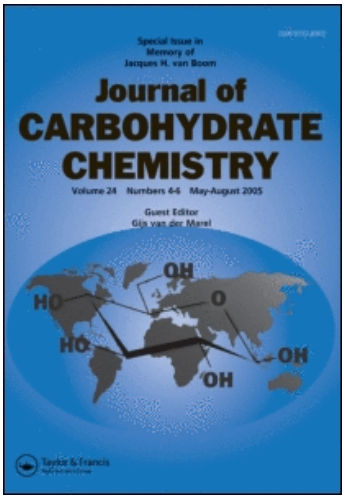

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A Facile Latex Agglutination Lectin Assay (LALA) for Weakly Binding Ligands

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To apply the latex agglutination lectin assay (LALA) to carbohydrate ligands, monosaccharide derivatives were incorporated onto latex beads by various methods, and the usefulness of the resulting beads was evaluated. The best outcome, which resulted in aggregation with lectin concentrations of 1 to 4 μg/mL, was obtained when latex beads coated with bovine serum albumin were treated with divinylsulfone, a linker agent, and then with 2-aminoethyl glycosides. Monosaccharides with an amino or anomeric hydroxyl group other than *N*-acetylglucosamine were applicable in this direct LALA. For example, mannose- and 5-thiomannose-coupled latex beads showed aggregation with minimum concanavalin (ConA) concentrations of 4 and 32 μg/mL, respectively. An inhibition assay was more versatile than the direct LALA, and the standardized inhibition activity (EC^0_{50}) was determined for several compounds. Representative EC^0_{50} data for mannose, methyl mannoside, and *p*-nitrophenyl mannoside (1, 0.12, and 0.06 mM, respectively) are consistent with those reported with other methods. We obtained EC^0_{50} values for some synthetic compounds with slightly different binding abilities to ConA, demonstrating a semiquantitative character of this method. The inhibition LALA can be performed without instrumentation or tedious derivatization and is thus suitable for the rapid evaluation of monovalent ligands prior to assemblage into multivalent ligands.

Keywords Latex beads, lectin, agglutination, monosaccharides, inhibition assay

INTRODUCTION

The creation of ligands that strongly and specifically bind to lectins has been a major goal for synthetic carbohydrate chemists.[1] A number of synthetic lectin ligands synthesized thus far are multivalent[2] and were designed on

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the monovalent basis and then assembled on scaffolds (e.g., bovine serum albumin [BSA]).[3] The underlying concept of this ligand design is that even a monovalent ligand with a millimolar dissociation constant can be recognized very specifically by a lectin, and the binding strength is increased by up to six orders of magnitude when the ligand acquires multivalency.[2]

To improve the specificity, stability in a living body, and solubility of these ligands in a short period of time, optimization of the monovalent ligand structure through a rapid lectin assay of a ligand library is essential because screening at the monovalent stage will avoid the assembly of less active multivalent ligands. Monosaccharide derivatives as monovalent ligands are more synthetically accessible than oligosaccharides, and indeed, a number of highly active lectin ligands consist of assembled monosaccharides. However, most monosaccharides have weak binding abilities to lectins, which decreases the power of many lectin assays.

Even the enzyme-linked lectin assay (ELLA),[4] the most popular lectin assay, may not be the method of choice for the direct evaluation of monosaccharides attached to a microplate because lectins that are weakly bound to monosaccharides on the microplate are easily detached during washing processes. In practice, we needed at least five mannosyl units assembled on a peptoid backbone to recognize concanavalin A (ConA) in the ELLA method,[5] whereas the mannose monomer was sufficient in a solution fluorescence anisotropy assay,[6] which is impractically time consuming for library screenings. Inhibition experiments with strong multivalent ligands such as glycoproteins are another option in ELLA,[7] but the availability of suitable glycoproteins and enzyme-linked lectins is limited, and a microplate reader is indispensable.

In this context, the latex bead-based agglutination lectin assay (LALA), which is facile, is sensitive, and requires neither special instruments nor lectin modifications, may be superior to ELLA. In a typical LALA, a glycoprotein such as a blood group substance[8] or ovalbumin[9] is adsorbed on latex beads, and the agglutination caused by multivalent binding between a lectin and the oligosaccharides is subjected to inhibition experiments with test compounds. Instead of glycoproteins, glycopeptides can be covalently bound to functionalized latex beads.[10] These LALA methods also suffer from limited availability of glycoproteins or glycopeptides. However, the attachment of synthetic or inexpensive saccharides on latex beads via covalent bonds or physical adsorption resolves the availability problem and enables direct measurement of the binding. Castellanos et al. reported the adsorption of monosaccharide-bound BSA on latex beads,[11] but most glycoproteins are quite expensive, or if unavailable, tedious preparation of the neoglycoprotein is necessary. The covalent coupling of carboxyazide-functionalized glycosides onto BSA-coated latex is the easiest reported protocol for LALA,[12] although synthesis of the functionalized sugars is still a little tedious. On the other hand, Kaul et al. reported the coupling

of *N*-acetyl-glucosamine with carboxyl-coated latex beads using a dehydrating agent,[13] but the origin of the unusual reactivity of the hydroxyl (or acetamido) groups toward the activated carbonyl function was not stated. This method seems to be limited to the reported *N*-acetylglycosamines.

After we had started the study of LALA, we found reports regarding an agglutination assay with gold nanoparticles immobilizing sugars.[14] In this gold nanoparticles assay, the aggregation formed can be visually observed and may be quantified by measuring the supernatant for absorbance at 530 nm. However, preparation of the modified gold nanoparticles involves several synthetic processes, which may limit versatility. We thus continued the study for LALA, focusing more on the easy preparation of latex-sugar conjugates.

