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Significant Other Half of a Glycoconjugate: Contributions of Scaffolds to Lectin−Glycoconjugate Interactions

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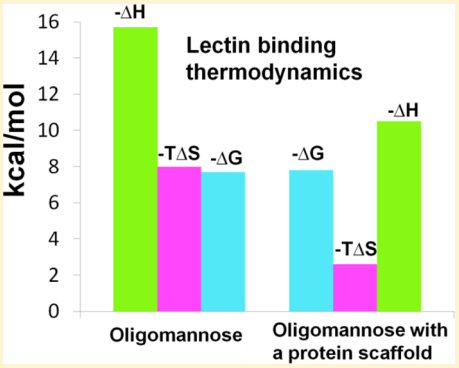
Supporting Information

ABSTRACT: The glycan epitopes of natural and synthetic glycoconjugates exist as covalent attachments of well-defined inner structures or scaffolds. Macromolecules such as proteins, peptides, lipids, and saccharides and synthetic structures serve as scaffolds of glycoconjugates. It is generally perceived that the biological activities of glycoconjugates are determined mainly by the attached glycans, while the seemingly inert inner scaffolds play a passive role by providing physical support to the attached glycan epitopes. However, our data show that scaffolds actively influence lectin recognition and can potentially modulate lectin-mediated signaling properties of glycoconjugates. Through *in vitro* experiments, we found that the scaffolds significantly altered the thermodynamic binding properties of the covalently attached glycan epitopes. When a free glycan was attached to a scaffold, its lectin binding entropy became more positive. The level of positive entropic gain was dependent on the types of scaffolds tested. For example, protein scaffolds of glycoproteins were found to generate more positive entropy of binding than synthetic scaffolds. Certain scaffolds were found to have limiting effects on glycoconjugate affinity. We also found that scaffold-bearing glycans with a similar affinity or an identical valence demonstrated different kinetics of lattice formation with lectins, when the scaffold structures were different. Our data support the view that scaffolds of glycoconjugates (i) help the covalently attached glycans become more spontaneous in lectin binding and (ii) help diversify the lattice forming or cross-linking properties of glycoconjugates.



A glycoconjugate is composed of glycan epitopes and a covalently linked core structure called a scaffold. Proteins, peptides, lipids, and saccharides can serve as scaffolds (Figure 1). Glycoproteins, proteoglycans, glycopeptides, and peptidoglycans are glycoconjugates that contain protein or peptide scaffolds, whereas glycolipids and lipopolysaccharides possess lipid scaffolds. Synthetic glycoconjugates bear a vast array of synthetic scaffolds.[1,2] Saccharides have been used as scaffolds of many synthetic glycoconjugates.[3−5]

Binding of lectins to the glycan epitopes of glycoconjugates is essential for numerous biological functions, such as cell−cell communication, cell adhesion, cell recruitment, intracellular trafficking, and immune recognition.[6−8] Multivalent lectin−glycoconjugate interactions have been studied thoroughly over the years.[9−13] The current paradigm is that the lectin-mediated biological functions of glycoconjugates are controlled primarily by the structures of the attached glycan epitopes and by the extent of their clustering,[14−18] while the inner scaffolds, which do not participate in lectin binding, provide physical support to the covalently linked glycan epitopes. Our thermodynamic and spectroscopic data, however, strongly suggest that the role of scaffolds in lectin−glycoconjugate interactions is not at all passive as perceived. Rather, scaffolds profoundly affect the thermodynamics of lectin binding by glycoconjugates and the kinetics of lectin−glycoconjugate cross-linking or lattice formation.

Multivalent glycoconjugates often form lattice structures upon their interaction with lectins. Lattice formation with lectins is a prerequisite for many biological functions of glycoconjugates, including their signaling properties.[19−21] Synthetic glycoconjugates with different scaffolds that contained different numbers of glycan epitopes have been tested for their lectin binding properties. Affinity and cross-linking properties of those glycoconjugates varied on the basis of their valence.[22]

However, in this study, synthetic glycoconjugates possessing identical valence and similar affinities showed different lattice formation kinetics. Differential kinetics of lattice formation, which is independent of the affinity and valence of the glycoconjugates, suggest that scaffolds can potentially diversify lectin-mediated biological functions of glycoconjugates. Glycoconjugates with equal valence and comparable affinities can

