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Microarray Analysis of Oligosaccharide-Mediated Multivalent Carbohydrate–Protein Interactions and Their Heterogeneity

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Carbohydrate–protein interactions (CPIs) are involved in a wide range of biological phenomena. Hence, the characterization and presentation of carbohydrate epitopes that closely mimic the natural environment is one of the long-term goals of glycosciences. Inspired by the multivalency, heterogeneity and nature of carbohydrate ligand-mediated interactions, we constructed a combinatorial library of mannose and galactose homo- and hetero-glycodendrons to study CPIs. Microarray analysis of these glycodendrons with a wide range of biologically important plant and animal lectins revealed that oligosaccharide structures and heterogeneity interact with each other to alter binding preferences.

Introduction

There is a wide interest in mimicking the highly complex cell-surface glycocalyx chemically to target crucial biological events, including cellular recognition and disease progression.[1] The major roadblock to delineating cell-surface carbohydrate epitopes is the weak monovalent binding affinity. However, multivalent probes now provide a robust strategy for mimicking the glycocalyx to study carbohydrate–protein interactions (CPIs).[1h,2] Although multivalency enhances the binding avidity, it is not the optimal presentation of the carbohydrate scaffolds of cell-surface glycans. Therefore approaches to targeting the glycocalyx have focused on tuning the properties of multivalency, including density, sequence control, heterogeneity and geometry.[3] For example, FimH bacterial binding studies with shape-dependent glyco-gold nanoparticles showed significant impact on bacterial binding and cellular uptake through specific CPIs.[4] Homo- and hetero-glycodendrons with variable carbohydrate density alter lectin binding.[3d,5] Similarly, programmable sequences of monodispersed hetero-glycooligomers showed higher affinity towards ConA lectin than did homo-multivalent analogues.[6] Janus glycodendrimers with sequence-defined glycodendrosomes revealed high activity and sensor capacity with human lectins.[7] However, despite the numerous examples of glycoprobes with heterogeneity, different topology and multivalency,[8] no systematic investigation with a broad lectin family to rationalize their contributions has been described. Herein, we present a combinatorial library of glycodendrons that use heterogeneity, the nature of the carbohydrate ligand and multivalency to discriminate the binding preferences with lectins. More specifically, we targeted biologically important plant and animal lectins that specifically bind to mannose and galactose ligands. Our results suggest that multivalent heterogeneous carbohydrate ligands serve as better carbohydrate epitopes for targeting lectins.

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Results and Discussion

To identify the optimal glycodendron ligand to target different mannose- and galactose-specific lectins, we designed and synthesized two distinct classes of tripodal glycodendrons,[9] namely, homo- and hetero-glycodendrons in which ten combinations of mannose and galactose ligands were displayed. These libraries comprise four homodendrons bearing monomannose (**M1**), α(1–2)-linked mannose di- (**M2**) and trisaccharides (**M3**) or galactose carbohydrate (**M4**), three heterodendrons bearing twin monomannose (**M5**) or oligosaccharide ligand (**M6** and **M7**) with a single galactose ligand and three heterodendrons bearing twin galactose ligands and a single mono- or oligo-mannose ligand (**M8**–**M10**). Manα(1–2)-linked oligosaccharides were selected because the high-mannose structure was expressed on the surface of the gp120 protein of HIV, which binds to human monoclonal antibody (mAb) 2G12.[10] In all glycodendrons, the fourth arm of the dendron was extended with an amine linker to allow them to be immobilized on the microarray epoxide surfaces. It is expected that