RESULTS AND DISCUSSION

From a survey of the literature as stated above, we found that there are no standard LALA protocols that can be generally used for weakly binding monovalent ligands. Thus, we sought an improved LALA method in which the only required operations would be mixing of reagents and centrifugation, and neither tedious syntheses nor expensive instruments would be required to complete the assay. We explored both a direct assay with a target ligand bound to latex beads and an inhibition assay that used a target ligand and a standard sugar-latex conjugate.

To this end, we exploited divinylsulfone (DVS)[15] and 1,5-difluoro-2,4-dinitrobenzene (DFDNB)[16] as homobifunctional cross-linkers that can bridge -OH and/or -NH_2 groups of a ligand and the latex bead surface. Nucleophiles react at two vinyl groups through a Michael addition for DVS or at the aromatic carbon adjacent to fluorine through a substitution reaction for DFDNB. For the latex bead surface, we used BSA- or amino-coated latex beads. The amino groups of lysine residues in BSA provide nucleophiles toward DVS or DFDNB.

2-Aminoethyl α-D-mannopyranoside (ManEtN or **Mn**),[17] 2-aminoethyl α-L-fucopyranoside (FucEtN or **Fn**),[18] 2-aminoethyl *N*-acetyl-β-D-glucosamine (GlcNAcEtN or **Nn**),[19] and methyl 5-thio-α-D-mannopyranoside (Me5SMan or **mS**)[20] were synthesized as reported (Fig. 1). 2-Aminoethyl 5-thio-α-D-mannopyranoside (5SManEtN or **Sn**) was synthesized as shown in Scheme 1. Per-*O*-acetyl-5-thiomannose (**1**), synthesized from mannose in nine steps,[21] was subjected to glycosidation with 2-bromoethanol to give 2-bromoethyl mannoside **2**, which was treated with sodium azide to give 2-azidoethyl mannoside **3**. Deacetylation and reduction of the azide group gave 5SManEtN (**Sn**).

For the first assay run, we used ManEtN (**Mn**) to immobilize mannose on latex beads (**L**) through DVS (**v**) and BSA (**B**) (Fig. 1), and the resultant conjugate (**MnvBL**) was mixed with varied concentrations of ConA in

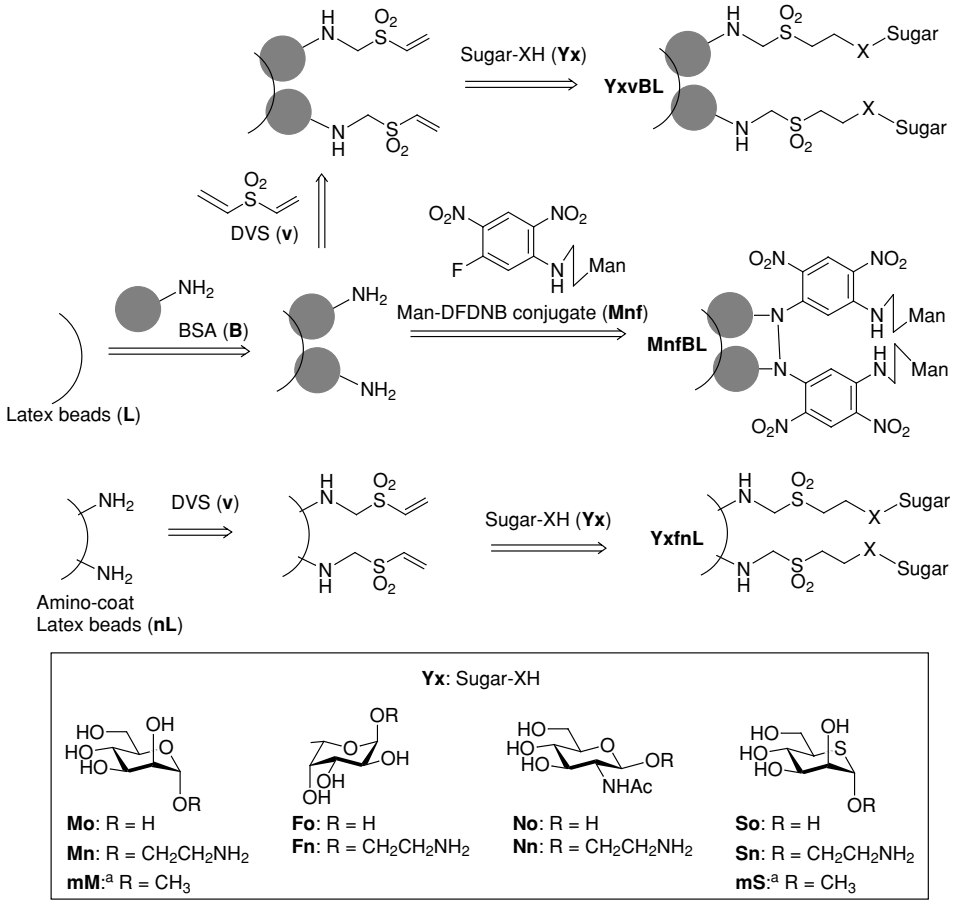
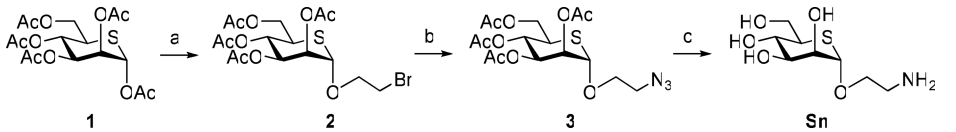


Figure 1: The structures of ligands and their immobilization on latex beads. [a]These compounds were used in the inhibition assays.

microtiter plate wells. Agglutination occurred at ≥ 1 μg/mL ConA, as demonstrated by two- or fourfold serial dilution (Fig. 2; Table 1, entry 2), which we hereafter refer to as a $[\text{ConA}]_{\text{ag}}$ value of 1 μg/mL. We also tested the ability of the amino-coated latex beads (**nL**) to directly immobilize ManEtN through DVS, and Tween 20 was added to prevent nonspecific binding. The covalently linked conjugate (**MnvnL**) caused rather ambiguous agglutination



Scheme 1: a) $HO(CH_2)_2Br$, $BF_3 \cdot OEt_2$, CH_2Cl_2 (79%); b) NaN_3, DMF, 60°C (98%); c) 1) NaOMe, 2) Ph_3P, THF-MeOH-H_2O (84%).

