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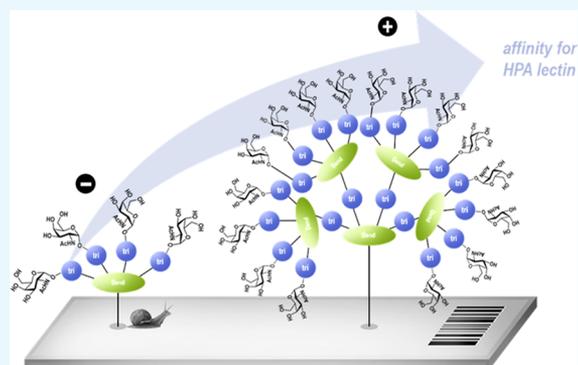
Identification of Nanomolar Lectin Ligands by a Glycodendrimer Microarray

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S Supporting Information

ABSTRACT: Carbohydrate–protein interactions play key roles in a wide variety of biological processes. These interactions are usually weak, with dissociation constants in the low millimolar to high micromolar range. Nature uses multivalency to reach high avidities via the glycoside cluster effect. Capitalizing on this effect, numerous synthetic multivalent glycoconjugates have been described and used as ligands for carbohydrate-binding proteins. However, valency is only one of the several parameters governing the binding mechanisms that are different for every biological receptor, making it almost impossible to predict. In this context, ligand optimization requires the screening of a large number of structures with different valencies, rigidities/flexibilities, and architectures. In this article, we describe a screening platform based on a glycodendrimer array and its use to determine the key parameters for high-affinity ligands of lectin. Several glycoclusters and glycodendrimers displaying varying numbers of α -N-acetylgalactosamine residues were covalently attached on glass slides, and their bindings were studied with the fluorophore-functionalized *Helix pomatia* agglutinin (HPA) used as a lectin model. This technique requires minimal quantities of glycoconjugate compared to those for other techniques and affords useful information on the binding strength. Building of the glycodendrimer array and quantification of the interactions with HPA are described.



INTRODUCTION

Multivalent glycoarchitectures have progressively emerged as relevant molecular systems for diagnostic and therapeutic applications.^{1–3} Strong interaction with carbohydrate-binding proteins (i.e., lectins and antibodies) by means of the glycoside cluster effect^{4–6} is the prerequisite to the development of efficient tools, such as diagnostic probes, antiadhesives, and antitumoral therapeutics or drug-delivery systems. However, despite major progresses in the deciphering of binding mechanisms, the design of high-affinity ligands remains almost impossible to predict because each biological receptor specifically responds to multivalent ligands according to its own structural parameters.^{7,8} For this reason, the development of active ligands often requires time-consuming synthesis of libraries of structures with diverse geometries and valencies and in a sufficient quantity (~ 10 mg) to allow reliable biological investigations.