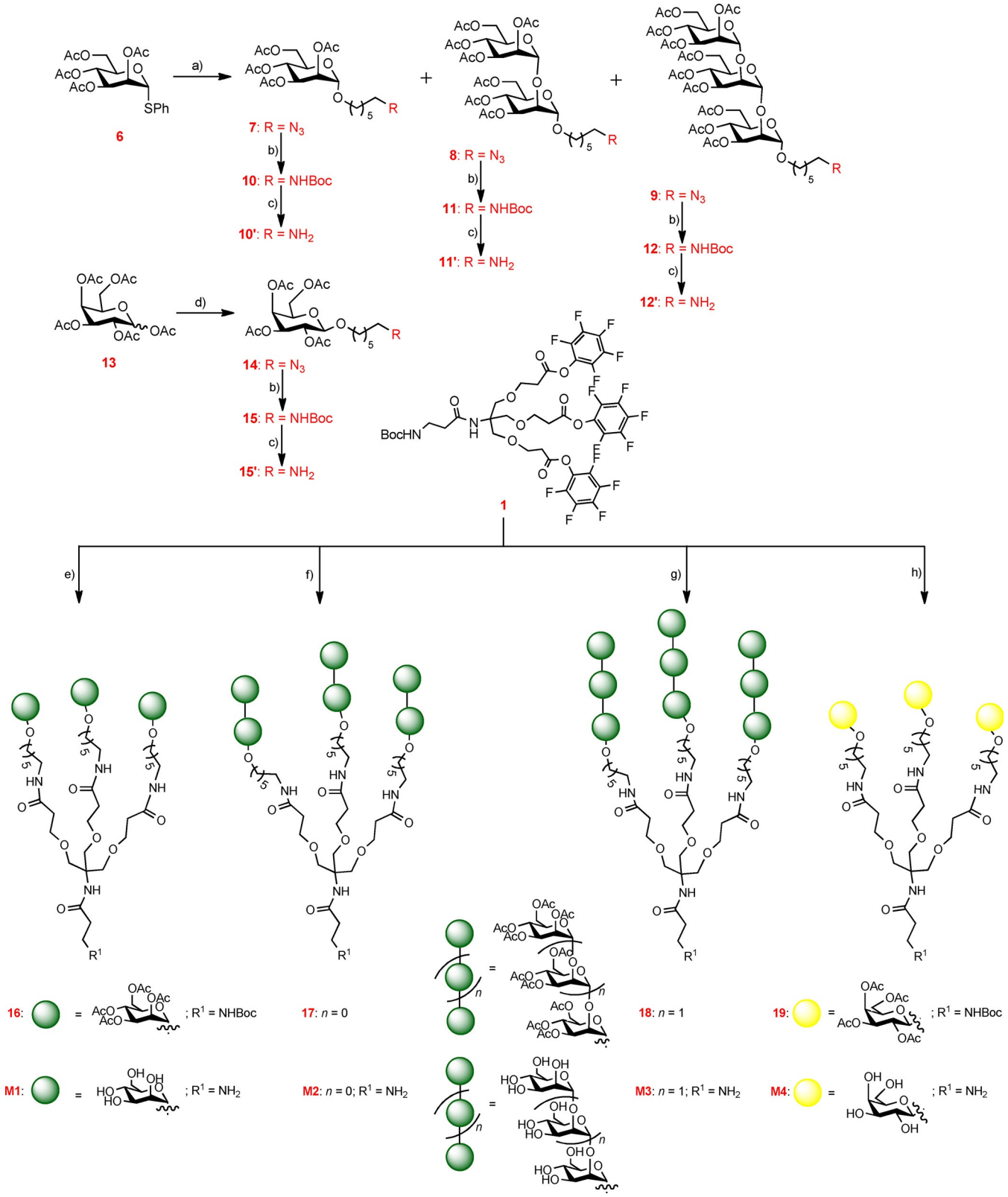
the heterogeneity, nature of the carbohydrate ligand and multivalency of these glycodendrons could distinctly affect plant and animal lectin binding, and combining these effects would provide a unique recognition signature for differentiating the specific lectin recognition. The synthesis of all these glycodendrons was carried out through a divergent strategy.

Tripod-active ester **1** was readily synthesized from Tris base, as described.[11] Acetylated mannose thioglycoside **6** was chosen as mannose donor to synthesize in one pot mono-, di- and tri-mannose ligands (**7**–**9**) in the presence of 0.8 equivalents of linker with NIS/TfOH activator.[12a] The amine-linked peracetylated galactose **14** was synthesized according to a published procedure.[12b] The azide-linker derivatives **7**–**9** were treated with triphenyl phosphine and Boc anhydride to give Boc-protected ligands, which were deprotected with TFA/CH_2Cl_2 to give mannose or galactose amine linker (**10′**–**12′** and **15′**). Pentafluorophenol (PFP) ester **1** was treated with these ligands to form the desired homo-glycodendrons (**M1**–**M4**) under basic conditions, before global Boc and acetate deprotection (Scheme 1). Bis-mannose and bis-galactose hetero-glycodendrons were generated by mixing stoichiometric amounts of mono-, di- or trimannose- or -galactose-containing amine linkers (**10′**–**12′** and **15′**) to **1** to afford disubstituted derivatives **20**–**23**. These disubstituted ligands were mixed with galactose or mannose ligand to afford hetero-glycodendrons (**M5**–**M10**). Finally, acetyl and Boc protecting groups were removed to give a library of new multivalent, homo- and hetero-glycodendrons (Scheme 2).