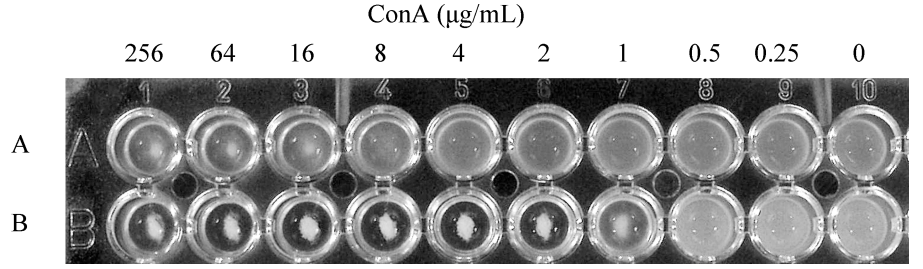


Figure 2: Agglutination assay for ConA with latex beads carrying directly coupled mannoside **MnvnL** (lane A) and BSA-mediated mannoside **MnvBL** (lane B).

that lacked distinct boundaries, with a $[ConA]_{ag}$ value of 16 μg/mL (Fig. 2A; Table 1, entry 1).

To quantify the aggregation, we defined aggregation extent as the standard deviation of the darkness (with values between 0 and 255) over all pixels within the digital image of each microtiter well.[22] The standard deviation of

Table 1: Aggregation extent[a] for LALA with various modified latex beads.[b]

Entry	Modification for latex beads	ConA or a specified lectin (μg/mL)												
		0	**0.1**	**0.3**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**256**	**512**
1	**MnvnL**	7	—	7	7	7	8	8	10	*15*	—	*18*	*19*	—
2	**MnvBL**	7	—	7	6	*26*	*39*	*46*	*44*	*45*	—	*43*	*41*	—
3	**PovnL**	5	—	5	—	6	—	7	—	7	—	9	*16*	—
4	**PovBL**	10	—	—	11	17	12	11	*26*	*40*	—	*44*	*44*	—
5	**MnfBL**	9	—	—	—	—	*7*	*20*	*34*	*41*	—	*32*	*25*	—
6	50 mg/mL **MnvBL**	13	—	12	12	*44*	*48*	*47*	*47*	*44*	*44*	*43*	—	—
7	5 mg/mL **MnvBL**	12	—	11	11	*41*	*49*	*47*	*47*	*46*	*43*	*42*	—	—
8	2.5 mg/mL **MnvBL**	12	—	11	11	*39*	*48*	*47*	*47*	*43*	*44*	*43*	—	—
9	0.5 mg/mL **MnvBL**	12	—	11	11	*25*	*45*	*44*	*45*	*44*	*40*	*42*	—	—
10	0.25 mg/mL **MnvBL**	12	—	11	11	11	*31*	*38*	*41*	*40*	*38*	*36*	—	—
11	**MovBL**	10	10	12	10	15	—	*46*	—	*46*	—	*48*	*47*	*44*
12	**SovBL**	14	—	—	—	—	13	13	11	15	*26*	*40*	*39*	*35*
13	**TovBL**	10	9	9	9	9	—	11		9	—	9	*26*	*31*
14	**FnvBL** + LTA	11	—	9	8	9	8	*24*	*41*	*46*	*48*	*50*	—	—
15	**FovBL** + LTA	11	—	9	10	10	10	9	9	9	*19*	*29*	—	—
16	**NnvBL** + WGA	10	10	10	8	*43*	—	*51*	—	*50*	—	*49*	*46*	*39*
17	**MnvBL**	8	—	9	10	*45*	*46*	*47*	*46*	*46*	—	*45*	*41*	—
18	Mannase + **MnvBL**	10	—	12	11	12	11	11	11	12	—	12	12	—
19	GlcNAcase + **MnvBL**	6	—	7	8	*49*	*49*	*48*	*48*	*46*	—	*44*	*41*	—
20	**NnvBL**	11	—	*23*	*34*	*36*	*35*	*39*	*40*	*41*	—	*36*	*35*	—
21	GlcNAcase + **NnvBL**	11	—	11	11	10	11	11	12	11	—	12	12	—
22	Mannase + **NnvBL**	15	—	*43*	*47*	*49*	*51*	*50*	*50*	*49*	—	*50*	*44*	—
23	**MovBL**	12	—	—	14	12	14	27	*37*	*43*	*43*	*46*	—	—
24	Mannase +**MovBL**	11	—	—	9	12	10	13	13	14	15	18	—	—
25	**SovBL**	14	—	—	15	14	12	14	19	*30*	*36*	*34*	—	—
26	Mannase +**SovBL**	13	—	—	12	15	25	*34*	*41*	*49*	*47*	*48*	—	—

[a]Standard deviation of pixels' darkness (0–255) for the digital image of each well.
[b]The numerals for the wells in which aggregation was observed are italicized.