Received: January 29, 2014
Revised: June 18, 2014
Published: June 20, 2014

dx.doi.org/10.1021/bi5001307 | *Biochemistry* 2014, 53, 4445−4454

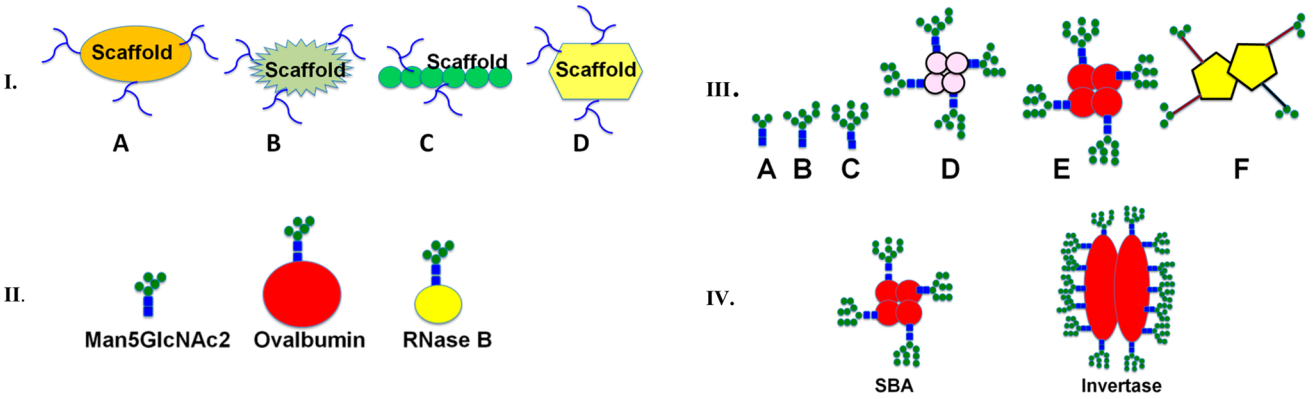


Figure 1. (I) Scaffolds are integral parts of natural and synthetic glycoconjugates. Glycoconjugates are glycans (blue lines) covalently attached to (A) protein (as in glycoproteins and proteoglycans) or peptide scaffolds (as in glycopeptides and peptidoglycans), (B) lipid scaffolds (as in glycolipids and lipopolysaccharides), (C) saccharide scaffolds (as in polysaccharides), and (D) synthetic scaffolds (as in synthetic analogues). Apart from glycoconjugates, a variety of non-glycan epitopes, functional groups, and/or pharmacophores in the natural and synthetic world need to be attached to scaffolds for their activities.[45−47] (II) Free glycans gain positive binding entropy when they are covalently attached with protein scaffolds. ConA binds to free $Man_5GlcNAc_2$. ConA also binds to $Man_5GlcNAc_2$ when it is a part of a protein scaffold, as in ovalbumin and RNase B. The binding entropy ($T\Delta S$) of ConA for $Man_5GlcNAc_2$ becomes more positive when the glycan is attached to a protein scaffold (Table 1). (III) Compared to synthetic scaffolds, protein scaffolds appear to help glycoconjugates gain more positive binding entropy. ConA binds to the "core" trimannoside region (green circles of structure A) of all N-linked glycans, including all hybrid-type and high-mannose oligosaccharides such as $Man_7GlcNAc_2$ (B) and $Man_9GlcNAc_2$ (C). This trimannoside structure is the complementary binding epitope of ConA. As a result, structures A−C and hybrid-type oligosaccharides have similar binding affinities for ConA, although the numbers of constituent monosaccharide residues are different in structures A−C and hybrid-type oligosaccharides. Avidin (D) is a tetrameric natural glycoprotein in which a protein scaffold (four fused circles) is linked to four high-mannose ($Man_7GlcNAc_2$)/hybrid-type oligosaccharides. Soybean agglutinin (SBA) (E) is another tetrameric natural glycoprotein in which a protein scaffold (four fused circles) is attached to four high-mannose ($Man_9GlcNAc_2$) oligosaccharides. Analogue **6** (F) contains four trimannoside residues on a synthetic scaffold (yellow). As the binding epitope of ConA is the trimannoside structure, SBA (E), avidin (D), and analogue **6** (F) can be viewed as structural analogues because each contains four similar binding epitopes for ConA. ConA binds to SBA and analogue **6** with similar affinity (K_a), but the binding entropy ($T\Delta S$) of ConA for SBA is significantly more positive than that of analogue **6** (Table 1). When compared with analogue **6**, avidin also showed a similar trend. (IV) Structures of protein scaffolds may limit the glycan density-dependent affinity effect. SBA has four accessible high-mannose glycans on its protein scaffold, whereas invertase contains 14 accessible high-mannose glycans. However, affinities (K_a) of both glycoproteins for ConA are comparable.

potentially show different levels of efficacy in lectin clustering, if the scaffold structures of the glycoconjugates are different. We also found that scaffolds modulated the thermodynamic binding properties of glycoconjugates. More specifically, scaffolds were found to regulate the positive binding entropy and thus the spontaneity of lectin binding by glycoconjugates. An increasing positive entropy is a hallmark of increasing spontaneity of interaction.

EXPERIMENTAL PROCEDURES

The strategies leading to the synthesis of glycoconjugate analogues (glycodendrimers) **1**−**5** (Figure 2) have been described.[23−25] The synthesis of analogue **6** (Figure 2) has been previously mentioned.[11] "Core" trimannoside and mannopentaose (Man_5) (Figure 3) were purchased from Sigma Chemical Co. and Dextra Laboratories Ltd., respectively. Following a previously described procedure,[26] the Man_9 glycopeptide was prepared from the Pronase digest of soybean agglutinin (SBA). Man_7 glycopeptides were isolated from the Pronase digest of quail ovalbumin as described previously.[27] Mannooligosaccharide structures are shown in Figure 3. The concentrations of carbohydrates were determined by modification of the Dubois phenol sulfuric acid method[28,29] using appropriate monosaccharides as standards. Estimation of the carbohydrate concentration is a challenging task. An accurate carbohydrate concentration is required to generate unambiguous ITC data. We have employed two different methods[28,29] to minimize the error in carbohydrate concentration. ITC experiments with accurately estimated protein and monovalent glycans produce an "*n*" value of 1 or very close to 1 when the glycan concentration is correctly determined. We have determined this via numerous ITC experiments over the years. In the study presented here, *n* values of all the ITC experiments with monovalent ligands are very close to 1 (Table 1). This indicates the accuracy of ligand concentration. The *n* values of multivalent interactions are fractional (Table 1), and the valence of a multivalent ligand is determined from the *n* value (valence = $1/n$).[13]

Glycoproteins were purchased from Sigma. Whenever necessary, they were further fractionated on ConA−agarose columns. ConA was purchased from Sigma and/or prepared from jack bean (*Canavalia ensiformis*) seeds. The concentration of ConA was determined spectrophotometrically at 280 nm using $A_{1\%,1\ cm}$ values of 13.7 and 12.4 at pH 7.2 and 5.2, respectively,[30] and expressed in terms of monomer (M_r = 25600).

Isothermal Titration Microcalorimetry. ITC experiments were performed with MCS-ITC and VP-ITC instruments from GE (Microcal, Inc., Northampton, MA). Injections of 4 μL of a carbohydrate solution were added from a computer-controlled microsyringe at an interval of 4 min into the sample solution of lectin (cell volume of 1.34 mL) with 350 rpm stirring. Control experiments performed by making identical injections of saccharide/glycoconjugate into a cell, containing buffer without protein, showed insignificant heats of dilution. The concentrations of lectins were from 10 to 100 μM and those of the glycans and glycoconjugates from 0.1−4.0 mM. A representative ITC profile is shown in Figure 4.

