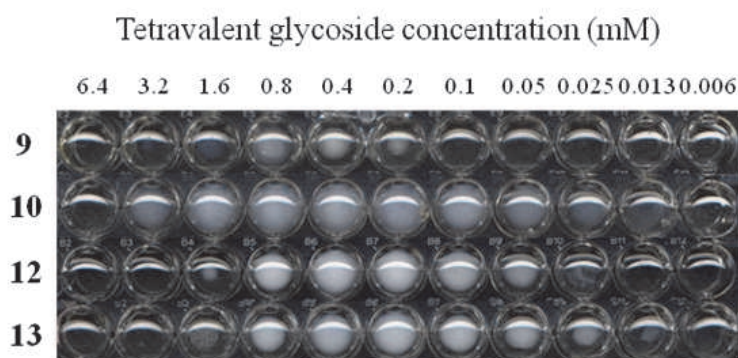

Fig. 6. Photographs of precipitate formed by addition of tetravalent glycosides to WGA-PBS solution.
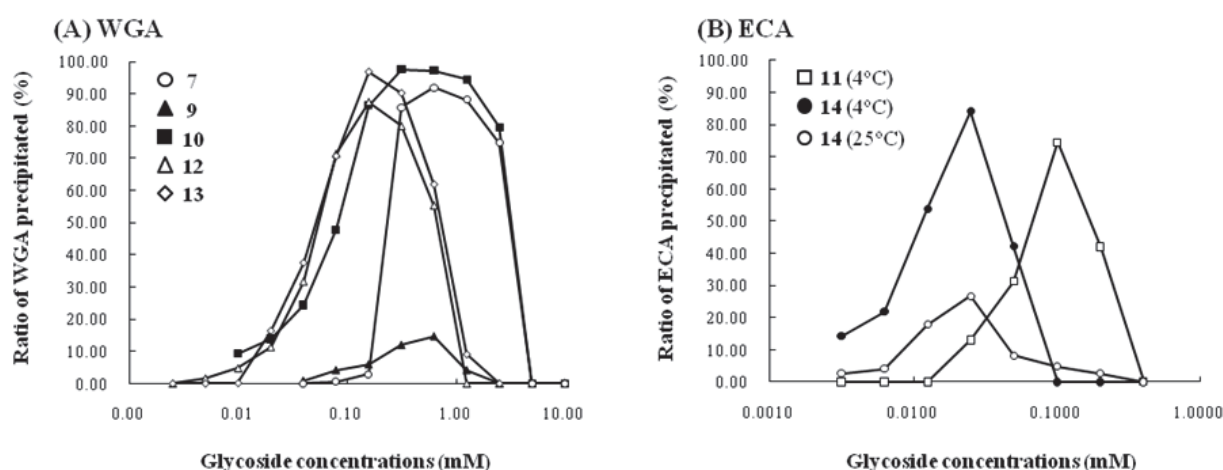

Fig. 7. Precipitin curves for the precipitation of WGA (A) and ECA (B) with tetravalent glycosides. The percentage of precipitated WGA or ECA was calculated by subtracting the amount of WGA/ECA in the supernatant from the total amount of WGA/ECA, respectively.