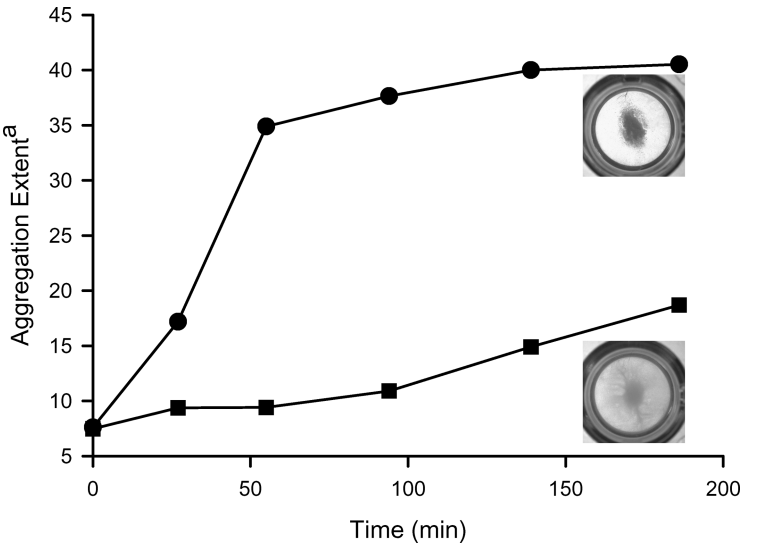


Figure 3: Plots showing the aggregation rate for **MnvBL** (solid circle) and **MnvnL** (solid square) at 256 μg/mL ConA. The inset pictures show the microscopic image of the aggregation at 186 min for each assay. [a]Standard deviation of pixels' darkness (0–255) for the digital image of each well.

darkness increased with the extent of aggregation. As a result, the BSA-coated latex, **MnvBL**, showed faster and greater aggregation than the amino-coated latex, **MnvnL** (Figs. 2B and 3). From the aggregation extents, the amount of aggregation for **MnvnL** at 186 min was estimated to be 46% of that for **MnvBL**. These results, together with the higher price of amino-coated latex beads, indicate that the BSA-mediated immobilization of sugars is preferable to direct covalent bond formation.

We also immobilized mannan (**Po**) on latex beads and compared the agglutination abilities. **PovnL** and **PovBL** caused agglutination with $[ConA]_{ag}$ values of 256 and 8 μg/mL, respectively (Table 1, entries 3 and 4), which were worse than those for the mannose-modified latex beads. This result was surprising because some ELLA studies have reported that mannan, as a strong ligand attached to microplates, persists through washing steps that monosaccharides would not tolerate.[3,23] Perhaps the long chain length of mannan is suitable to embrace a lectin molecule but is likely to exclude mannans attached on other latex beads from binding to the same lectin, thereby weakening the aggregation ability. This phenomenon seems to be related to the observation termed "steric stabilization" found for the inhibition of influenza virus by polymeric compounds.[2c]

We also tested DFDNB (**f**) as a linker between ManEtN and BSA because this linker is less expensive and demonstrated better properties than other generally used linkers in a study of affinities between mannose-BSA

conjugates and ConA.[3] We prepared Man-DFDNB conjugate as previously reported,[3] and it was coupled to latex beads (**MnfBL**). Agglutination occurred at a $[ConA]_{ag}$ value of 4 μg/mL (Table 1, entry 5), which is worse than that for **MnvBL**. Furthermore, the extent of aggregation for **MnfBL** was estimated to be 57% less than that for **MnvBL** at 256 μg/mL ConA. Thus, the merit of using DVS as a linker was justified.

The **MnvBL** conjugate described above was made with 50 mg/mL ManEtN (0.2 mL). This amount is quite large and wasteful for synthetic ligands. Thus, the amount of the sugar was varied (down to 0.25 mg/mL), and 0.5 mg/mL was found to cause agglutination of ConA (1 μg/mL) as well as 50 mg/mL ligand (Table 1, entries 6–10). Therefore, the minimum amount of ManEtN required for 10 agglutination test wells was calculated to be 0.1 mg. The required ConA amount was estimated to be 0.3 μg per assay, for which 4 μg/mL ConA is serially diluted.

We next examined the direct LALA for two related monosaccharides, mannose (Man or **Mo**) and 5SMan (**So**). These compounds were incorporated successfully onto latex beads and caused agglutination with $[ConA]_{ag}$ values of 4 and 32 μg/mL, respectively (Table 1, entries 11 and 12). The difference in aggregation ability between Man and 5SMan is probably due to the weaker binding ability of 5-thiomannose, as observed for 5-thiomannose-containing oligosaccharides.[24] However, incorporation of trehalose (**To**) caused agglutination (entry 13) with a higher $[ConA]_{ag}$ value (256 μg/mL), and a trial with methyl mannoside (MMan or **mMo**) resulted in no agglutination. Thus, the presence of an amino or anomeric hydroxyl group is a prerequisite for ligands in the direct LALA. A trial with 5SManEtN (**Sn**) was also unsuccessful, probably because in this case the monosaccharide was scarcely recognized, as discussed in the section describing inhibition assays.

FucEtN (**Fn**) and fucose (**Fo**) were incorporated successfully onto latex beads (**FnvBL** and **FovBL**) and caused agglutination with Lotus tetragonolobus agglutinin (LTA), a fucose-binding lectin, with $[LTA]_{ag}$ values of 4 and 32 μg/mL, respectively (Table 1, entries 14 and 15). As expected, mannose-bound latex beads, **MovBL**, caused no aggregation with LTA. A trial with *N*-acetylglucosamine (GlcNAc) resulted in no agglutination with wheat germ agglutinin (WGA), a GlcNAc-specific lectin, probably because GlcNAc was not incorporated. On the other hand, the incorporation of GlcNAcEtN (**Nn**) was successful (**NnvBL**) and caused aggregation with a $[WGA]_{ag}$ value of 1 μg/mL (entry 16).