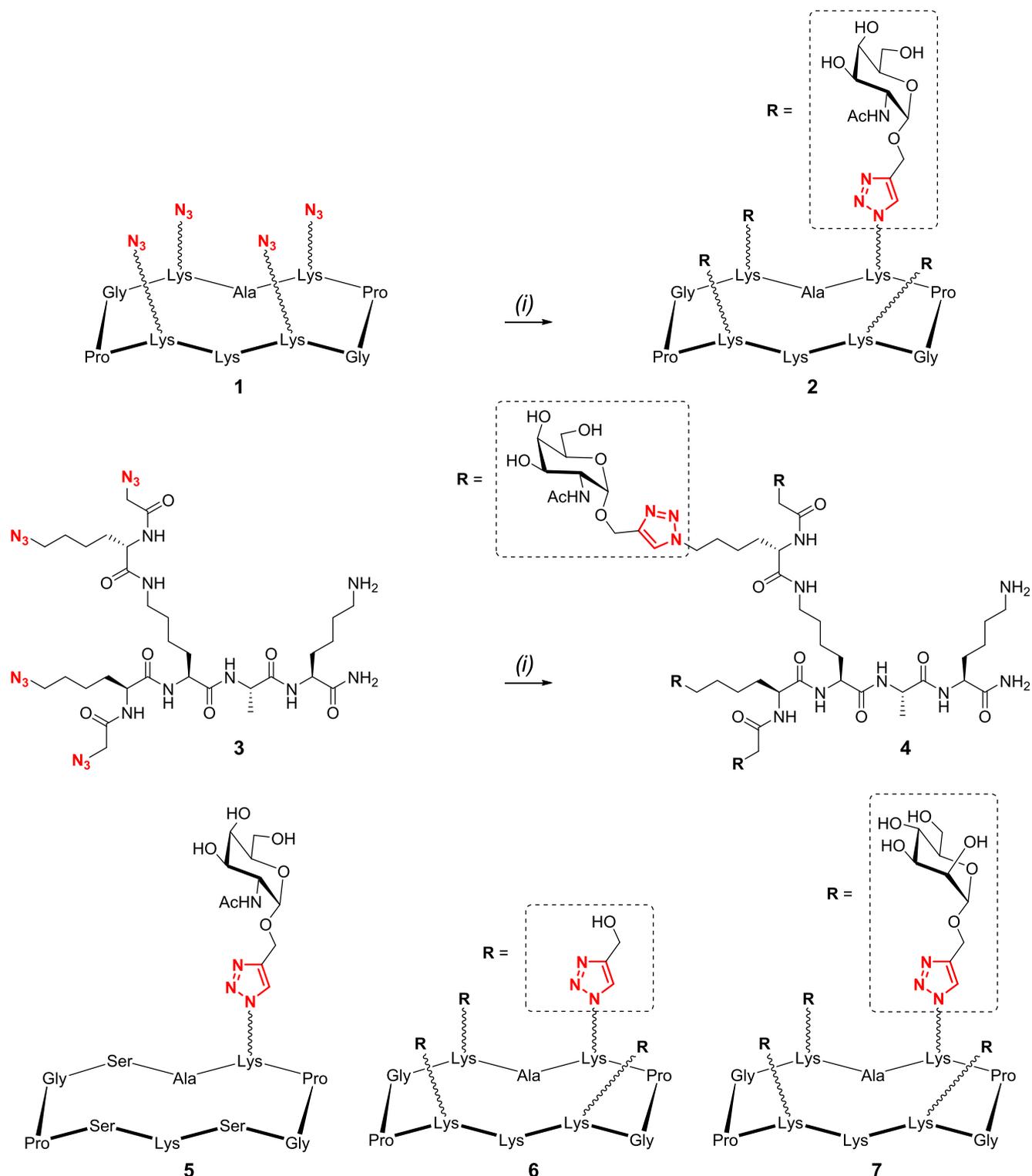
Microarray technology has clearly demonstrated its efficacy to probe interactions between carbohydrates and biological targets^{9–17} (i.e., proteins, pathogens, or cells) because the microarray format requires lower quantities of both ligand and protein than those required for standard experiments such as isothermal titration calorimetry (ITC) or enzyme-linked immunosorbent assay-type assays,^{18,19} is easy to set up, and is reusable.²⁰ If covalent immobilization of carbohydrates on surfaces allows multivalent presentation, the resulting two-

dimensional disposition only partially reflects their natural display, which strongly limits the access of reliable information to design potent multivalent ligands. In addition, even though surface density can be tuned easily, intermolecular chelation of lectins with monovalent ligands may occur, thus leading to an uninterpretable surface cluster effect. More recently, glyco-cluster-based microarrays have been developed to both overcome these limitations and improve sensitivity of detection. The controlled presentation of sugars in a well-defined three-dimensional arrangement and at low surface density indeed gives access to the direct analysis and binding properties of the immobilized compound. For example, Pieters et al. have immobilized 1- to 8-valent structures covalently onto porous aluminum oxide chips to monitor multivalency effects in real time with fluorescent lectins.²¹ In another study, the same group has drawn binding profiles for a series of lectins, which has highlighted both specific recognition and distinct binding patterns.²² The groups of Morvan and Chevolut have used the noncovalent DNA-directed immobilization method for immobilizing glycocluster–DNA conjugates on DNA microarrays by double-helix formation.^{23,24} Fluorescence endpoint detection was used to screen these