To differentiate between the lectins, we constructed a microarray platform of synthetic glycodendrons. The rationale of the interface platform is to generate multivalent arrays to facilitate the analysis of lectin binding and to mimic cell-surface carbohydrate presentations.[13] Synthetic glycodendrons **M1**–**M10** were printed onto epoxide-functionalized microarray slides at an optimal concentration of 50 μM in replicates of four, as described in the Experimental Section.[14] Plant lectins such as concanavalin A (ConA), peanut agglutinin (PNA) and galanthus nivalis lectin (GNL), which are specific for mannose and galactose moieties, were tested first at an optimal concentration of 20 ng μL^{-1} under optimized buffer conditions.[15] As expected, ConA lectin bound to all homo- and heteromannose glycodendrons, but did not bind to galactose glycodendron **M4** (Figure 1 A). Interestingly, **M2** showed higher binding preferences than **M1** and **M3**, presumably as a result of the nature of the mannose ligand preferences of ConA lectin. Figure 1 A further illustrates that, within the hetero-glycodendrons, **M6**, which has twin α(1–2)-linked mannose disaccharide moiety, showed stronger binding preferences to ConA than **M5** or **M7**; its binding preference was close to that of **M2**. The twin galactose glycodendrons (**M9** and **M10**) also showed a stronger binding preference to ConA than **M8**, thus indicating that heterogeneity with a specific mannose scaffold induced strong binding preferences. Overall, ConA preferred α(1–2)-linked mannose disaccharide-capped homo- and hetero-glycodendrons, and heterogeneity in the form of one or two galactose substitutions in the glycodendrons increased the special arrangement of mannose ligands and induced strong binding preferences. These results correlate to the ConA–mannose heterogeneity binding trend found in the literature.[8c] In contrast, GNL, another mannose-specific lectin displayed weak reactivity with all mannose glycodendrons (Figure 1 B). However, similar to ConA lectin, homo- and hetero-glycodendrons with α(1–2)-linked mannose disaccharide ligands (**M2** and **M6**) showed preferential binding compared to other analogues (**M1** or **M3** and **M5** or **M7**). In contrast to ConA lectin binding, twin galactose glycodendrons exhibited no binding activity, thus indicating that GNL glycodendron epitope preference is different from that of ConA. Finally, PNA, a galactose-specific plant lectin, showed a preference for binding to homogalactose glycodendron **M4** and twin galactose hetero-glycodendron such as **M8** and **M9** (Figure 1 C). These results indicate that ConA and GNL, which have one and three mannose-binding motifs in each tetrameric protein, displayed variable structural–activity relationship with mannose glycodendron binding pattern. In addition, the heterogeneity of glycodendrons influences the CPIs microenvironment.