Unfortunately, we have been unable to quantify the amounts of sugars loaded onto latex beads because of some technical problems. To prove that the desired monosaccharides are actually immobilized on latex beads and that these sugars are recognized specifically by lectins, we treated **MnvBL** and **NnvBL** with α-mannosidase (mannase) and β-N-acetylglucosaminidase (GlcNAcase), and the resultant latex beads were mixed

with ConA and WGA, respectively (Table 1, entries 17–22). Mannase treatment of **MnvBL** (entry 18) resulted in no agglutination with ConA, whereas treatment with GlcNAcase caused agglutination (entry 19), which indicates that α-mannosides existed on the latex beads before enzyme treatment (entry 17) and that the sugars were specifically recognized by ConA. The same conclusion can be made from the reverse result with **NnvBL**, in which mannase and GlcNAcase treatments caused agglutination and no agglutination, respectively (entries 20–22). It should be noted that LALA after glycosidase treatment could be considered a novel method to elucidate the specificity of unknown glycosidases.

We also examined the mannosidase tolerance of 5-thiomannoside on **SovBL**. The mannose-latex conjugate, **MovBL**, caused aggregation with ConA (entry 23) and it was inhibited by pretreatment with mannase (entry 24). This result indicates that the DVS linker is attached mainly at the anomeric hydroxyl of mannose in **MovBL**,[15b] forming mannase-cleavable mannosides instead of uncleavable ethers with nonanomeric hydroxyls. In contrast, mannase treatment of **SovBL** showed no inhibition against aggregation formation (entries 25 and 26), probably because the 5-thiomannoside of **SovBL** is not cleavable with mannase. 5-Thioglycosides, the glycoside analogs with sulfur in the rings, are glycosidase resistant, while they can be recognized by lectins.[25] This property of the 5-thiglycosides as ligands is advantageous for bio samples, which may include glycosidases as well as lectins.

The above results indicated that the direct assay of monovalent ligands is limited to compounds with amino or reactive hydroxyl groups. To expand the applicability of LALA, we examined the ability of some monosaccharide derivatives to inhibit the agglutination of the Man-latex conjugates by ConA. We first conducted inhibition LALA with Man, MeMan, *p*-nitrophenyl α-D-mannopyranoside (pNPMan), and *p*-nitrophenyl α-D-glucopyranoside (pNPGlc) against ConA of various concentrations using **MnvBL** (Table 2). Aggregation occurred at Man inhibitor concentrations $\leq$2 mM with 2 μg/mL of ConA (Fig. 4). The aggregation pattern at 2 mM Man is blurred because the inhibitor concentration is on the boundary of complete inhibition. Thus, the effective concentration (EC_{50}) of Man to inhibit aggregation by $\sim$50% is 2 mM. In the same way, EC_{50} at 2 μg/mL of ConA was 0.25, 0.125, and 1 mM for MeMan, pNPMan, and pNPGlc, respectively.

Because the EC_{50} value is dependent on both ConA concentration ([ConA]) and the aggregatability of the sugar-modified latex beads ($[ConA]_{ag}$), the inhibitory parameter should be standardized so that the same value is obtained for an inhibitor regardless of experimental conditions. For the monosaccharides tested above, the EC_{50} value appeared to be proportional to [ConA], at least within the small practical range (2–8 μg/mL, Table 2). Also, if we assume a competitive inhibition, the EC_{50} value should be inversely

Table 2: Aggregation extent[a] for the LALA inhibition study with **MnvBL**[b]

Inhibitor	ConA (μg/mL)	Inhibitor concentration (mM) 0	0.125	0.25	0.5	1	2	4	8	16	EC_{50}^{0} [c] (mM)
Man	8	*37*	*36*	*36*	*37*	*37*	*34*	*28*	*19*	8	1
	4	*38*	*37*	*37*	*37*	*35*	*33*	*24*	7	7	1
	2	*34*	*35*	*35*	*34*	*28*	*18*	6	6	—	1
MeMan	8	*37*	*38*	*36*	*33*	*26*	7	6	6	10	0.12
	4	*38*	*36*	*35*	*32*	9	6	6	5	9	0.12
	2	*35*	*32*	*25*	8	7	7	5	5	—	0.12
pNPMan	8	*38*	*38*	*35*	*28*	7	6	6	—	—	0.06
	4	*37*	*34*	*30*	9	6	6	11	—	—	0.06
	2	*36*	*26*	7	7	6	6	5	—	—	0.06
pNPGlc	8	*39*	*42*	*41*	*40*	*36*	*32*	*16*	—	—	0.5
	4	*38*	*36*	*35*	*33*	*31*	*19*	12	—	—	0.5
	2	*37*	*37*	*35*	*29*	*16*	6	—	—	—	0.5

[a]Standard deviation of pixels' darkness (0–255) for the digital image of each well.
[b]The numerals for the wells in which aggregation was observed are italicized. [c]Standardized inhibition (EC_{50}^{0}) = EC_{50}*$(ConA)_{ag}$/ (ConA), where EC_{50} is the inhibitor concentration (mM) required to inhibit the aggregation, $(ConA)_{ag}$ is the ConA concentration (μg/mL) required to aggregate the latex beads, and (ConA) is the ConA concentration used in the inhibition assay.

proportional to $[ConA]_{ag}$. Therefore, the standardized EC_{50} (EC_{50}^{0}) is derived from the equation:

$$EC_{50}^{0} = EC_{50} * [ConA]_{ag}/[ConA](i)$$

The EC_{50}^{0} values for Man, MeMan, and pNPMan were calculated to be 1, 0.12, and 0.06 mM, respectively. The reported EC_{50} value for the inhibition of ConA-mannan binding with Man, MeMan, and pNPMan are approximately 5, 1, and 0.2 mM, respectively.[23] Thus, the LALA inhibition experiments were semi-quantitative with regard to relative inhibition activity. Furthermore, the stronger binding ability of a mannose derivative (pNPMan) than the glucose counterpart (pNPGlc; EC_{50}^{0} = 0.5 mM) toward