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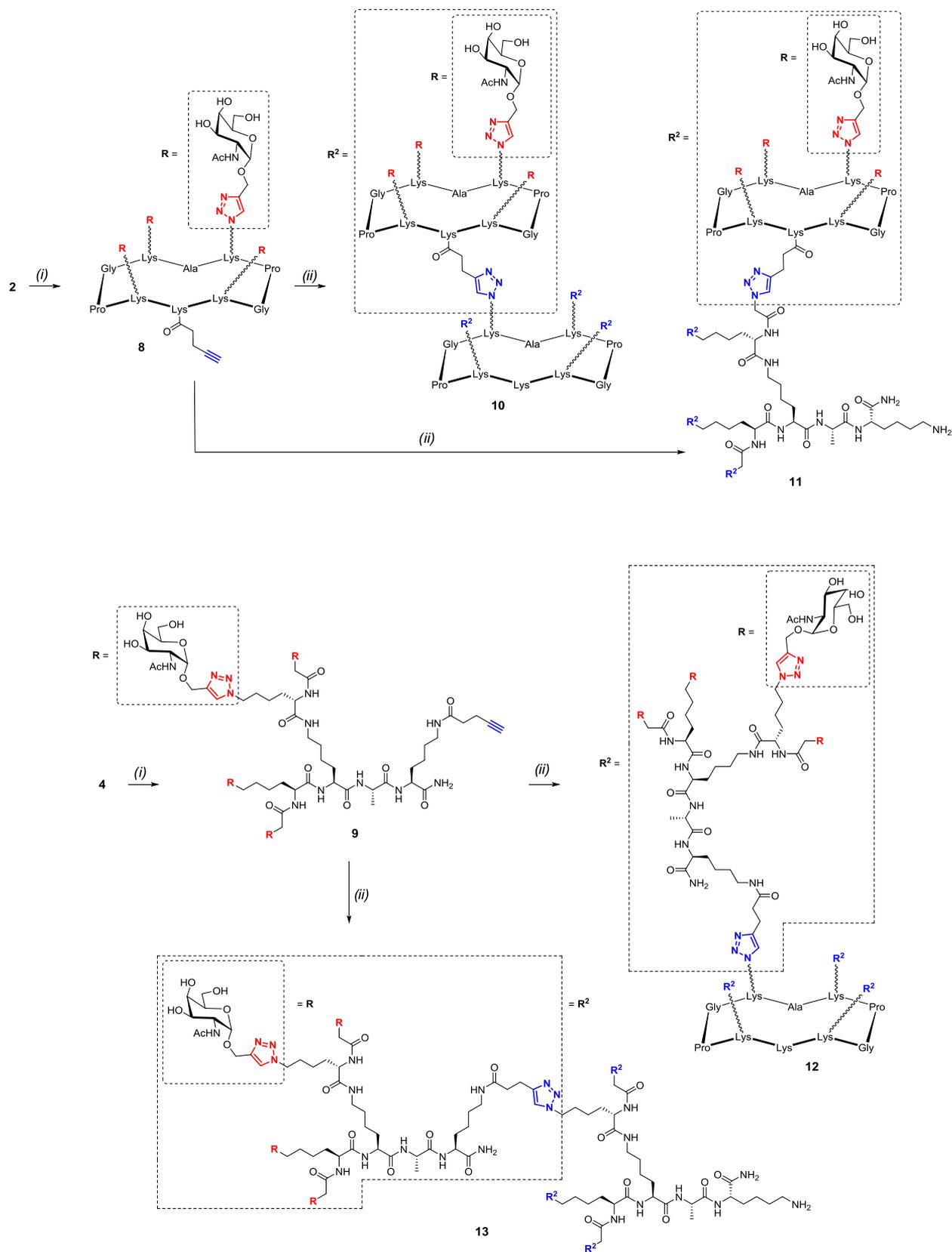
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Scheme 1. Synthesis of Tetravalent Glycoclusters^a

^aReagents and conditions: (i) propargyl 2-deoxy-2-acetamido- α -D-galactopyranoside, CuSO₄·5H₂O, tris(3-hydroxypropyltriazolylmethyl)amine (THPTA), sodium ascorbate, dimethylformamide (DMF)/phosphate-buffered saline (PBS) (1:3, pH 7.5), room temperature (rt), 1 h. All amino acids have the L-configuration.

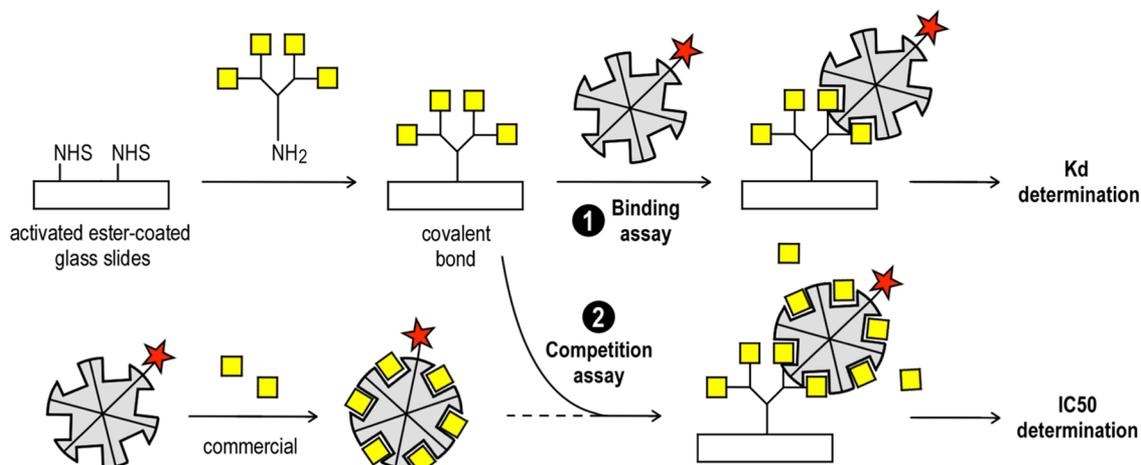
ligands toward lectins. After studying the impact of the cluster density, they have been able to identify nanomolar inhibitors for the lectin LecA from *Pseudomonas aeruginosa*.²⁵ All of these studies clearly demonstrated that the spatial arrangement of multivalent structures on surfaces favors stronger interactions

with lectins than with monovalent ligands. However, these experiments allowed discrimination of ligands with significant differences in valency, thus leading to predictable data in the majority of cases. On the contrary, systematic evaluation of ligands with close structural features in terms of rigidity/