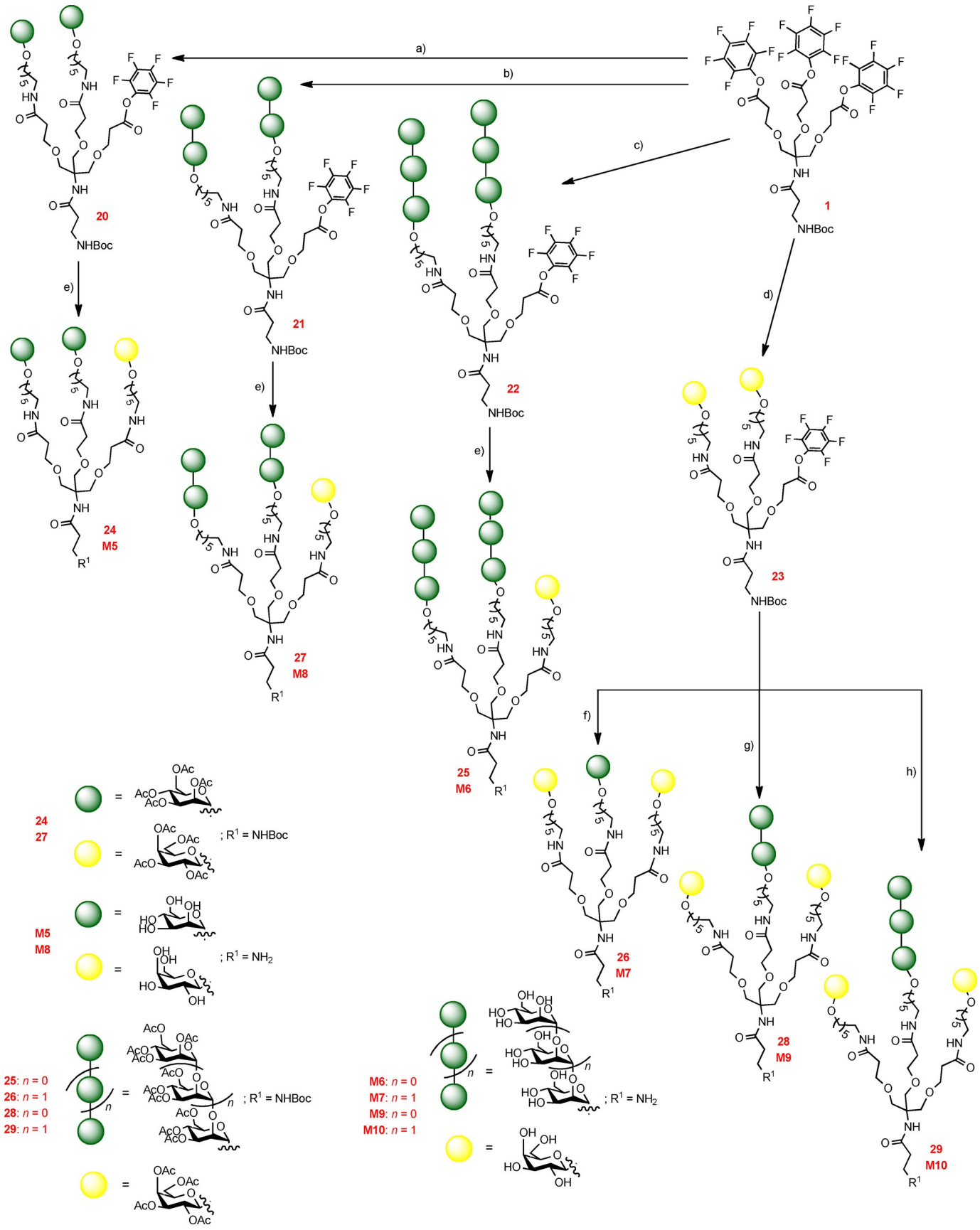
To demonstrate the potential of the heterogeneity, oligomeric structure and multivalency of glycodendrons in CPIs, we compared the microarray binding profile with C-type lectins (human IgG Fc fusion proteins; CLR-hFc: DC-SIGN, SIGNR3, Mincle and MGL-1) and human galectins (galectin-1 and -3). Targeting C-type lectin receptors (CLRs) and galectins with designed complex glycans is like a "Trojan horse" strategy for developing next-generation drugs for treating cancer, infections and autoimmune diseases.[16] We tested CLR–hFc proteins on the microarray slide at 20 ng μL^{-1} in 50 mM HEPES buffer with 5 mM $CaCl_2$, 5 mM $MgCl_2$, 0.005% Tween-20 and 1% ovalbumin, followed by the secondary antibody (Cy3-anti-human IgG). Divalent cations were maintained during all the binding and washing steps. In control blocks, divalent cations were omitted from the binding and washing steps. The slides were scanned, and the binding was determined by fluorescence intensity, as described in the Experimental Section. Notably, all four CLRs displayed unique binding preferences that depended on the heterogeneity and oligosaccharide structure on the glycodendrons (Figure 1).