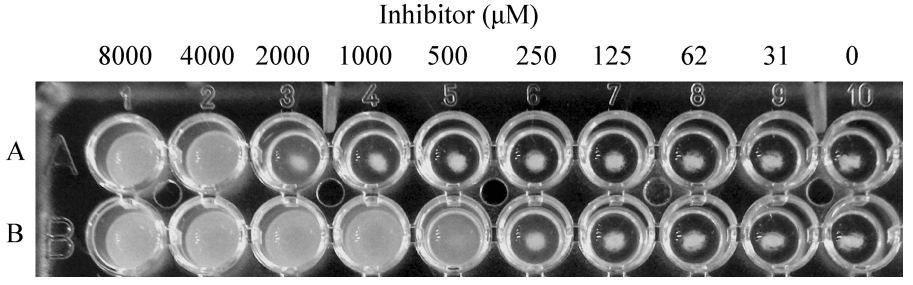


Figure 4: A typical agglutination inhibition assay for ConA (2 μg/mL) with **MnvBL** and inhibitors mannose (A) and methyl α-D-mannopyranoside (B).

Table 3: Aggregation extent[a] for the LALA inhibition study with **MovBL**.[b]

	Inhibitor concentration (mM)												
Inhibitor	**0**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	EC_{50}^{0} [c] (mM)
Man	*36*	*36*	*34*	*34*	*23*	6	6	5	5	7			0.5
MeMan	*38*	*36*	*27*	11	6	4	5	5	5	5			0.12
5SMan	*36*	—	*36*	*37*	*37*	*36*	*35*	*34*	*30*	12	6		8
Me5SMan	*33*	—	*34*	*34*	*34*	*33*	*29*	*22*	9	7	5		4
5SManEtN	*33*	—	—	*31*	*30*	*30*	*23*	*19*	*23*	*24*	14	6	16

[a]Standard deviation of pixels' darkness (0–255) for the digital image of each well.
[b]The numerals for the wells, in which aggregation were observed, are italicized.
[c]Standardized inhibition (EC_{50}^{0}) = EC_{50}*$(ConA)_{ag}$/ (ConA), where EC_{50} is the inhibitor concentration (mM) required to inhibit the aggregation, $(ConA)_{ag}$ is ConA concentration (μg/mL) required to aggregate the latex beads, and (ConA) is the ConA concentration used in the inhibition assay. In this assay (ConA) was 8 μg/mL.

ConA was reflected in the assay results, as also demonstrated by a sophisticated method.[26]

The EC_{50}^{0} value could be varied slightly with the use of differently modified latex beads. For example, an EC_{50}^{0} value of 1 mM was calculated for Man in the inhibition assay with **MnvBL** (Table 2). On the other hand, the inhibition experiment with **MovBL** demonstrated an EC_{50}^{0} value of 0.5 mM for Man (Table 3). In the case of MeMan, the EC_{50}^{0} value of 0.12 mM was consistent for two types of latex beads. 5SMan showed an EC_{50}^{0} value of 8 mM with the use of **MovBL**, while Me5SMan and 5SManEtN had EC_{50}^{0} values of 4 and 16 mM, respectively. The relative inhibitory activity of Man versus 5SMan (16:1) is consistent with the relative aggregation activity of the latex beads immobilizing these sugars (**MovBL** and **SovBL**). The poor binding ability of 5-thiomannose derivatives could be expected from our previous study of 5-thiomannose-containing oligosaccharides.[24] It should be noted that **MovBL** is more convenient than **MnvBL** because no synthetic labors are required to prepare this mannose-latex conjugate. The other reducing sugars, except for glycosamines, can be similarly incorporated, as demonstrated for fucose.

CONCLUSION

We demonstrated that the direct LALA, in which a test ligand compound is attached to the surface of latex beads, is useful when the test compound has an amino or reactive hydroxyl group and the coupling has no effect on the binding to lectins. In cases that do not satisfy these criteria, an inhibition experiment with a synthetic or reducing natural sugar as a standard ligand on latex beads is the method of choice. Inhibition experiments for compounds with previously reported EC_{50} values established the semi-quantitative character of this method. Overall, the LALA experiment is facile, sensitive, and

semi-quantitative and does not require expensive instruments, and the capability of immobilizing reducing sugars without any tedious syntheses is especially advantageous over the other aggregation assays. Improvements in sensitivity and automation will be possible if the amount of latex beads is scaled down and if the beads are encapsulated into arrays of a tiny glass compartment equipped with a filter, which would allow rapid washing of latex beads and observation of aggregation.