Scheme 2. Synthesis of Hexadecaivalent Glycodendrimers^a

^aReagents and conditions: (i) 2,5-dioxopyrrolidin-1-yl pent-4-ynoate, diisopropylethylamine, DMF, rt, 30 min. (ii) 1 or 2, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, THPTA, sodium ascorbate, DMF/PBS (1:3, pH 7.5), rt, 1 h.

Scheme 3. Glycoconjugate Immobilization and Binding and Competition Assays



flexibility, size, and valency that are key parameters to identify potent inhibitors has only rarely been described so far.²⁶ In this study, we report the immobilization and screening of a series of *N*-acetyl- α -D-galactosamine-functionalized (α GalNAc) tetra- and hexadecaivalent glycodendrimers on microarray slides. Using this screening platform, different binding assays were performed, to quantify their affinity toward the α GalNAc-specific *Helix pomatia* agglutinin (HPA) lectin. HPA is a hexameric lectin produced by a roman snail. This lectin displays two trimers constituted of monomers linked by disulfide bridges. This β -sandwich fold leads to two domains distant of 100 Å, each displaying three carbohydrate recognition domains located between two adjacent monomer strands, with 20 Å distance between two neighboring binding sites.²⁷

RESULTS AND DISCUSSION

In a preliminary report, our group has shown that covalent immobilization of glycoclusters on glass slides using both direct and indirect oxime ligation strategies affords surfaces displaying well-defined structures capable of interacting selectively with fluorescent lectins.²⁸ In particular, we have reported that a tetravalent α GalNAc-cluster showed promising but moderate interaction with HPA. To improve its recognition potency, several elements have to be tuned in the structure. We first decided to increase the valency to 16 copies onto scaffolds with variable flexibilities and shapes. Alternate combination of cyclopeptides and/or polylysine dendrons was indeed proved to be successful to identify nanomolar inhibitors of the bacterial lectin LecB from *P. aeruginosa*.²⁹ Similar structures have thus been synthesized following a strategy based on copper-catalyzed azide–alkyne cycloaddition (CuAAC).^{30,31}

Synthesis of Multivalent Glycoconjugates. First, tetravalent glycoclusters **2** and **4** were prepared by CuAAC conjugation of propargyl 2-deoxy-2-acetamido- α -D-galactopyranoside³² on azido-functionalized cyclodecapeptide **1** and polylysine dendron **3** (Scheme 1).³⁰ Monofunctionalized compound **5** was also prepared, as a monomeric reference, and clusters **6** and **7** displaying hydroxymethyl and α -mannoside residues, respectively, were chosen to serve as negative controls in the binding studies.

Next, hexadecaivalent compounds were built in a convergent manner. Clusters **2** and **4** were further functionalized on their

remaining free lysine residue with an *N*-hydroxysuccinimide (NHS)-activated pentynoic acid linker³³ to afford intermediates **8** and **9**. A second CuAAC ligation with scaffolds **1** and **2** yielded glycodendrimers **10–13** displaying 16 copies of α GalNAc (Scheme 2).

Glycoarray Fabrication. To ensure a reproducible and controlled display of the multivalent glycoconjugates, covalent immobilization was chosen, exploiting the free amino group on their lysine side chain and NHS-activated glass slides to obtain an amide linkage. Compounds were spotted in triplicate using a piezoelectric microspotter at various concentrations in buffered solutions (PBS 1 \times with 5% glycerol). The volume of each drop was monitored by spotter software to obtain final spots with a controlled diameter of 200 μ m. The experiments have been performed while monitoring the humidity to prevent evaporation. With this platform in hand, qualitative binding assays as well as K_d and IC_{50} determination could be performed (Scheme 3).

Binding Assays. To facilitate the binding assays, the functionalized glass slide was inserted into a rigid mask with silicon separators, creating 16 isolated wells and allowing us to evaluate 14 different experimental conditions in parallel. Lectin solutions (PBS 1 \times pH 7.5 with 0.1% BSA) ranging from 7.5 μ M to 0.15 nM were poured in the mask wells and incubated for 1 h. After washing with PBS and drying with argon, the slide was scanned and the fluorescence intensities were quantified using a fluorescent scanner (Figure 1).