4.2.3 Double diffusion test

Figure 8 shows the results of double diffusion reactions using tetravalent glycosides **9-14** with WGA and ECA lectins. In general, precipitate is formed by the reaction between two specific high molecular weight compounds, such as lectin-glycoprotein and lectin-polysaccharide (Ogata et al., 2007; Zeng et al., 2000; Usui et al., 1981). Interestingly, sharp precipitin bands are seen between the central well with WGA and the surrounding wells containing tetravalent glycosides **10**, **12** and **13** (Fig. 8A). Precipitin bands were not observed with **9**, **11** and **14**. Weak precipitin bands were observed between the central well with ECA and the surrounding wells containing **11** and **14** bearing LacNAc at 4°C (Fig. 8B). These reactivities correlate well with the results of the hemagglutination inhibitory assay.
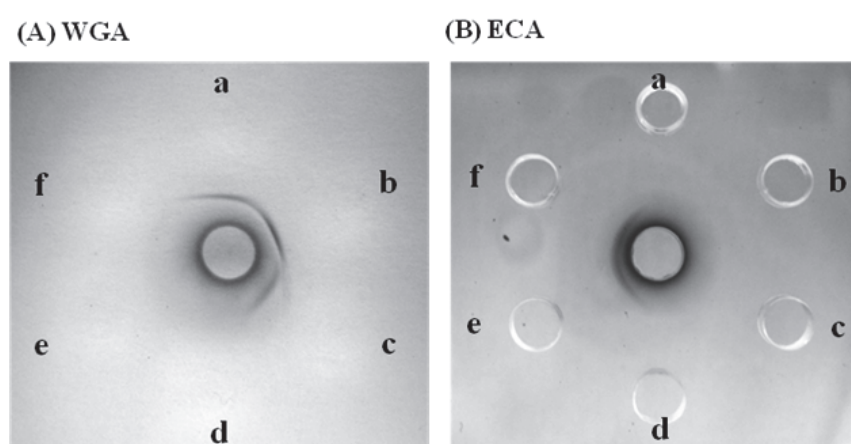
Fig. 8. Double diffusion reaction of tetravalent glycosides with WGA (A) and ECA (B) in agar gel. Lectin solutions were put in the center wells. Portions of tetra-headed glycoside solutions were added to the peripheral wells. a: **13**; b: **10**; c: **12**; d: **9**; e: **14**; f: **11**.

4.2.4 SPR analysis

SPR competition binding assays were used to monitor the effect of tetravalent glycosides on the interaction of soluble WGA with surface-bound WGA. The dissociation rate of **10** and **13** at maximal RU bearing tandem GlcNAc was much slower than that of **9** and **12** bearing single GlcNAc (Fig. 9). The dissociation rates can be summarized as: **13 > 10 >> 12 > 9**. The slow down of the dissociation rate enhances binding affinity, and is due to multivalency effects (Lee & Lee, 2000). This relationship is consistent with the hemagglutination inhibition activity mention above. Thus, it indicates that the binding affinity is not only enhanced by the existence of a spacer on the tetravalent glycosides, but also a sugar length. In our study, RU increased as the concentration of the tetravalent glycosides increased from 0.1 µM to 0.1 mM. Thus, the tetravalent glycosides promoted, rather than inhibited, binding of WGA to a surface-bound WGA. Our data suggests co-injection of tetravalent glycoside and tetravalent WGA as analyte results in simultaneous cross-linking. The cross-linking complexes presumably bind to the surface-bound WGA through unbound tetravalent glycosides. Compounds **12** and **13**, with flexible spacer, clearly show maximum binding at 0.1 mM and spacer-N-linked divalent GlcNAc-glycoside (**5**) displayed maximum binding at 1 mM (Fig. 10). The results of SPR analysis corresponded with the results of the precipitation assay.