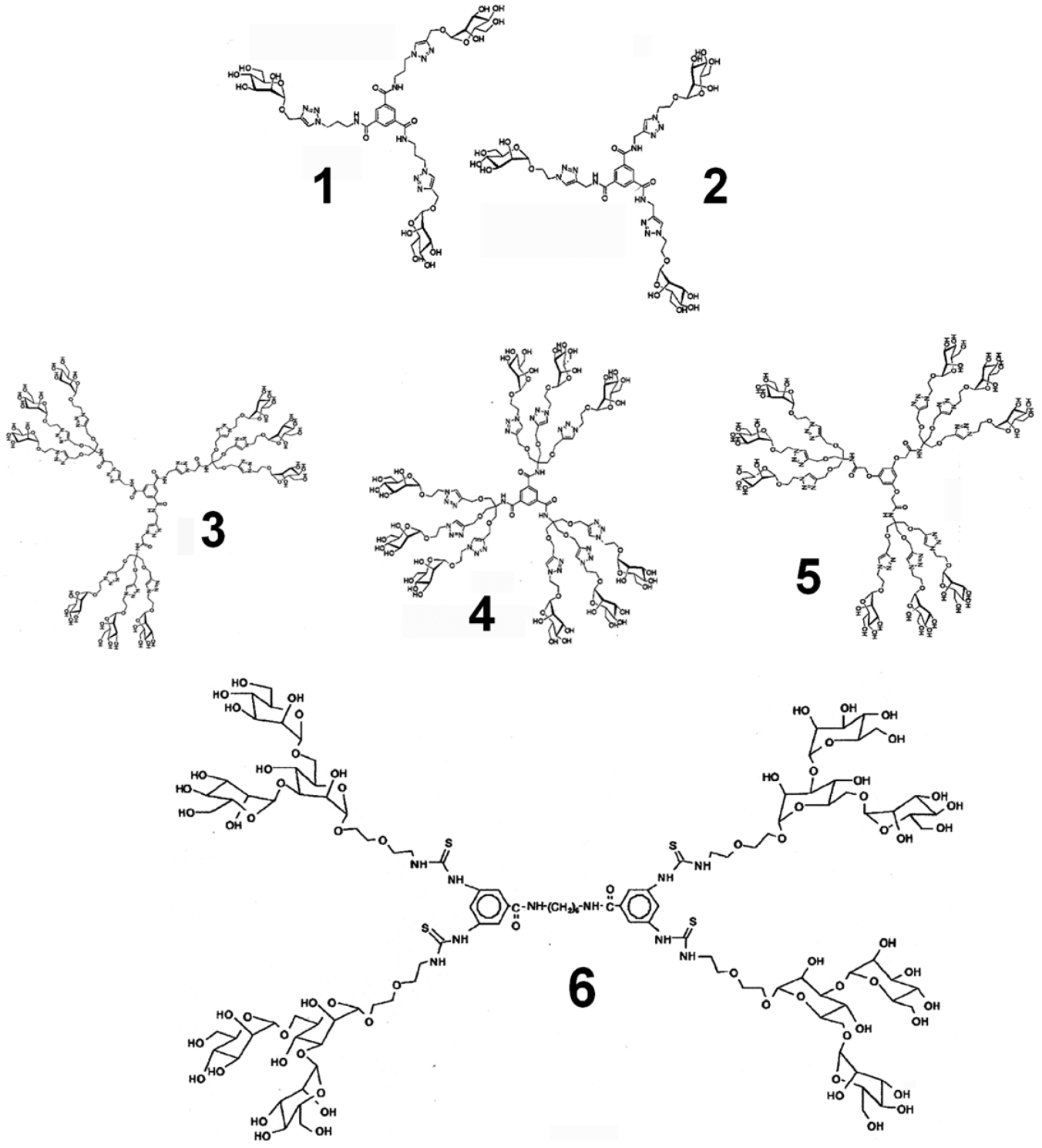


Figure 2. Structures of the synthetic glycoconjugates used in this study. Analogues **1** and **2** each contain three mannose residues on structurally different synthetic scaffolds. Analogues **3**−**5** each contain nine mannose residues on synthetic scaffolds, which are structurally dissimilar. Analogue **6** contains four trimannoside residues on a synthetic scaffold.

Titrations were conducted at pH 5.0−5.2 and NaCl concentrations ranging from 0.05 to 0.15 M (100 mM sodium acetate buffer containing 5 mM $CaCl_2$ and 5 mM $MnCl_2$). Titrations were also repeated at pH 7.2 (100 mM HEPES with 150 mM NaCl, 5 mM $CaCl_2$, and 5 mM $MnCl_2$) to check if binding parameters change with pH. The experimental data were fit to a theoretical titration curve using software supplied by Microcal, with ΔH (enthalpy change in kilocalories per mole), K_a (association constant in inverse molar), and n (number of binding sites per monomer) as adjustable parameters. The quantity $c = K_a\mathrm{Mt}(0)$, where $\mathrm{Mt}(0)$ is the initial macromolecule concentration, is important in titration microcalorimetry.[31] All experiments were performed with c values between 1 and 200. The instrument was calibrated using the calibration kit containing ribonuclease A (RNase A) and cytidine 2′-monophosphate (2′-CMP) supplied by the

dx.doi.org/10.1021/bi5001307 | *Biochemistry* 2014, 53, 4445−4454

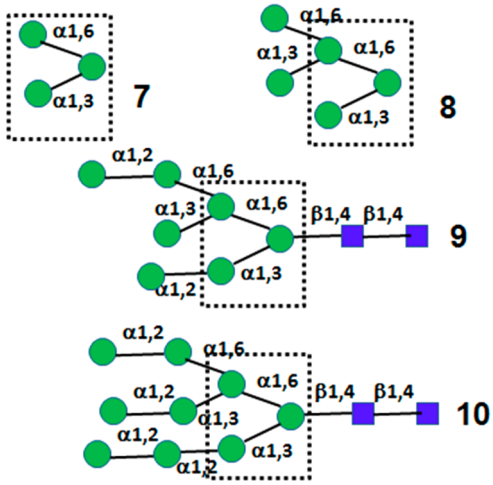


Figure 3. Structures of mannooligosaccharides. Structures of trimannoside (**7**), Man_5 (**8**), $Man_7GlcNAc_2$ (**9**), and $Man_9GlcNAc_2$ (**10**) are shown (the circles represent mannose residues and the squares GlcNAc). ConA binds to the internal "core" trimannoside (shown within the boxes with dashed sides) of the oligosaccharides.

manufacturer. Thermodynamic parameters were calculated from the equation $\Delta G = \Delta H - T\Delta S = RT \ln K_a$, where ΔG, ΔH, and ΔS are the changes in free energy, enthalpy, and entropy of binding, respectively, T is the absolute temperature, and R = 1.98 cal mol^{-1} K^{-1}.

Under proper stoichiometric conditions, multivalent ligands and lectins form insoluble cross-linked complexes. Such complex formation during ITC measurement was prevented by using a low lectin concentration, a buffer with a low pH (pH 5.0), and a low salt concentration. Under such conditions, ConA exists as a dimeric protein, and thus, insoluble complex formation is significantly prevented. In a previous study, we obtained unambiguous ITC data using this approach.[11] We have also found that just by using a lower concentration of ConA (10−25 μM), multivalent interactions could be measured by ITC even at pH 7.2. Thus, the ITC experiments reported in this study were performed under conditions under which the formation of insoluble cross-linked complexes was arrested or significantly slowed. As a result, the quality of the ITC-driven thermodynamic data was not compromised.

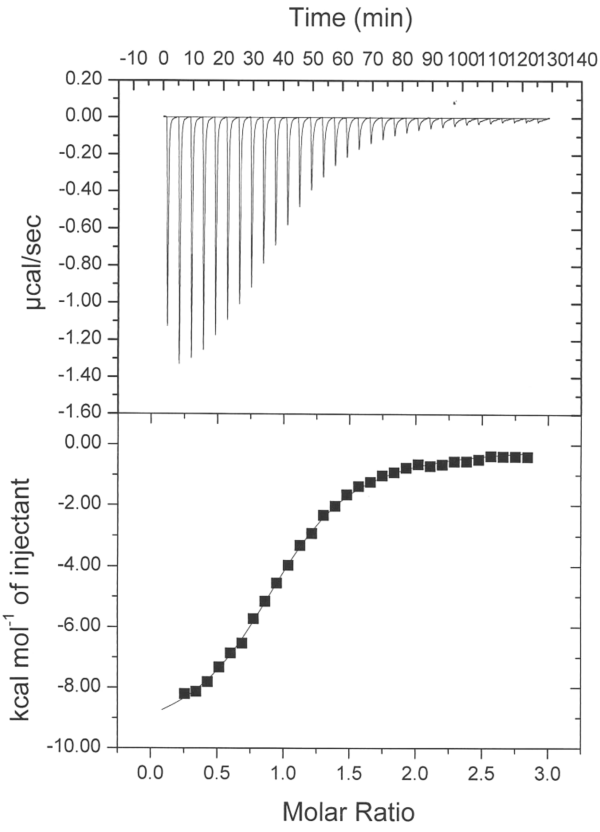


Figure 4. Calorimetric titration profile of ConA (30 μM) with RNase B (1250 μM) at 27 °C and pH 5.2. The top panel shows data obtained for automatic injections (4 μL each) of RNase B. The integrated curve showing experimental points and the best fit are shown in the bottom panel. The calorimetric profile of the ConA−RNase B interaction at pH 7.2 is shown in the Supporting Information.