First, the absence of signal for tetravalent compounds **6** and **7**, functionalized with hydroxymethyl and α -mannoside, respectively, confirms that nonspecific interactions either with the surface or with a sugar other than α GalNAc are not observed. This assay also showed a significant difference between glycoclusters **2** and **4**, suggesting that the interaction with the latter, based on the lysine dendron, is much stronger. The data obtained for hexadecaivalent glycodendrimers also show a more favorable binding when this lysine dendron is used whether as the central (higher intensities for **11** and **13** compared to those for **10** and **12**, respectively) or peripheral (higher intensities for **12** and **13** compared to those for **10** and **11**, respectively) scaffold. Finally, on correcting the intensity values with regard to the compounds' valencies (Figure 1C), compound **13** shows the highest relative potency per sugar residue over other hexadecaivalent structures. It should also be mentioned that when glycoclusters are diluted with com-

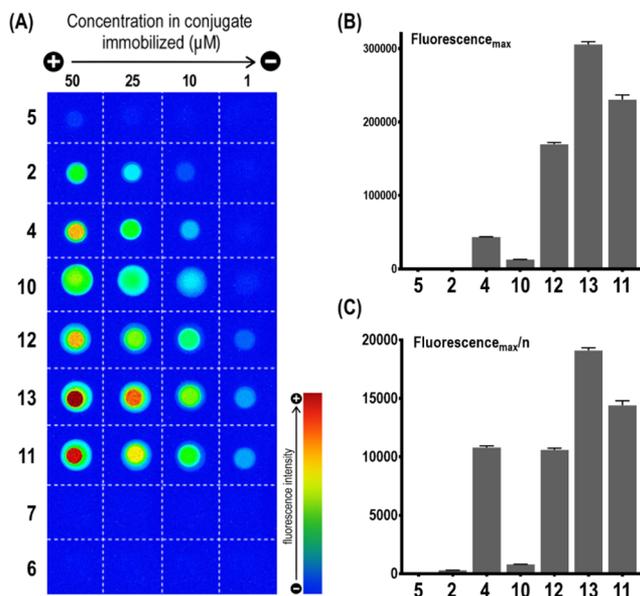


Figure 1. Fluorescence intensity evaluation for the interaction of multivalent glycoconjugates with HPA: (A) rainbow picture displaying fluorescence intensity (HPA: 1.9 nM, λ_{ex} = 635 nm); (B) comparison of the maximum of fluorescence (HPA: 7.5 nM; immobilization concentration of ligand: 40 μ M); and (C) comparison of the maximum of fluorescence normalized per monosaccharide unit (HPA: 7.5 nM; immobilization concentration of ligand: 40 μ M).

pounds devoid of GalNAc instead of free buffer, no difference of interaction is observed. This result is in good agreement with previous observations and suggests that glycoclusters are immobilized in a homogenous manner on the surface.³⁴

Measurement of K_d Values. The strength of the interaction and the influence of relative surface densities of glycoconjugates were assessed in the measurement of dissociation constants (K_d) values.^{24,35,36} To this end, compounds were spotted at eight different concentrations, ranging from 0.3 nM to 40 μ M. Binding isotherms were obtained for each set of concentrations by increasing the concentration of lectin, and K_d values were obtained by linear regression (Figure 2).³⁷ To have a progressive isotherm reaching a maximum plateau region, lectin has to be introduced in excess relative to the glycocluster. In the case of the monovalent compound 5 and the tetravalent compounds 2 and 4, this plateau could not be reached

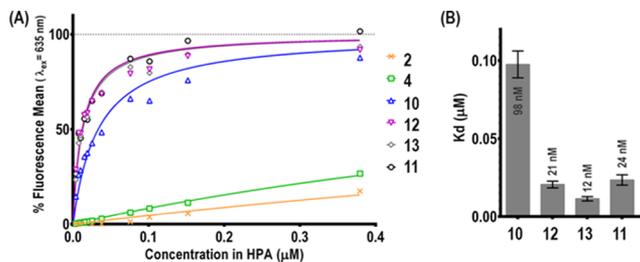


Figure 2. Determination of dissociation constants: (A) binding curves for the tetra- and hexadecavalent glycoconjugates (immobilization concentration: 10 μ M); (B) comparison of the dissociation constants for the hexadecavalent structures (mean of the K_d determined for a range of immobilization concentration of 40–0.3 μ M; see the Supporting Information).

therefore K_d 's could only be precisely calculated for hexadecavalent molecules 10–13.

The dissociation constant is a thermodynamic parameter that should not be dependent on the experimental conditions (as opposed to IC_{50}). However, some authors have reported that above a given surface density a receptor can interact with epitopes from two neighboring glycoconjugates, leading to a surface cluster effect and a decrease of K_d .^{38,39} In our case, this phenomenon was observed at spotting concentrations above 10 μ M (see the Supporting Information). Therefore, only K_d 's calculated at lower concentrations were considered.