Our results show flexible tetravalent **13** has particularly high-affinity for WGA. Gour and Verma with the help of AFM (atomic force microscope) imaging demonstrated strong,
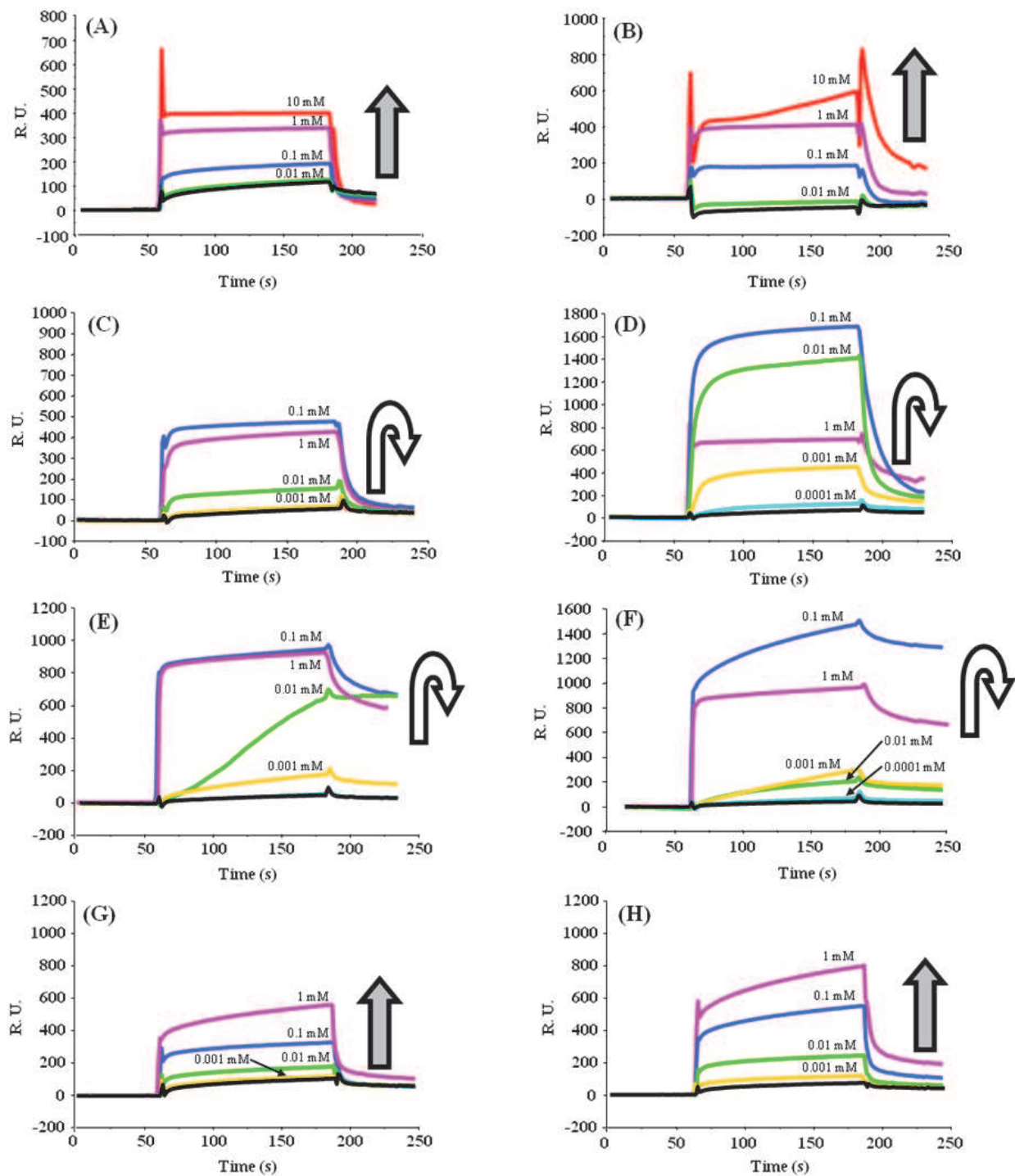
Fig. 9. Sensorgrams showing the interactions of the glycosides with WGA. WGA corresponding to 8100 (A and B) or 6700 (C to H) response units was directly immobilized onto the sensor chip. WGA was co-injected with glycoside: (A) (GlcNAc)₂, (B) (GlcNAc)₃, (C) **9**, (D) **12**, (E) **10**, (F) **13**, (G) **11**, and (H) **14** [Color lines show increased and decreased RU, or injected without glycoside as a negative control (black line)].