Kinetics of Noncovalent Cross-Linking or Lattice Formation. Measured volumes of lectin and saccharide solutions were mixed in a 1 mL quartz cuvette, and the time-dependent development of turbidity was measured at 420 nm with a Shimadzu UV-2450 spectrophotometer.[32] The buffer was HEPES [100 mM HEPES, 0.15 M NaCl, 5 mM $CaCl_2$, and 5 mM $MnCl_2$ (pH 7.2)]. The same experiments were repeated in 100 mM sodium acetate buffer containing 5 mM $CaCl_2$ and

Table 1. Thermodynamic Binding Parameters of ConA Obtained with Free Mannooligosaccharides, Mannooligosaccharide-Containing Glycoproteins, and a Mannooligosaccharide-Containing Synthetic Analogue at pH 5.2 and 27 °C[a]

ligand	K_a ($\times 10^{-4}$ M^{-1})	c value	$-\Delta G$ (kcal/mol)	$-\Delta H$ (kcal/mol)	$-T\Delta S$ (kcal/mol)	n (sites/monomer)
trimannoside	38 ± 2.03	14 ± 0.9	7.6 ± 0.03	14.6 ± 0.18	7.0 ± 0.21	1.01 ± 0.03
Man_5	43.2 ± 2.33	15 ± 1.7	7.7 ± 0.04	15.6 ± 0.23	8.0 ± 0.23	1.02 ± 0.02
Man_7Gn_2	31.4 ± 3.02	12 ± 0.6	7.5 ± 0.05	17.6 ± 0.42	10.7 ± 0.33	0.99 ± 0.03
Man_9Gn_2	51.0 ± 3.41	17 ± 1.2	7.8 ± 0.02	17.9 ± 0.31	10.2 ± 0.41	1.03 ± 0.02
RNase B	64.6 ± 3.17	19 ± 0.5	7.9 ± 0.04	10.2 ± 0.33	2.2 ± 0.27	0.98 ± 0.02
ovalbumin	29.6 ± 2.93	16 ± 0.2	7.5 ± 0.05	8.9 ± 0.24	1.5 ± 0.19	1.03 ± 0.03
analogue **6**	1378 ± 34	150 ± 5.8	9.7 ± 0.02	54.1 ± 0.62	44.4 ± 0.54	0.25 ± 0.005
SBA	1606 ± 23	160 ± 6.9	9.8 ± 0.02	38.2 ± 0.49	28.4 ± 0.43	0.26 ± 0.008
avidin	1050 ± 32	140 ± 3.0	9.6 ± 0.03	42.0 ± 0.24	32.5 ± 0.37	0.26 ± 0.003
invertase	1750 ± 29	150 ± 7.2	9.9 ± 0.01	91.3 ± 0.75	81.5 ± 0.91	0.08 ± 0.002

[a]Each value represents a mean value of a particular parameter obtained from three different experiments. K_a, $-\Delta H$, c, and n values are mean values of from three experiments. Values of $-\Delta G$ and $-T\Delta S$ were calculated separately for each experiment, and then the mean values of $-\Delta G$ (from three experiments) and $T\Delta S$ (from three experiments) were determined. The table shows those mean values and standard errors. A hybrid-type oligosaccharide showed an inhibitory potency, comparable to that of trimannoside.

dx.doi.org/10.1021/bi5001307 | *Biochemistry* 2014, 53, 4445−4454

5 mM $MnCl_2$ (pH 5.2). All experiments were conducted at room temperature. The absorbance was monitored continuously until the OD became constant. After each experiment, a portion of the precipitate was treated with 200 mM Me-α-mannose to determine whether the precipitation was due to the binding of the saccharides.

The invertase/ConA stoichiometry was determined by incubating invertase with different amounts of ConA. The amount of cross-linked complex formation was measured at 420 nm with a Shimadzu UV-2450 spectrophotometer.

RESULTS AND DISCUSSION

Well-characterized lectin concanavalin A (ConA), glycoproteins with known glycan profiles, and strategically designed synthetic glycan analogues have been used in this study to ensure unambiguous quantitative analysis.

Rationale behind the Selection of Glycoconjugates. To test the role of scaffolds (protein and synthetic) of glycoconjugates in lectin binding and postbinding events, we have selected three different groups of ligands: (a) glycans without scaffolds [free oligomannose/high-mannose oligosaccharides without scaffolds, such as "core" trimannoside, mannopentaose (Man_5), Man_7/Man_8, Man_9 (Figures 1 and 3), and a hybrid glycan], (b) glycans with natural protein scaffolds (i.e., glycoproteins) [high-mannose oligosaccharides bearing glycoproteins RNase B, chicken ovalbumin, avidin, invertase, and soybean agglutinin (SBA) (Figure 1)], and (c) glycans with synthetic scaffolds [mannose-containing analogues (**1**−**6**) with synthetic scaffolds (Figure 2)].

Synthetic glycoconjugates with identical valences and comparable affinities but different scaffold structures were chosen to determine if the influence of scaffolds is independent of affinity and valence. For example, analogues **1** and **2** are trivalent, their affinity for ConA is comparable, but their scaffold structures are different. Similarly, **3**−**5** are nonavalent analogues possessing identical affinity for ConA. Analogues **1** and **2** are mannopyranoside trimers differing by the linker length between the mannoside anomeric carbons and the aromatic carbon. They are 10 and 9 atoms in length, respectively. Glycodendrimers **3**−**5** have nine mannopyranoside residues each attached to the central benzene ring. They differ by the linker distance between the anomeric carbon and the first focal point of attachment and by the number of atoms between the focal point and the aromatic carbon. The different numbers of atoms in the linkers were strategically designed to present better flexibility and accessibility. Despite the subtle differences in the scaffold structures within the groups (**1** and **2** and **3**−**5**), individual group members showed similar binding affinities. However, the cross-linking (lattice formation) kinetics of **1** was different from that of **2**. A similar trend was observed in the other group (**3**−**5**) (described below). Synthetic analogue **6** was chosen because its valence and affinity were similar to those of two glycoproteins [soybean agglutinin (SBA) and avidin]. The only difference between **6** and the glycoproteins lies in their scaffold structures: analogue **6** contains a synthetic scaffold, whereas SBA and avidin possess protein scaffolds. **6**, SBA, and avidin were selected to examine how synthetic and protein scaffolds differentially influence lectin binding properties. Invertase and SBA have similar affinities for ConA; however, the former has a valence higher than that of SBA. Invertase was used to show that a higher valence (higher level of epitope clustering) may not necessarily yield a higher affinity. RNase B and ovalbumin were chosen because they contain one copy of $Man_5GlcNAc_2$, covalently attached to their respective protein core (scaffold). Binding data obtained with a scaffold-free $Man_5GlcNAc_2$ and scaffold-linked $Man_5GlcNAc_2$ (as in RNase B and ovalbumin) would show how binding properties change when a free glycan is covalently attached to a protein scaffold (as in a glycoprotein).

Glycoprotein Heterogeneity. We used ConA and a carefully selected panel of ligands to avoid the glycan/glycoprotein heterogeneity problem. We chose ConA for its exceptional ability to bind high-mannose (oligomannose) and hybrid-type glycans through their internal "core" trimannoside residues.[33] The "core" trimannoside is the binding epitope of ConA because the lectin's binding site is complementary to this trimannoside structure. This "core" structure is present in all N-linked glycans, including high-mannose and hybrid-type glycans. RNase B and ovalbumin are monomeric glycoproteins with a single glycosylation site that contains either oligomannose oligosaccharides (in RNase B) or oligomannose and/or hybrid-type oligosaccharides (in ovalbumin)[34,35] (Figure 1). Our studies show that oligomannose, hybrid-type chain, and trimannoside possess similar affinities for ConA (Table 1) as the internal trimannoside "core" of all N-linked oligosaccharides (including high-mannose and hybrid-type oligosaccharides) is the binding epitope of ConA and the trimannoside epitope is present in both high-mannose and hybrid-type chains. Therefore, oligosaccharide heterogeneity in RNase B and ovalbumin did not alter the binding data as the binding epitope for ConA is a common structure of all N-linked oligosaccharides. The same is applicable to avidin[36] (that possesses four oligomannose, hybrid-type glycans) and invertase that contains 14 accessible high-mannose glycan chains.[37] Soybean agglutinin (SBA) is a naturally homogeneous plant glycoprotein with four high-mannose glycans.[38] Thus, oligosaccharide heterogeneity did not influence the data presented here. Whenever necessary, glycoproteins used in this study were chromatographed on a lectin affinity column (such as a ConA−agarose column).