The values obtained confirmed what was observed in the qualitative binding assays. Compound 13 composed of two layers of lysine dendron showed the best affinity with a K_d of 12 nM. Compounds 11 and 12 alternating the two scaffolds gave very similar results with K_d values of 24 and 21 nM, respectively. However, compound 10 seemed to be a weaker ligand with a K_d of 98 nM. Although these four compounds have the same valency, a significant difference of affinity of almost 1 order of magnitude could be evidenced with our assay.

Measurement of IC_{50} Values. To determine IC_{50} values, assays were performed using GalNAc as a competitor. Glycoconjugates were immobilized at 10 μ M to avoid the surface cluster effect that could affect the outcome of the experiment. Solutions of HPA (7.5 nM) and GalNAc at various concentrations, from 50 mM to 1 μ M, were preincubated for 1 h. These solutions were then incubated for 1 h in the slide's wells functionalized with glycodendrimers. The resulting IC_{50} values (Figure 3) are the concentrations in

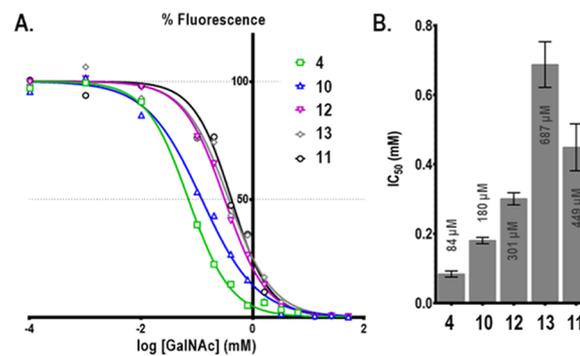


Figure 3. IC_{50} determination by competition with GalNAc incubated with HPA at 7.5 nM (immobilization concentration: 10 μ M): (A) inhibition curves; (B) IC_{50} values determined for the competitor.

the competitor required to inhibit half of the HPA–glycoconjugate interaction. At this immobilization concentration (10 μ M), IC_{50} values could only be obtained for the hexadecavalent compounds and tetravalent molecule 4. When comparing the affinities of hexavalent dendrimers, the same relative potencies were observed as with K_d measurements. Compound 10 with an IC_{50} of 180 μ M showed the weakest binding as opposed to compound 13 (IC_{50} = 687 μ M). Once again molecules 11 and 12 showed no significant difference with intermediate values of 449 and 301 μ M, respectively.

Enzyme-Linked Lectin Assay (ELLA). To confirm the reliability of the screening method, enzyme-linked lectin assays (ELLA) have been performed (Figure 4). In contrast to the IC_{50} values evaluated by the microarray, in the ELLA experiment, the half-inhibition concentration was determined

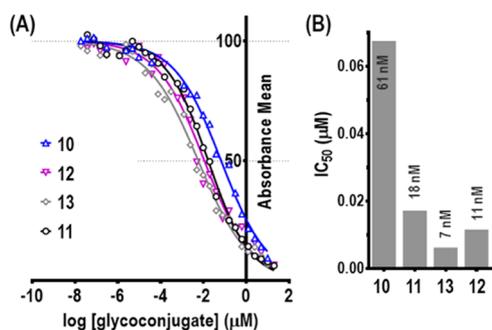


Figure 4. ELLA experiments: (A) inhibition curves; (B) IC₅₀ values determined for the hexadecavalent conjugates.

for the glycodendrimers with an α GalNAc-functionalized polymer used as the immobilized reference ligand. The lowest value was determined for compound **13** with an IC₅₀ of 7 nM and the highest one for **10** with 61 nM that corresponds to a difference of approximately 1 order of magnitude. Compounds **11** and **12** display similar IC₅₀ values with, respectively, 18 and 11 nM. These results are in excellent agreement with the trend previously observed by microarray evaluations.

CONCLUSIONS

In this study, we demonstrate that glycodendrimer arrays allow rapid screening of multivalent glycostructures toward lectin. By grafting multivalent architectures in a controlled, covalent manner on a glass slide, we have evaluated the binding of α GalNAc-ligands with the lectin HPA both qualitatively, with a simple binding assay, and quantitatively, allowing for K_d and IC₅₀ values. All of the collected data have shown consistent results, thus confirming the reliability of this method (Table 1). Compared with other techniques such as ITC, surface plasmon resonance, or ELLA inhibition assays, the microarray format requires very low quantities of ligands, is versatile and easy to handle, and can be used to screen a large library of ligands in parallel. In addition, this approach offers the advantage to allow interaction studies with no risk of aggregation that often occurs with multivalent compounds. Together with the previous “indirect method” that allows us to synthesize glycostructures on surfaces using successive conjugation steps,²⁸ we expect to develop an expedient method to assemble glycodendrimers on surfaces with a larger quantity and diversity of platforms and sugar units. The resulting arrays would represent ideal tools to discover high-affinity ligands for relevant bacterial lectins or antibodies for both therapeutic and diagnostic applications.