Human DC-SIGN, a mannose- and fucose-specific lectin,[17] displayed preferential binding to mannose glycodendrons and weak binding to **M4**. Next, we compared whether the CPIs of DC-SIGN would generate a unique pattern different from those of ConA and GNL lectins. As shown in Figure 1 E, DC-SIGN exhibited stronger binding affinity for **M3** than **M1** or **M2**, thus indicating that **M3**, which mimics the high-mannose *N*-glycan structure, is the preferred ligand of DC-SIGN. Among the hetero-glycodendrons, **M7** induced strong binding preferences and is close to **M3,** thus demonstrating that heterogeneity indeed influences the binding affinity of DC-SIGN. These results clearly illustrated that the DC-SIGN binding pattern is different from that of ConA lectin. Notably, ConA displayed strong binding to α(1–2)-linked mannose disaccharide as a potential carbohydrate ligand, whereas DC-SIGN showed preferential binding to the α(1–2)-linked mannose trisaccharide on homo- (**M3**) and hetero-glycodendrons (**M7**). We next tested the glycodendron binding pattern of SIGNR3, a murine orthologue of DC-



Scheme 1. Synthesis of glycodendrons **M1** to **M4**. a) NIS, TfOH, 6-azidohexan-1-ol; b) PPh_3, THF, water, Boc_2O; c) TFA (20%) in CH_2Cl_2; d) 6-azidohexan-1-ol, $BF_3{\cdot}Et_2O$; PPh_3, THF, water, Boc_2O; e) **10′**, Et_3N, CH_2Cl_2, RT; TEA in CH_2Cl_2; NaOMe, MeOH; f) **11′**, Et_3N, CH_2Cl_2, RT; TEA in CH_2Cl_2; NaOMe, MeOH; g) **12′**, Et_3N, CH_2Cl_2, RT; TEA in CH_2Cl_2; NaOMe, MeOH; h) **15′**, Et_3N, CH_2Cl_2, RT; TEA in CH_2Cl_2; NaOMe, MeOH.

SIGN (Figure 1F). Interestingly, SIGNR3 showed weak binding to all mannose glycodendrons. Amongst them, homo-glycodendron **M3** showed stronger preferences than other glycodendrons, thus highlighting that the structure–activity relation-



Scheme 2. Synthesis of glycodendrons **M5** to **M10**. a) **10**′, Et_3N, CH_2Cl_2, RT; b) **11**′, Et_3N, CH_2Cl_2, RT; c) **12**′, Et_3N, CH_2Cl_2, RT; d) **15**′, Et_3N, CH_2Cl_2, RT; e) **15**′, Et_3N, CH_2Cl_2, RT; TEA in CH_2Cl_2; NaOMe, MeOH; f) **10**′, Et_3N, CH_2Cl_2, RT; TEA in CH_2Cl_2; NaOMe, MeOH; g) **11**′, Et_3N, CH_2Cl_2, RT; TEA in CH_2Cl_2; NaOMe, MeOH; h) **12**′, Et_3N, CH_2Cl_2, RT; TEA in CH_2Cl_2; NaOMe, MeOH.