Binding of ConA to Oligomannose and Oligomannose-Containing Glycoproteins Is pH-Independent. Binding of ConA to trimannoside, Man_5, RNase B, and ovalbumin was studied by ITC at pH 5.2 and 7.2 [Figure 4, Figure S5 (RNase B), Figure S2 (Man_5), Figure S6 (ovalbumin), and Figure S1 of the Supporting Information, and ref 10 (trimannoside)]. The thermodynamic binding parameters did not change with pH (Table S1 of the Supporting Information).

The Lectin Binding Entropy of a Glycan Becomes More Positive When It Contains a Covalently Linked Protein Scaffold. Thermodynamic binding data of intact RNase B were compared with those obtained with its constituent oligosaccharides in free form (trimannoside, Man_5, Man_7, and Man_8). The K_a and ΔG values were found to be comparable, whereas the binding entropy was more positive for intact RNase B. Similar results were also found in experiments with intact ovalbumin and its constituent oligosaccharides. The binding entropy ($T\Delta S$) of ConA for free Man_5 was −8.0 kcal/mol, whereas the $T\Delta S$ values for Man_5 attached to protein scaffolds, as in ovalbumin and RNase B, were −1.5 and −2.2 kcal/mol, respectively (Table 1). It is interesting to note that the K_a values (affinities), and thus the ΔG values, of free Man_5 and the glycoproteins (RNase B and ovalbumin) were similar but the relative contributions of enthalpies and entropies differed because of the presence of

dx.doi.org/10.1021/bi5001307 | Biochemistry 2014, 53, 4445−4454

protein scaffolds. In the presence of protein scaffolds (as in ovalbumin and RNase B), the entropy of binding becomes more positive. At the same time, the enthalpy of the system becomes less negative as a compensatory measure (Table 1). Enthalpy−entropy compensation is an integral part of binding thermodynamics. The spontaneity of a binding interaction increases either with an increase in positive binding entropy or with an increase in negative binding enthalpy. Our data strongly suggest that the positive entropy-driven binding spontaneity of a glycan increases when it is covalently attached to a protein scaffold.

ConA binds to a $Man_9GlcNAc_2$ oligosaccharide and a trimannoside with comparable affinities (Table 1) because ConA recognizes only the core trimannoside structure of this oligosaccharide. SBA contains four $Man_9GlcNAc_2$ oligosaccharides (Figures 1 and 3) and is a tetravalent ligand of ConA. ConA would bind to the trimannoside epitope of each $Man_9GlcNAc_2$ of SBA. The synthetic analogue **6** has four trimannoside epitopes (Figures 1 and 2) and is a tetravalent ligand of ConA. Therefore, it is a structural analogue of SBA. Indeed, the affinity (K_a) and ΔG values of ConA for analogue **6** and SBA are comparable, but their $T\Delta S$ values are significantly different (−44.4 kcal/mol for analogue **6** and −28.4 kcal/mol for SBA) (Table 1). In other words, the relative contribution of enthalpy and entropy to the binding free energy is very different for these two ligands. For SBA, the entropy of binding is significantly more positive than that of analogue **6**. The data suggest that the thermodynamic binding mechanism changes with the structures of the scaffolds of the ligands. Analogue **6** and SBA have similar glycan epitopes and similar valences for ConA. While analogue **6** contains a synthetic scaffold, SBA possesses a protein scaffold. Therefore, the difference in the entropic values could be attributed to the structural differences of the scaffolds. The data suggest that the thermodynamic binding mechanism changes with the structures of the scaffolds of the ligands. Data obtained with ConA and another tetravalent glycoprotein avidin (Table 1) further support this view. It is difficult to draw a general conclusion on the basis of data obtained from three ligands (analogue **6**, SBA, and avidin), but it is tempting to speculate form our limited data that compared to synthetic scaffolds, protein scaffolds provide more an entropic advantage.

Overall, these findings support the notion that compared to their free states, glycans and possibly glycosaminoglycans would bind to their receptors more spontaneously when they are covalently linked with protein scaffolds (as in glycoproteins and proteoglycans, respectively). This explains, at least partially, why structural glycans in nature almost always need to be attached to scaffolds (for example, glycoproteins and proteoglycans). Protein glycosylation is a natural way of attaching glycans to protein scaffolds. It seems that one of many objectives of glycosylation is to make glycans functionally more spontaneous. Addition of a scaffold (protein or otherwise) to a glycan increases its hydrodynamic radius and changes its rotational and tumbling properties. This, in turn, may influence the chance of a lectin molecule to "find" and bind to the glycan epitopes. Addition of a scaffold to a glycan may also alter glycan conformation and flexibility. It remains to be seen how these scaffold-dependent physical changes contribute to favorable lectin binding entropy (studies are underway in our lab). One could speculate that in a system with favorable entropy, breaking of hydrogen bonds may result in smaller numbers of overall hydrogen bonds. The reduced negative enthalpy values support this speculation.

Structures of Protein Scaffolds May Limit Glycan Density-Dependent Affinity Effects. Invertase is a high-mannose glycan-containing glycoprotein with 14 accessible high-mannose glycans, whereas SBA possesses four high-mannose glycans (Figure 1). Thermodynamic binding data and precipitation studies show that, compared to SBA, invertase has a higher valence for ConA (Table 1 and Figure S11 of the Supporting Information). [Valence is $1/n$ (ref 13). The valence of SBA for ConA is 1/0.25 = 4; the valence of invertase for ConA is 1/0.08 = 12.5.] However, the affinities of invertase and SBA for ConA are comparable (Table 1). The glycan density on invertase is higher than that of SBA, but that does not translate into a higher affinity. The structures of the protein scaffolds may play a role in limiting the affinity. We have seen similar effects with other globular glycoproteins (data not shown).

The Scaffold Structure of a Relatively Low-Affinity Multivalent Ligand Can Turn It into a Very Potent Lattice-Forming (cross-linking) Agent of Lectins. Depending on their scaffold structures, some synthetic multivalent ligands with relatively weak affinity for lectins can cross-link the same lectins with surprising efficiency. In other words, ligand scaffolds can make cross-linking kinetics (or the rate of lattice formation) independent of ligand affinity. For example, synthetic glycoconjugate **1** possesses an affinity (62 μM) much weaker than that of invertase (0.07 μM), but the lattice forming efficiency of the former is comparable to that of invertase (Figure 5). This result indicates that even lower-affinity multivalent ligands with proper scaffolds have the potential to form a lattice with cell surface receptors.

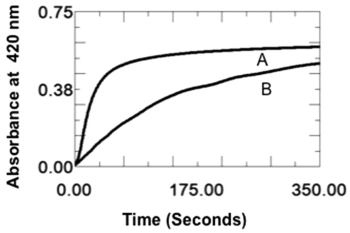


Figure 5. Scaffolds can make lattice formation kinetics independent of ligand affinity. Lattice formation kinetics of ConA with low-affinity [analogue **1** (A)] and high-affinity [invertase (B)] ligands.