EXPERIMENTAL SECTION

General Methods. All chemical reagents were purchased from Aldrich (Saint Quentin Fallavier, France) or Acros (Noisy-Le-Grand, France) and were used without further purification. All protected amino acids and Fmoc-Gly-Sasrin resin were obtained from Advanced ChemTech Europe (Brussels, Belgium), BachemBiochimie SARL (Voisins-Les-Bretonneux, France) and France Biochem S.A. (Meudon, France). For peptides and glycopeptides, analytical reverse phase high-performance liquid chromatography (RP-HPLC) was performed on a Waters alliance 2695 separation module, equipped with a Waters 2489 UV/visible detector. Analyses were carried out at 1.23 mL/min (Interchim UPTISPHERE X-SERIE, C₁₈, 5 μ m, 125 \times 3.0 mm²) with UV monitoring at 214 and 250 nm using a linear A–B gradient (buffer A: 0.09% CF₃CO₂H in water; buffer B: 0.09% CF₃CO₂H in 90% acetonitrile). Preparative HPLC was performed on Waters equipment consisting of a Waters 600 controller and a Waters 2487 Dual Absorbance Detector. Purifications were carried out at 22.0 mL/min (VP 250 \times 21 mm² nucleosil 100-7 C₁₈) with UV monitoring at 214 and 250 nm using a linear A–B gradient. Progress of reactions was monitored by thin layer chromatography using silica gel 60 F254-precoated plates (Merck). Spots were visualized by charring with 10% H₂SO₄ in EtOH. Silica gel 60 (0.063–0.2 mm or 70–230 mesh, Merck) was used for column chromatography. ¹H and ¹³C NMR spectra were recorded on a BrukerAvance III 500 MHz spectrometer, and chemical shifts (δ) were reported in parts per million (ppm). Spectra were referenced to the residual proton solvent peaks relative to the signal of D₂O (4.79 ppm for ¹H). ESI mass spectra of peptides and glycopeptides were measured on an Esquire 3000 spectrometer from Bruker or on an Acquity UPLC/MS system from Waters equipped with a SQ2 detector. high-resolution mass spectrometry analyses were performed on a Waters Xevo G2-S QTOF at mass spectrometry facility, PCN-ICMG, Grenoble.

General Procedures. *General Procedure A for Copper-Catalyzed Azide Alkyne Cycloaddition (CuAAC).* A solution of CuSO₄·5H₂O (0.1 equiv per alkyne), THPTA (0.2 equiv per alkyne), and sodium ascorbate (1 equiv per alkyne) in PBS buffer (400 μ L, pH 7.5) was added to a solution of azidated scaffold (1 equiv) and propargyl 2-deoxy-2-acetamido- α -D-galactopyranoside (6 equiv) or propargylated glycocluster (4.4 equiv) in 400 μ L of a 1:1 mixture of DMF/PBS buffer (pH 7.5). The mixture was degassed under argon and stirred at room temperature for 1 h, after which ultra performance liquid chromatography (UPLC) analysis showed complete coupling. Chelex resin was added to the reaction mixture that was stirred for an additional 30 min and purified by semipreparative RP-

Table 1. Compilation of the Data Obtained by Microarray and ELLA

conjugate	valency	$K_{d,microarray,isoT}$ (nM)	SEM ($K_{d,microarray,isoT}$) (nM)	IC _{50,microarray} (μ M) ^a	SEM (IC _{50,microarray}) (μ M) ^a	IC _{50,ELLA} (nM) ^b
5	1	na	na	na	na	na
2	4	na	na	na	na	na
4	4	na	na	84	9	na
10	16	98	9	180	9	61
12	16	24	3	301	17	18
13	16	12	1	687	69	7
11	16	21	2	449	68	11

^aDetermined for the competitor and for an immobilization concentration of 10 μ M for the ligand. ^bDetermined for the conjugate.

HPLC (5–40% solvent B in 15 min) to afford the desired compound as a white fluffy solid after lyophilization.

General Procedure B for Coupling of NHS-Activated Pentynoic Acid. To a solution of glycocluster (1 equiv) in dry DMF (200 μL) were added diisopropylethylamine (3 equiv) and *N*-succinimidyl pentynoate (1.5 equiv). The mixture was stirred at room temperature (rt) for 1 h, after which UPLC analysis showed complete conversion. H_2O (3 mL) was then added to the mixture, which was purified by semipreparative RP-HPLC (5–40% solvent B in 15 min) to afford the desired compound as a white fluffy solid after lyophilization.