EXPERIMENTAL

General Methods

The following materials were obtained from Sigma: latex beads (10% suspension, 0.82 μm diameter), amino-coated latex beads (2.5% suspension, 0.8 μm diameter), ConA (C2010, powder), WGA (L0636, powder), LTA (L9254, powder), α-mannosidase (suspension, 95 units/mL), $\beta - N$-acetylglucosaminidase (suspension, 63.6 units/mL), *p*-nitrophenyl α-D-mannopyranoside, *p*-nitrophenyl α-D-glucopyranoside, and mannan. DVS and DFDNB were purchased from Aldrich. All solvents and reagents were reagent grade, and in cases in which further purification was required, standard procedures[27] were followed. Column chromatography was performed on Merck Kieselges 60 (Art 7734), Wako gel C-300, or Kanto Silica gel 60N (spherical, neutral) with the specified solvent systems. Optical rotations were determined with a Horiba SEPA-200 polarimeter using a 1-dm-length cell. ^{1}H NMR spectra were recorded at 400 MHz (Varian Unity-400) or 270 MHz (JEOL EX-270). Internal tetramethylsilane (δ 0 ppm) in $CDCl_3$ or a DOH peak (δ 4.80 ppm) in D_2O was used as a standard. Chemical shifts were expressed in ppm referenced to the standard. The multiplicity of signals was abbreviated as follows: s = singlet, d = doublet, dd = doublet of doublets, t = triplet, dt = doublet of triplets, ddd = doublet of doublets of doublets, br = broad signal, and m = multiplet. ^{13}C NMR spectra were recorded at 67.8 MHz (JEOL JNM-EX-270) or 100.6 MHz (Varian Unity-400), and a solvent peak (δ 77.0 ppm) in $CDCl_3$ or internal acetone (δ 29.8 ppm) in D_2O was used as a standard. High-resolution mass spectra (HRMS) were recorded on a Mariner Biospectrometry Workstation ESI-TOF mass spectrometer.

Synthesis of Monosaccharide Derivatives

*2-Bromoethyl 2,3,4,6-tetra-O-acetyl-5-thio-α-D-mannopyranoside (**2**)*

To a stirred solution of 1,2,3,4,6-penta-*O*-acetyl-5-thio-α-D-mannopyranose (**1**; 108 mg, 0.266 mmol) and 2-bromoethanol (97 μL, 1.41 mmol) in CH_2Cl_2 (5.4 mL) was added BF_3OEt_2 (87 μL, 0.707 mmol) and the solution was stirred at

rt for 12 h. The reaction was stopped by adding triethylamine (129 μL, 0.47 mmol) and the solution was evaporated and chromatographed on silica gel column (toluene:AcOEt, 5.5:1) to give **2**; colorless crystals (99 mg, 79%); mp 61–65°C; $[\alpha]_D^{29}$+51.0° (c 0.51, $CHCl_3$); R_f 0.55 (toluene:EtOAc, 1:1). ^{1}H NMR (270 MHz, $CDCl_3$): 5.48 (t, 1H, J = 10.1 Hz, H-4), 5.42 (dd, 1H, J = 3.1 Hz, J = 3.8 Hz, H-2), 5.33 (dd, 1H, J = 3.1 Hz, J = 10.1 Hz, H-3), 4.64 (d, 1H, J = 3.8 Hz, H-1), 4.33 (dd, 1H, J = 5.5 Hz, J = 12.0 Hz, H-6a), 4.06–4.20 (m, 2H, H-6b, CH_2O), 3.47–3.83 (m, 4H, H-5, CH_2O, CH_2Br), 2.00, 2.04, 2.09, 2.19 (4s, 12H, 4Ac); ^{13}C NMR (67.8 MHz, $CDCl_3$): 170.6, 170.1, 169.8, 169.6 (4C=O), 81.3 (C-1), 71.1, 70.1, 69.1, 68.4, 61.8 (C-2, C-3, C-4, C-6, CH_2O), 39.4 (C-5), 29.6 (CH_2Br), 21.0, 20.8, 20.7, 20.6 ($4CH_3$); HRMS (ESI) *m/z* $[M + Na]^+$, Calcd for $C_{16}H_{23}O_9SBrNa$: 493.0145 and 495.0124. Found: 493.0122 and 495.0093.

*2-Azidoethyl 2,3,4,6-tetra-O-acetyl-5-thio-α-D-mannopyranoside (**3**)*

To a stirred solution of **2** (81 mg, 0.172 mmol) in DMF (18 mL) was added NaN_3 (38 mg, 0.585 mmol) and the solution was stirred at 60°C for 1 h. The solution was evaporated and partitioned between $CHCl_3$ and H_2O. The $CHCl_3$ layer was evaporated and chromatographed on silica gel column (toluene:AcOEt, 3:1) to give **3**; colorless syrup (73 mg, 98%); $[\alpha]_D^{27}$+59.3° (c 0.94, $CHCl_3$); R_f 0.51 (toluene:EtOAc, 1:1). ^{1}H NMR (270 MHz, $CDCl_3$): 5.48 (t, 1H, J = 10.1 Hz, H-4), 5.43 (dd, 1H, J = 3.0 Hz, J = 3.8 Hz, H-2), 5.33 (dd, 1H, J = 3.0 Hz, J = 10.1 Hz, H-3), 4.64 (d, 1H, J = 3.8 Hz, H-1), 4.34 (dd, 1H, J = 5.5 Hz, J = 12.0 Hz, H-6a), 4.06–4.13 (m, 2H, H-6b, CH_2O), 3.58 (ddd, 1H, J = 4.0 Hz, J = 5.5 Hz, J = 10.1 Hz, H-5), 3.41–3.51 (m, 3H, CH_2O, CH_2N_3), 2.00, 2.04, 2.10, 2.19 (4s, 12H, 4Ac); ^{13}C NMR (67.8 MHz, $CDCl_3$): 170.6, 170.1, 169.8, 169.5 (4C=O), 81.6 (C-1), 71.1, 70.0, 69.1, 67.5, 61.8 (C-2, C-3, C-4, C-6, CH_2O), 50.2 (CH_2N_3), 39.2 (C-5), 21.0, 20.7, 20.6 ($4CH_3$); HRMS (ESI) *m/z* $[M + Na]^+$, Calcd for $C_{16}H_{23}N_3O_9SNa$: 456.1053. Found: 456.1035.