Scaffold Structures Influence the Lattice Formation (cross-linking) Kinetics. Receptor clustering (lattice formation) by multivalent ligands often leads to receptor-mediated signaling events. Such signaling processes are sensitive to the kinetics of clustering.[22,39−43] Hence, factors that influence this kinetics can potentially modulate signaling events. An *in vitro* turbidometric assay is a reliable way to measure the kinetics of cross-linking.[14,22,44] Turbidometric assays performed at pH 5.2 and 7.2 showed a similar trend of cross-linking kinetics (Figure 6). The kinetics of complex formation at pH 5.2 was comparatively slower because ConA is a dimer at pH 5.2 (ConA is a tetramer at pH 7.2).

Trivalent mannose analogues (**1** and **2** in Figure 2) that differ only in linker length (i.e., 10 atoms for **1** and 9 atoms for **2**) (Figure 2) showed very different lattice formation kinetics with ConA (Figure 6, top) at pH 5.2 and 7.2. The affinity (0.2 mM) and valence (trivalent) of these analogues were similar.

dx.doi.org/10.1021/bi5001307 | *Biochemistry* 2014, 53, 4445−4454

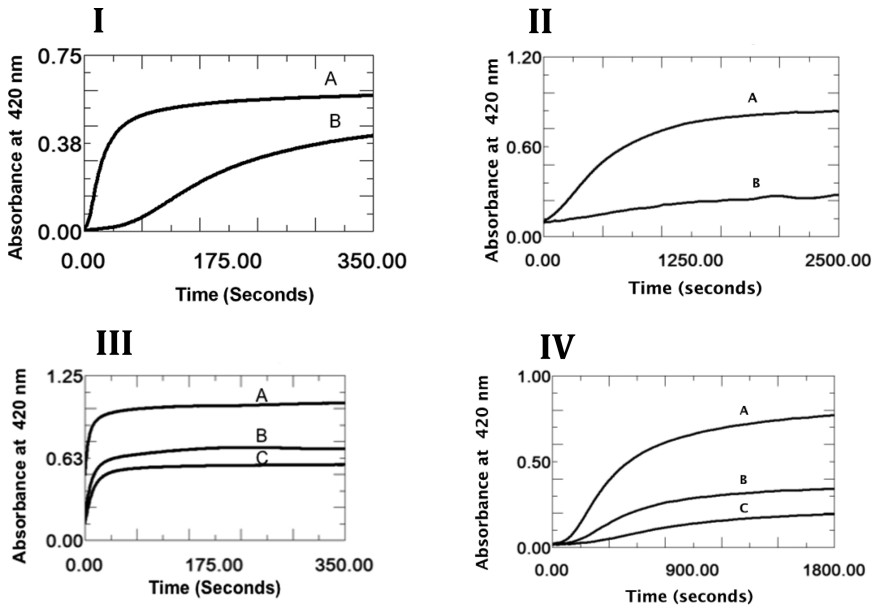


Figure 6. Scaffolds of synthetic glycoconjugates modulate their lattice forming kinetics. Cross-linking or lattice forming kinetics of ConA with (top) trivalent analogues **1** (A) and **2** (B) at pH 7.2 (I) and pH 5.2 (II) and (bottom) nonavalent analogues **3** (A), **4** (B), and **5** (C) at pH 7.2 (III) and pH 5.2 (IV).

Therefore, these differences in lattice formation kinetics were independent of binding affinity and valence but dependent on the structures of the scaffolds. Similar observations were made with three nonavalent analogues (**3**–**5** in Figure 2) with identical valences (nonavalent) and affinities (0.03 mM). Differential lattice formation kinetics of **3**–**5** at two different pH values (5.2 and 7.2) (Figure 6, bottom) were due to the subtle structural differences in their scaffolds. In addition to these synthetic glycoconjugates, natural glycoproteins possessing similar affinities for ConA demonstrated different lattice formation kinetics with ConA. For example, SBA and avidin possess similar affinities and valences for ConA (Table 1), but their cross-linking kinetics are different (Figure S12 of the Supporting Information). On the surface of cells, glycoconjugate-mediated lattice formation kinetics may be a determining factor of lectin–glycoconjugate lattice structures and subsequent signal initiation. If scaffold structures of glycoconjugates can differentially influence lattice formation kinetics, scaffolds can potentially modulate lectin-mediated signal initiation and diversify the signaling properties of glycoconjugates.

■ CONCLUSIONS AND IMPLICATIONS

Scaffolds of glycoconjugates do not directly interact with lectins and are perceived as mere support structures that help the multivalent display of glycans, but our data show that scaffolds play a much more critical role in lectin–ligand interactions. The following points summarize the potential roles of scaffolds as well as the implications of the findings presented here.

Thermodynamic Advantage of Glycosylation. We found that the binding entropy of a glycan became more positive when a glycan contained a scaffold. The degree of entropic change was found to depend on the nature of the scaffolds. Protein scaffolds were responsible for an entropy change that was more positive than that of synthetic scaffolds. An increasing positive entropy is a hallmark of an increasing spontaneity of interaction. It seems that one of many purposes of glycosylation (which is a natural process of adding a scaffold to glycans) is to provide glycoconjugates with an entropic advantage, so that they can interact with lectins more spontaneously.

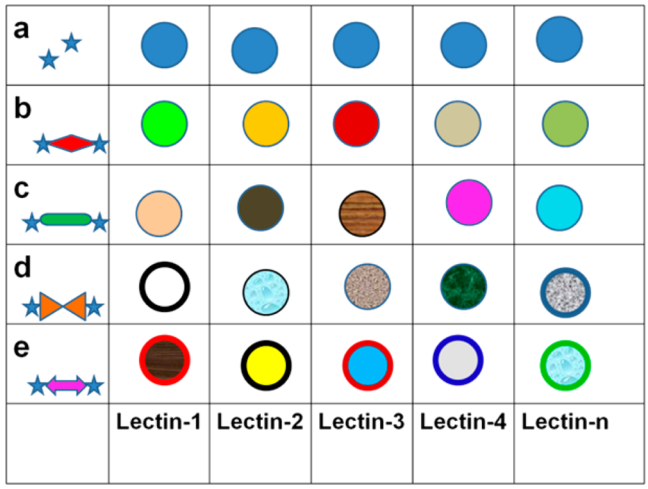


Figure 7. Schematic presentation of the proposed scaffold-mediated functional diversity of glycoconjugates. Interactions of a group of homologous lectins (lectin-1 to lectin-*n*) possessing similar carbohydrate binding specificities with a particular glycan [free (a) and on different scaffolds (b–e), *Y* axis, first column] are shown with circles. Different colors represent differences in binding and postbinding properties (binding thermodynamics, lattice formation properties, and signaling properties). The binding specificities of all the homologous lectins for the free glycan (a) will be comparable (denoted as blue circles). The same glycan (a) on different scaffolds (b–e) will show different properties with a single lectin. Each scaffold-containing glycoconjugate (b–e) will show different properties with individual members of the homologous lectin family. LacNAc specific lectins galectins form such a family of homologous lectins.