Microarray Fabrication. Samples were dissolved in ultrapure H_2O to prepare stock solutions (1 mM) and then diluted in PBS 1 \times (140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4 , 2 mM KH_2PO_4 , pH 8.5) containing 5% glycerol. Glycerol was used to prevent evaporation during spotting steps. The solutions of glycoconjugates (20 μL , 100 μM to 0.03 μM) were transferred in a 384-well plate and then spotted in triplicate using a piezoelectric microspotter (sciFLEXAR-RAYER S3, Scienion AG, Germany) on NHS-coated glass slides (Nexterion, Slide H, Schott). During the printing step, the humidity was monitored by a humidifier in the spotter enclosure (75% humidity) to prevent the evaporation of the spots. Each spot position was fixed with the software of the spotter. A distance of 480–500 μm between the centers of adjacent spots was imposed, and each probe was spotted in triplicate (6–7 drops, 400 pL/drop, spot size 200 μm). After completion of the printing step, glass slides were incubated in a humidity chamber (70% humidity imposed by saturated NaCl) for 17–20 h. The slides were dipped into the blocking solution (PBS 1 \times , 100 mM boric acid, 25 mM ethanolamine, 0.01% Tween 20, pH 8.5) to deactivate unreacted NHS-functions and agitated for 1 h at 37 $^\circ\text{C}$. Slides were washed in PBS 1 \times containing 0.1% Tween 20 (3 \times , 3 min) and then were rinsed in PBS 1 \times (3 \times , 3 min) and in ultrapure H_2O to remove salt. Slides were dried with argon and directly used for interaction or competition assays. Slides can also be conserved in H_2O in a fridge for 1 month without observing any alteration.

Binding Studies. Interaction Assay Procedure. Printed slides were disposed into 16-well masks. Fields containing immobilized glycoconjugates were probed with Alexa Fluor 647-labeled *H. pomatia* agglutinin lectin (HPA) (80 μL , 2 $\mu\text{g}/\text{mL}$ to 2 ng/mL) in PBS 1 \times containing 0.1% BSA for 1 h at 37 $^\circ\text{C}$. Bovine serum albumin was added to prevent nonspecific interaction of lectin with the slide and minimize the background signal. Slides were washed in PBS 1 \times containing 0.1% Tween 20 (3 \times , 10 min) under gentle agitation to remove unbound lectins and then were rinsed in PBS 1 \times (3 \times , 3 min) and in ultrapure H_2O to remove salt. Slides were finally dried under argon and scanned (Labomix Innoscan 710, red laser, excitation wavelength 635 nm).

K_d Evaluation. Interaction assays were performed using the same protocol. Immobilized glycoconjugates were probed with 25 $\mu\text{g}/\text{mL}$ to 50 ng/mL Alexa Fluor 647-labeled *H. pomatia* agglutinin lectin (HPA) to ensure an excess of lectin.

Competition Assay Protocol and IC_{50} Evaluation. In protein low-bind eppendorf tubes, the concentration range of commercial GalNAc (50 mM to 1 μM) was prepared in a solution containing Alexa Fluor 647-labeled *H. pomatia* agglutinin lectin (HPA) (1 ng/mL) in PBS 1 \times containing 0.1% BSA. Samples were incubated for 1 h at 37 $^\circ\text{C}$ under agitation. Printed slides were disposed into 16-well masks. Fields containing immobilized glycoconjugates were probed

with the previously incubated solutions containing GalNAc/HPA lectin. Slides were incubated for 1 h at 37 $^\circ\text{C}$ under slow agitation. Slides were washed in PBS 1 \times containing 0.1% Tween 20 (3 \times , 10 min) under gentle agitation to remove unbound lectins and the competitor and then were rinsed in PBS 1 \times (3 \times , 3 min) and in ultrapure H_2O to remove salt. Slides were finally dried under argon and scanned.

Data Analyses. Scans were analyzed with Mapix. To analyze the entire range of spots including inhomogeneous ones, a fixed diameter was imposed (200 μm), equivalent to the minimal diameter observed on the slide. Data were treated with Excel and GraphPad Prism 6. To evaluate the interaction and approximatively determine K_d values, a hyperbole model was used to fit the data, with Y being the mean fluorescence and X being the concentration in lectin. To obtain an accurate value of K_d , a linear regression $Y = AX + B$ was plotted (see equation below) and K_d was determined. To evaluate IC_{50} values, a sigmoidal model (4PL model), which is a variable slope model, was used to fit the data, with Y being the mean fluorescence and X being the log of the concentration in competitor introduced.

$$\frac{[\text{lectin}]}{\text{fluorescence}} = [\text{lectin}] \frac{1}{\text{fluorescence}_{\text{max}}} + \frac{K_d}{\text{fluorescence}_{\text{max}}}$$

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b01526.

General synthetic procedures; compound characterization (RP-HPLC, ESI-TOF, and ^1H NMR), experimental procedure for determination of dissociation constants (K_d) by the microarray, IC_{50} evaluation by the microarray and enzyme-linked lectin assays (PDF)

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Notes

The authors declare no competing financial interest.

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