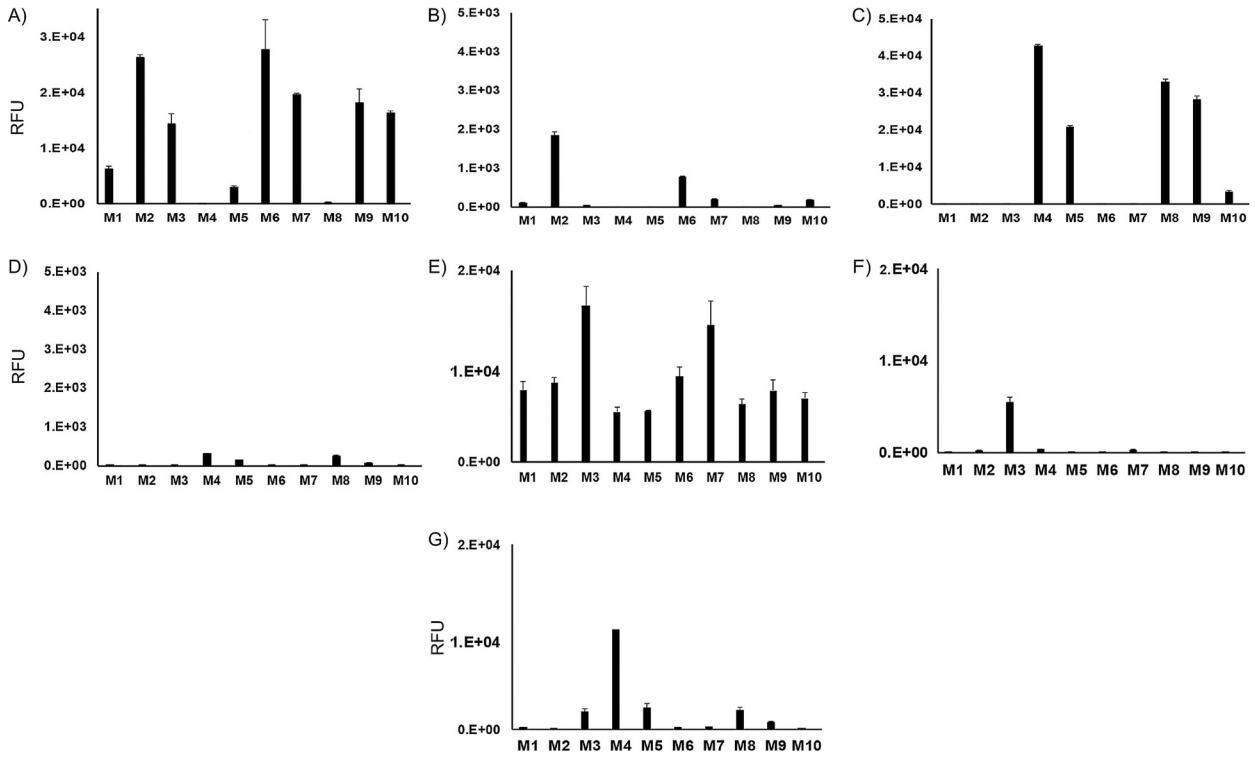


Figure 1. Microarray analysis of **M1** to **M10** with plant and animal lectins. A) Bio-ConA, B) Bio-GNL, C) Bio-PNA, D) galectin-1, E) DC-SIGN-Fc, F) SIGNR3-Fc and G) MGL-1-Fc.

ship of SIGNR3 is different from that of DC-SIGN. Based on these results, **M7** was identified as the preferential ligand for DC-SIGN rather than SIGNR3. MGL-1 lectin, which is known to bind galactose ligands, displayed stronger binding to **M4** than other ligands, thus revealing the significance of homo-glycodendron structure in lectin binding (Figure 1 G). In contrast, Mincle, which is known to bind trehalose derivatives,[18] showed no binding towards the glycodendrons (data not shown).

To analyze the Ca^{2+} dependency of the interactions of CLR-hFc with glycodendrons, we screened binding preferences in the absence of Ca^{2+} (and Mg^{2+}) ions. As expected, glycodendron binding to the CLR-hFc fusion proteins was reduced thereby supporting Ca^{2+}-dependent binding (Figure S1 in the Supporting Information). These results illustrate that the oligomeric effect and heterogeneity dominate in CLR-Fc binding. Finally, to rationalize the galactose preferences of the homo- and hetero-glycodendrons, we tested the binding preference with galectin-1 and -3.[19] We observed that galectin-1 showed weak binding preference with **M4** and **M8** (Figure 1 D), whereas galectin-3 exhibited no binding, thus indicating that the carbohydrate ligand for galectin might be different from that of MGL-1 lectin. Although specific to terminal galactose moieties, the low binding to galectins could suggest that they prefer more extended moieties with a terminal galactose. Overall, microarray analysis clearly showed that **M4** could be a potential ligand for targeting both PNA and MGL-1, and **M8** might be another preferential ligand for PNA lectin.

To uncover changes in CPIs, principal component analysis (PCA) was applied for further discrimination. PCA is a conventional algorithm designed to capture different variances in a given set into two- or three-dimensional parameters (i.e., respective ligands and binding preferences).[20] PCA classified lectins into two groups: galactose-specific lectins (MGL-1, galectin-1 and PNA) and mannose-specific lectins (SIGNR3, DC-SIGN, ConA and GNL; Figure 2). Glycodendrons **M4**, **M5**, **M8** and **M9** were also separated from the rest of the components with positive factor loading mainly due to high preferences to galactose-specific lectins such as MGL1-Fc, human galectin-1 and PNA lectins. **M4** differed from the others in its high affinity for MGL1-Fc and PNA; **M8** was close to PNA due to its preferential binding. Similarly, **M3** was in the region of DC-SIGN and SIGNR3; this indicated the binding preference of mannose-specific CLR lectins. In contrast, **M2** and **M6** were located in the region of GNL and ConA; this confirms the ability of the glycodendron system to discriminate the microenvironment among isostructural and carbohydrate-specific lectins. Notably, two principle PCA components of the glycodendrons displayed 72% variation in the data with different lectins (Figure 2, inset). In summary, PCA can serve as a useful tool for discriminating among CPIs based on different combinations of the heterogeneity and carbohydrate ligands of glycodendrons