Functional Diversity of Glycans and Their Receptors (lectins). The huge arrays of functionally diverse glycoconjugates in nature are basically made with a limited number of structurally different monosaccharides. In addition to other factors (linkages, modifications, arrangements, and clustering),

dx.doi.org/10.1021/bi5001307 | *Biochemistry* 2014, 53, 4445–4454

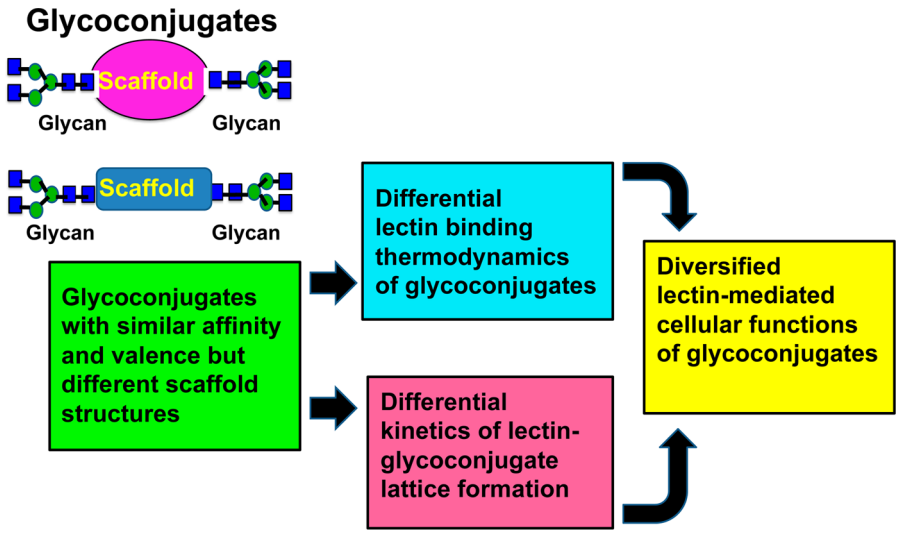


Figure 8. Proposed propagation of effects caused by scaffolds. Glycoconjugates with similar glycan epitopes, comparable affinities, and identical valences but different scaffold structures bind to the same lectin with different binding thermodynamics and display differential kinetics of lattice formation with the lectin. Via the modulation of lectin binding thermodynamics and lectin−glycoconjugate lattice formation kinetics, scaffolds can potentially diversify lectin-mediated cellular functions.

we believe that scaffolds make significant contributions to this functional diversity. Glycoconjugates with similar or overlapping glycan structures can potentially show different lectin-mediated functions if the glycoconjugates contain structurally different scaffolds (Figure 7). Our data strongly suggest that even a subtle difference in the structures of scaffolds could significantly influence the kinetics of lattice formation and thus can potentially diversify the signaling properties of glycoconjugates. The functional ability of multitasking lectins and the ability of lectins to respond differently to self-glycoconjugates and non-self-glycoconjugates might be dependent, at least in part, on the structures of the scaffolds of the glycoconjugates with which they interact. On the basis of our data, we propose that glycoconjugates with similar glycan epitopes, comparable affinities, and identical valences but different scaffold structures will bind to the same lectin with different binding thermodynamics and display differential kinetics of lattice formation with the lectin. Via the modulation of lectin binding thermodynamics and lectin−glycoconjugate lattice formation kinetics, scaffolds can potentially diversify lectin-mediated cellular functions (Figure 8).

Beyond Affinity and Valence Effects. Affinity and valence are the major determinants of ligand−receptor interactions. Our data showed that the influence of scaffolds on lectin−glycoconjugate interaction could be independent of the affinity and valence of the glycoconjugates involved. Along with affinity and valence, the scaffold of a ligand could be another factor that determines the outcome of ligand−receptor interaction.

The observations made in this study are based on thermodynamic data. Therefore, it is very likely that they will be relevant to other lectin−glycoconjugate systems and even to non-glycan macromolecules that possess scaffolds.[45−47]

■ ASSOCIATED CONTENT

Supporting Information

Additional ITC profiles, figures, and a table. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This work was supported by start-up funds (T.K.D., P.B.) and by the Research Excellence Fund (T.K.D.) of Michigan Technological University and by NERSERC (Canada) to R.R.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

Man_5, oligomannose with five mannose residues; Man_7, oligomannose with seven mannose residues; Man_9, oligomannose with nine mannose residues; ConA, concanavalin A lectin; SBA, soybean agglutinin; ITC, isothermal titration calorimetry.

■ REFERENCES

(1) Chabre, Y. M., and Roy, R. (2010) Design and creativity in synthesis of multivalent neoglycoconjugates. *Adv. Carbohydr. Chem. Biochem. 63*, 165−393.

(2) Chabre, Y. M., and Roy, R. (2013) Multivalent glycoconjugate syntheses and applications using aromatic scaffolds. *Chem. Soc. Rev. 42*, 4657−4708.

(3) Kitov, P. I., Sadowska, J., Mulvey, M., Armstrong, G. D., Ling, H., Pannu, N. S., Read, R. J., and Bundle, D. R. (2000) Shiga-like toxins are neutralized by tailored multivalent carbohydrate ligands. *Nature 403*, 669−672.

(4) Collins, M. N., and Birkinshaw, C. (2013) Hyaluronic acid based scaffolds for tissue engineering: A review. *Carbohydr. Polym. 92*, 1262−1279.

(5) Turnbull, W. B., and Stoddart, J. F. (2002) Design and synthesis of glycodendrimers. *J. Biotechnol. 90*, 231−255.

(6) Varki, A. (1993) Biological roles of oligosaccharides: All of the theories are correct. *Glycobiology 3*, 97−130.

(7) Gabius, H.-J. (2006) Cell surface glycans: The why and how of their functionality as biochemical signals in lectin-mediated information transfer. *Crit. Rev. Immunol. 26*, 43−79.