complex interactions between a trivalent mannose conjugate and Con A (Gour & Verma, 2007). Their results suggest the orientation of a tetravalent glycoside as ligand was highly compatible with the formation of cross-linked complexes, which is the likely cause of binding enhancement for this class of lectin. The chelate effect leads to large rate enhancement in tetravalent systems, with favorable orientation of ligands. In the chelation mechanism, neighboring binding sites are simultaneously occupied by ligands bridged by suitable spacers (Rao et al., 1998). Typically, binding of the first ligand is thought to reduce the entropic barriers for binding the second and further ligands. The chelate effect can lead to a large rate enhancement for the tetravalent system of **13**, so that the spacer is optimal in terms of flexibility. On closer inspection, it was found that binding enhancement was due to a combination of multivalency effects and spacer effects.
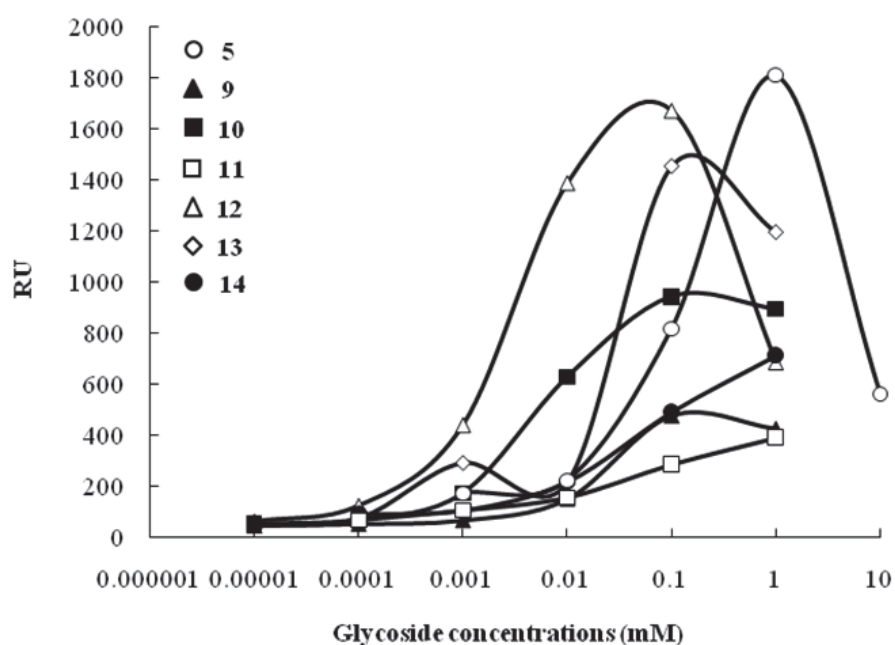
Fig. 10. Cross-linking formation of WGA with tetravalent glycosides on the sensor chip. WGA corresponding to 6700 response units was immobilized onto the sensor chip.

5. Summary

We designed various type di- and tetravalent glycosides to increase the binding strength of synthetic ligands for WGA and ECA. Synthesized di- and tetravalent glycosides bearing GlcNAc, (GlcNAc)$_2$ and LacNAc were found to be capable of binding and precipitating specific lectins as di- or tetravalent ligand. Especialy, spacer-N-linked tetravalent glycoside bearing flexible tandem GlcNAc (**13**) showed a strong multivalency effect for WGA, as studied through lectin-ligand interactions. Our results suggest that even small synthetic conjugates could act as potential ligands for lectin binding, producing similar multivalent interactions with glycoprotein. Future studies on the interaction of synthetic multivalent glycoside conjugates with lectins for the generation of cross-linking complexes are planned and are expected to complement other biophysical studies of lectin-multivalent carbohydrate interactions.

6. Acknowledgment

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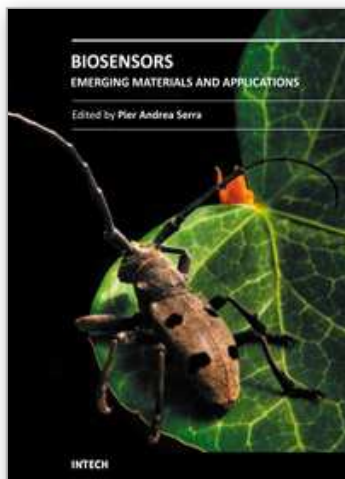
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