Conclusion

Multivalency, heterogeneity and oligomeric structures were harnessed in glycodendrons to identify optimal carbohydrate

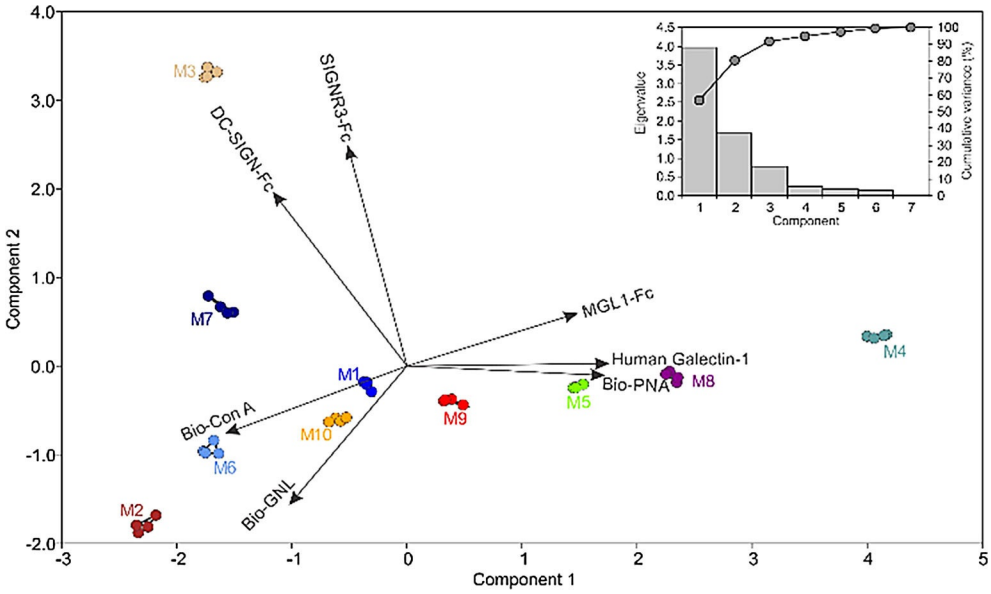


Figure 2. Principal component analysis of the fluorescence intensity of glycodendron binding with lectins. Inset: A screen plot of the percentage variance explained by each principal component.

epitopes for biologically relevant mannose- and galactose-specific plant, murine and human lectins. Microarray analysis revealed that the heterogeneity and carbohydrate ligands of the glycodendrons synergistically influence CPIs, thereby allowing lectin-specific glycodendron ligands and sensor molecules to be identified. Expanding the scope of this platform and synthesizing lectin specific homo- and hetero-glycodendrons could be used to develop biosensors for medical diagnostics and the molecular-level study of CPIs.

Experimental Section

General procedure for the production of the CLR-Fc library: The general procedure for producing a human and mouse CLR-Fc library has been described previously.[20] The primers shown in Table 1 were used for PCR amplification of cDNA fragments encoding the extracellular part of the respective CLR.

The cDNA fragments were cloned into pDrive cloning vector (Qiagen) and further ligated into the pFuse-hIgG1-Fc expression vector (InvivoGen). Next, the CLR-hFc encoding vectors were transiently transfected by using the FreeStyle Max CHO-S expression system (Life Technologies). The cell supernatant containing the CLR-hFc fusion proteins was collected, and the CLR-hFc fusion proteins were purified on HiTrap Protein G HP columns (GE Healthcare). The identity and purity of the CLR-Fc fusion proteins were confirmed by SDS-PAGE with subsequent Coomassie staining as well as by western blotting. The concentration was determined by using the Micro BCA protein assay kit (Thermo Scientific).

Table 1. Primers used for PCR amplification.