(8) Varki, A., Cummings, R. D., Esko, J. D., Freeze, H. H., Stanley, P., Bertozzi, C. R., Hart, G. W., and Etzler, M. E. (2009) *Essentials of Glycobiology*, 2nd ed., p 784, Cold Spring Harbor Laboratory Press, Plainview, NY.
(9) Lundquist, J. J., and Toone, E. J. (2002) The cluster glycoside effect. *Chem. Rev. 102*, 555−578.
(10) Dam, T. K., and Brewer, C. F. (2002) Thermodynamic studies of lectin-carbohydrate interactions by isothermal titration calorimetry. *Chem. Rev. 102*, 387−429.
(11) Dam, T. K., Roy, R., Das, S. K., Oscarson, S., and Brewer, C. F. (2000) Binding of multivalent carbohydrates to concanavalin A and *Dioclea grandiflora* lectin. Thermodynamic analysis of the "multivalency effect". *J. Biol. Chem. 275*, 14223−14230.
(12) Mammen, M., Choi, S.-K., and Whitesides, G. M. (1998) Polyvalent interactions in biological systems: Implications for design and use of multivalent ligands and inhibitors. *Angew. Chem., Int. Ed. 37*, 2754−2794.
(13) Dam, T. K., Gerken, T. A., Cavada, B. S., Nascimento, K. S., Moura, T. R., and Brewer, C. F. (2007) Binding studies of α-GalNAc-specific lectins to the α-GalNAc (Tn-antigen) form of porcine submaxillary mucin and its smaller fragments. *J. Biol. Chem. 282*, 28256−28263.
(14) Cairo, C. W., Gestwicki, J. E., Kanai, M., and Kiessling, L. L. (2002) Control of Multivalent Interactions by Binding Epitope Density. *J. Am. Chem. Soc. 124*, 1615−1619.
(15) Perrimon, N., and Bernfield, M. (2000) Specificities of heparan sulphate proteoglycans in developmental processes. *Nature 404*, 725−728.
(16) Mammen, M., Dahmann, G., and Whitesides, G. M. (1995) Effective inhibitors of hemagglutination by influenza virus synthesized from polymers having active ester groups. Insight into mechanism of inhibition. *J. Med. Chem. 38*, 4179−4190.
(17) Horan, N., Yan, L., Isobe, H., Whitesides, G. M., and Kahne, D. (1999) Nonstatistical binding of a protein to clustered carbohydrates. *Proc. Natl. Acad. Sci. U.S.A. 96*, 11782−11786.
(18) Dam, T. K., and Brewer, C. F. (2008) Effects of clustered epitopes in multivalent ligand-receptor interactions. *Biochemistry 47*, 8470−8476.
(19) Garner, O. B., and Baum, L. G. (2008) Galectin−glycan lattices regulate cell-surface glycoprotein organization and signaling. *Biochem. Soc. Trans. 36*, 1472−1477.
(20) Grigorian, A., Torossian, S., and Demetriou, M. (2009) T-cell growth, cell surface organization, and the galectin glycoprotein lattice. *Immunol. Rev. 230*, 232−246.
(21) Demetriou, M., Granovsky, M., Quaggin, S., and Dennis, J. W. (2001) Negative regulation of T-cell activation and autoimmunity by Mgat5 N-glycosylation. *Nature 409*, 733−739.
(22) Gestwicki, J. E., Cairo, C. W., Strong, L. E., Oetjen, K. A., and Kiessling, L. L. (2002) Influencing Receptor-Ligand Binding Mechanisms with Multivalent Ligand Architecture. *J. Am. Chem. Soc. 124*, 14922−14933.
(23) Chabre, Y. M., Brisebois, P., Abassi, L., Kerr, S., Fahy, J., Marcotte, I., and Roy, R. (2011) Hexaphenylbenzene as a rigid template for the straightforward syntheses of "star-shaped" glycodendrimers. *J. Org. Chem. 76*, 724−727.
(24) Chabre, Y. M., Contino-Pépin, C., Placide, V., Shiao, T. C., and Roy, R. (2008) Expeditive synthesis of glycodendrimer scaffolds based on versatile TRIS and mannoside derivatives. *J. Org. Chem. 73*, 5602−5605.
(25) Chabre, Y. M., Shiao, T. C., Vidal, S., and Roy, R. (2012) Synthesis of Prop-2-ynyl 2,3,4,6-tetra-O-acetyl-α-D-mannopyranoside. In *Carbohydrate Chemistry: Proven Synthetic Methods* (Kovac, P., Ed.) Vol. *1*, Chapter 31, pp 275−278, CRC Press/Taylor & Francis Group, Boca Raton, FL.
(26) Lis, H., and Sharon, N. (1978) Soybean agglutinin—a plant glycoprotein. Structure of the carboxydrate unit. *J. Biol. Chem. 253*, 3468−3476.
(27) Mandal, D. K., and Brewer, C. F. (1992) Interactions of concanavalin A with glycoproteins: Formation of homogeneous glycoprotein-lectin cross-linked complexes in mixed precipitation systems. *Biochemistry 31*, 12602−12609.
(28) Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1951) A colorimetric method for the determination of sugars. *Nature 168*, 167.
(29) Saha, S. K., and Brewer, C. F. (1994) Determination of the concentrations of oligosaccharides, complex type carbohydrates, and glycoproteins using the phenol-sulfuric acid method. *Carbohydr. Res. 254*, 157−167.
(30) Goldstein, I. J., and Poretz, R. D. (1986) in *The Lectins* (Liener, I. E., Sharon, N., and Goldstein, I. J., Eds.) pp 35−244, Academic Press Inc., New York.
(31) Wiseman, T., Williston, S., Brandt, J. F., and Lin, L.-N. (1989) Rapid measurement of binding constants and heats of binding using a new titration calorimeter. *Anal. Biochem. 179*, 131−135.
(32) Bhattacharyya, L., Fant, J., Lonn, H., and Brewer, C. F. (1990) Binding and precipitating activities of *Lotus tetragonolobus* isolectins with L-fucosyl oligosaccharides. Formation of unique homogeneous cross-linked lattices observed by electron microscopy. *Biochemistry 29*, 7523−7530.
(33) Gupta, D., Dam, T. K., Oscarson, S., and Brewer, C. F. (1997) Thermodynamics of lectin-carbohydrate interactions. Binding of the core trimannoside of asparagine-linked carbohydrates and deoxy analogs to concanavalin A. *J. Biol. Chem. 272*, 6388−6392.
(34) Williams, B. D., and Lennarz, W. J. (1984) Control of asparagine-linked oligosaccharide chain processing: Studies on bovine pancreatic ribonuclease B. An in vitro system for the processing of exogenous glycoproteins. *J. Biol. Chem. 259*, 5105−5114.
(35) Tai, T., Yamashita, K., Ogata-Arakawa, M., Koide, N., Muramatsu, T., Iwashita, S., Inoue, Y., and Kobata, A. (1975) Structural studies of two ovalbumin glycopeptides in relation to the endo-β-N-acetylglucosaminidase specificity. *J. Biol. Chem. 250*, 8569−8575.
(36) Hiller, Y., Gershoni, J. M., Bayer, E. A., and Wilchek, M. (1987) Biotin binding to avidin. Oligosaccharide side chain not required for ligand association. *Biochem. J. 248*, 167−171.
(37) Ziegler, F. D., Maley, F., and Trimble, R. B. (1988) Characterization of the glycosylation sites in yeast external invertase. II. Location of the endo-β-N-acetylglucosaminidase H-resistant sequins. *J. Biol. Chem. 263*, 6986−6992.
(38) Mandal, D. K., and Brewer, C. F. (1993) Differences in the binding affinities of dimeric concanavalin A (including acetyl and succinyl derivatives) and tetrameric concanavalin A with large oligomannose-type glycopeptides. *Biochemistry 32*, 5116−5120.
(39) Germain, R. N., and Stefanová, I. (1999) The dynamics of T cell receptor signaling: Complex orchestration and the key roles of tempo and cooperation. *Annu. Rev. Immunol. 17*, 467−522.
(40) Weintraub, B. C., Jun, J. E., Bishop, A. C., Shokat, K. M., Thomas, M. L., and Goodnow, C. C. (2000) Entry of B cell receptor into signaling domains is inhibited in tolerant B cells. *J. Exp. Med. 191*, 1443−1448.
(41) Sugiyama, J. E., Glass, D. J., Yancopoulos, G. D., and Hall, Z. W. (1997) Laminin-induced acetylcholine receptor clustering: An alternative pathway. *J. Cell Biol. 139*, 181−191.
(42) Petrie, R. J., Schnetkamp, P. P., Patel, K. D., Awasthi-Kalia, M., and Deans, J. P. (2000) Transient translocation of the B cell receptor and Src homology 2 domain-containing inositol phosphatase to lipid rafts: Evidence toward a role in calcium regulation. *J. Immunol. 165*, 1220−1227.
(43) Cochran, J. R., Aivazian, D., Cameron, T. O., and Stern, L. J. (2001) Receptor clustering and transmembrane signaling in T cells. *Trends Biochem. Sci. 26*, 304−310.
(44) Kitano, H., Sumi, Y., and Tagawa, K. (2001) Recognition of novel lipopolypeptides with many pendent sugar residues by lectin. *Bioconjugate Chem. 12*, 56−61.
(45) Hessler, G., and Baringhaus, K.-H. (2010) The scaffold hopping potential of pharmacophores. *Drug Discovery Today: Technol. 7*, e263−e269.

(46) Endo, M., and Sugiyama, H. (2009) Chemical Approaches to DNA Nanotechnology. *ChemBioChem 10*, 2420 −2443.
(47) Straley, K. S., Wong, C., Foo, P., and Heilshorn, S. C. (2010) Biomaterial design strategies for the treatment of spinal cord injuries. *Journal of Neurotrauma 27*, 1−19.