*2-Aminoethyl 5-thio-α-D-mannopyranoside (**Sn**)*

To a stirred solution of **3** (72 mg, 0.166 mmol) in methanol (7 mL) was added 0.1 M NaOMe (0.2 mL) and the solution was stirred at rt for 1 h. The solution was neutralized with Dowex50(H^+) and evaporated. The residue (45 mg) was dissolved in THF-MeOH-H2O (19:24:3, 4.6 mL) and Ph_3P (95 mg, 0.362 mmol) was added. After 6 h, the solution was evaporated and partitioned between $CHCl_3$ and H_2O. The H_2O layer was evaporated and chromatographed on 60N silica gel column (*i*PrOH:H_2O:28%NH_3, 25:5:1) to give **Sn**; yellow syrup (32 mg, 84%); $[\alpha]_D^{26}$+119° (c 0.54, H_2O); R_f 0.19 (*i*PrOH:H_2O, 5:1). ^{1}H NMR (400 MHz, D_2O): 4.67 (d, 1H, J = 3.7 Hz, H-1), 4.24 (dd, 1H, J = 3.0 Hz, J = 3.7 Hz, H-2), 4.03 (ddd, 1H, J = 4.0 Hz, J = 6.4 Hz, J = 10.7 Hz, CH_2O), 3.93 (dd, 1H, J = 3.4 Hz, J = 11.9 Hz, H-6a), 3.80 (dd, 1H, J = 6.7 Hz, J = 11.9 Hz, H-6b), 3.78 (t, 1H, J = 9.8 Hz, H-4), 3.72 (dd, 1H, J = 3.0 Hz, J = 9.8 Hz,

H-3), 3.56 (ddd, 1H, $J = 3.8$ Hz, $J = 6.1$ Hz, $J = 10.7$ Hz, CH_2O), 3.06 (ddd, 1H, $J = 3.4$ Hz, $J = 6.7$ Hz, $J = 9.8$ Hz, H-5), 2.97–3.09 (m, 2H, CH_2N); ^{13}C NMR (67.8 MHz, D_2O): 85.0 (C-1), 72.5, 70.2, 67.7, 61.1 (C-2, C-3, C-4, C-6, CH_2O), 44.5 (CH_2N), 40.1 (C-5); HRMS (ESI) *m/z* $[M + H]^+$, Calcd for $C_8H_{18}NO_5S$: 240.0906. Found: 240.0909.

Preparation of Sugar-DVS-BSA-Latex Bead Conjugates

Concentration of the latex bead suspensions was carried out by centrifugation at 5,000 rpm for 15 min (GBS) or 30 min (0.25 M Na_2CO_3) and removal of the supernatant. Suspensions of latex beads (17.5 μL) were placed in 1.5-mL Eppendorf tubes and washed with 350 μL 0.1 M glycine-buffered saline (GBS, 0.1% Tween 20, pH 7.0). After concentration, the residual beads were treated with BSA solution in GBS (10 mg/mL, 350 μL) for 1 h and washed twice with 350 μL GBS (0.1% Tween 20, pH 7.0) and once with 350 μL 0.25 M Na_2CO_3 (0.1% Tween 20, pH 11.0). After concentration, the residual beads were treated with 5% DVS in 200 μL 0.25 M Na_2CO_3 (0.1% Tween 20, pH 11.0) for 1 h and washed with 350 μL 0.25 M Na_2CO_3 (0.1% Tween 20, pH 11.0). After concentration, the residual beads were treated with a sugar solution (5 or 50 mg/mL) in 200 μL 0.25 M Na_2CO_3 (0.1% Tween 20, pH 11.0) for 18 h and washed three times with 350 μL GBS (0.1% Tween 20, pH 8.0). To the residual beads was added 350 μL GBS (0.1% Tween 20, pH 8.0) to afford a latex bead suspension for LALA studies. For the preparation of sugar-DVS-amino-latex bead conjugates, 70 μL of a suspension of amino-coated latex beads was treated with DVS and sugar as described above.

Preparation of Sugar-DFDNB-BSA-Latex Bead Conjugates

Concentration of latex bead suspensions was carried out by centrifugation at 5,000 rpm for 15 min and removal of supernatant. The BSA-coated latex beads prepared above were washed with 350 μL 0.25 M Na_2CO_3 (0.1% Tween 20, pH 11.0). After concentration, the residual beads were treated with Man-DFDNB conjugate (22.6 mg/mL) in 175 μL 97 mM borate buffer (pH 9.0) for 96 h and washed three times with 350 μL GBS (0.1% Tween 20, pH 8.4). To the residual beads was added 350 μL GBS (0.1% Tween 20, pH 7.4) to afford a latex bead suspension for LALA studies.

Glycosidase Treatment of the Sugar-Latex Bead Conjugates

Concentration of latex bead suspensions was carried out by centrifugation at 5,000 rpm for 15 min and removal of supernatant. The sugar-modified latex beads prepared above were concentrated, the residual beads were suspended in 200 μL 20 mM citrate buffer (pH 4.7), and a glycosidase suspension (20 μL

for mannosidase or 10 μL for glucosaminidase) was added. After 18 h, the suspension was concentrated and washed three times with 350 μL GBS (0.1% Tween 20, pH 8.4). To the residual beads was added 350 μL GBS (0.1% Tween 20, pH 7.4) to afford a latex bead suspension for LALA studies.

Latex-Bead-Based Agglutination Lectin Assay (LALA)

ConA in 0.15 M NaCl that included 5 mM each of $CaCl_2$, $MgCl_2$, and $MnCl_2$ was diluted to an appropriate concentration. For one assay set, 10 microplate wells were filled with 35 μL of the series of ConA solution, which was prepared by two- or fourfold serial dilution. Each of the latex bead suspensions (35 μL) prepared above was added to the wells, and the microplate was allowed to stand for 3 h with occasional mild shaking. For inhibition experiments, 17.5 μL each of ConA solution and inhibitor solution of the same solute was used.

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