Primer		Sequence
DC-SIGN	DC-SIGN-fw	5′-GAATTCGTCCAAGGTCCCCAGCTCCAT-3′
	DC-SIGN-rev	5′-CCATGGACGCAGGAGGGGGGTTTGGGGT-3′
SIGNR3	SIGNR3-fw	5′-GAATTCCATGCAACTGAAGGCTGAAG-3′
	SIGNR3-rev	5′-AGATCTTTTGGTGGTGCATGATGAGG-3′
Mincle	Mincle-fw	5′-CCATGGGGCAGAACTTACAGCCACAT-3′
	Mincle-rev	5′-AGATCTGTCCAGAGGACTTATTTCTG-3′
MGL-1	MGL-1-fw	5′-CCAGTTAAGGAGGGACCTAGGCAC-3′
	MGL-1-rev	5′-AGCTCTCCTTGGCCAGCTTCATC-3′

Microarray fabrication: Arrays were fabricated with NanoPrint LM-60 Microarray Printer (Arrayit) on epoxide-derivatized slides (PolyAn 2D) with 16 subarray blocks on each slide. Glycoconjugates were distributed into 384-well source plates with four replicate wells per sample and 8 μL per well (Version Vr.). Each glycoconjugate was prepared at 50 μM in an optimized print buffer (300 mM phosphate buffer, pH 8.4, supplemented with 0.005% Tween-20). To monitor printing quality, Alexa Flour 555-hydraside (Invitrogen, 2 ng μL^{-1} in 178 mM phosphate buffer, pH 5.5) was used for each printing run. The arrays were printed by using four SMP3 pins (5 μm tip, 0.25 μL sample channel, ≈100 μm spot diameter; Arrayit), with spot to spot spacing of 275 μm. The humidity level in the arraying chamber was maintained at about 70% during printing. Printed slides were left on arrayer deck overnight to allow the humidity to drop to ambient levels (40–45%). Next, slides were packed, vacuum-sealed and stored at room temperature until used.

Microarray binding assay: Slides were developed and analyzed as previously described[1h,2,3] with some modifications. Slides were rehydrated with dH_2O and incubated for 30 min in a staining dish with prewarmed (50 °C) ethanolamine (0.05 M) in Tris·HCl (0.1 M, pH 9.0) to block the remaining reactive epoxy groups on the slide surface, then washed with prewarmed (50 °C) dH_2O. Slides were centrifuged at 200 *g* for 5 min then fitted with a ProPlate multiarray 16-well slide module (Grace Bio-lab) to divide them into the subarrays (blocks). The slides were washed with washing buffer (50 mM HEPES, pH 7, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 0.005% Tween 20 for C-type lectins and Bio-PNA, PBS + 0.1% Tween 20 for Bio-GNL, Bio-ConA and galectin-1), aspirated and blocked with blocking buffer (200 μL/subarray; 50 mM HEPES, pH 7, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 0.005% Tween 20 and 1% *w/v* ovalbumin for C-type lectins and Bio-PNA, PBS + 1% ovalbumin for Bio-GNL, Bio-ConA and galectin-

1) for 1 h at RT with gentle shaking. Next, the blocking buffer was aspirated, and C-type lectins, plant lectins (Vector Labs) and galectin-1 (Peprotech; 200 µL/block; 20 ng µL^{-1}; all diluted in blocking buffer, except for Bio-PNA, which was diluted in blocking buffer with 0.1 mM $CaCl_2$ and 0.01 mM $MnCl_2$) were incubated overnight at 4 °C (for C-type lectins and Bio-PNA) or with gentle shaking for 2 h at RT (for Bio-GNL, Bio-ConA and galectin-1). The slides were then washed four times with washing buffer. Bound antibodies were detected by incubation with secondary detection agents Cy3-anti-human IgG, H + L (1 µg mL^{-1}) or Cy3-SA (1.5 µg mL^{-1}) diluted in washing buffer (200 µL/block) at RT for 1 h. The slides were washed four times with washing buffer, then with washing buffer for 10 min, removed from the ProPlate multiarray slide module and immediately dipped in a staining dish with dH_2O (Bio-GNL, Bio-ConA and galectin-1) or with dH_2O supplemented with $CaCl_2$ (5 mM; for C-type lectins and Bio-PNA) for 10 min with shaking. The slides then were centrifuged at 200 *g* for 5 min, and the dry slides were immediately scanned. (Full data and developing conditions in the Supporting Information.)

Array slide processing: Processed slides were scanned and analyzed as described[21,22] at 10 µm resolution by using a Genepix 4000B microarray scanner (Molecular Devices) with 350 gain (ConA 500 gain was used). Images were analyzed with Genepix Pro 6.0 analysis software (Molecular Devices). Spots were defined as circular features with a variable radius as determined by the Genepix scanning software. Local background was subtracted.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: C-type lectin · heterogeneity · mannose · microarray · oligosaccharides

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