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Report

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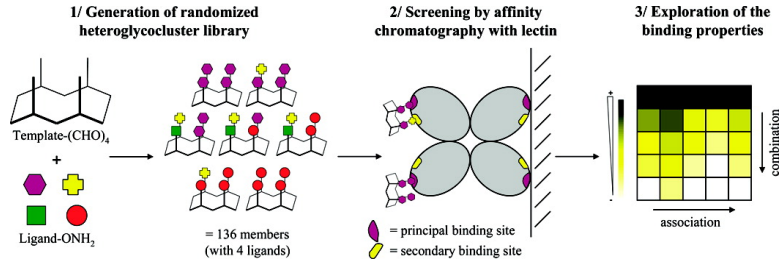

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Randomized Combinatorial Library of Heteroglycoclusters (hGC)

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Carbohydrate−protein interactions are closely involved in complex biological events including fertilization, cell−cell communication, host−pathogen interactions, and cancer metastasis.[1] With the recent emergence of glycomics, considerable progress has been made on understanding the structure−function relationships that regulate these processes and on deciphering the glycocode.[2] In particular, the increasing number of crystallographic data and structural studies has tremendously clarified the carbohydrate−protein recognition mechanisms.[3] Indeed, it is henceforth well established that unlike monovalent ligands, a cluster presentation of carbohydrate allows multiple and simultaneous contacts of identical sugar moieties with specific protein-binding sites, increasing the avidity of ligands by virtue of the "glycoside cluster effect".[4] More interestingly, a few reports have highlighted that some proteins contain secondary binding sites close to the sugar-binding pocket which may influence the recognition mechanisms.[5] While their structural basis remains to be explored, the recent interest in these surrounding regions suggests that secondary interactions should be considered to address not only the affinity and specificity of multivalent ligands but also the crucial question of selectivity.

Typically, synthetic multivalent conjugates are designed to exhibit multiple copies of identical sugar (namely, homoglycoclusters) to the surface of a scaffold with variable topology, valency, or density (e.g., calixarenes,[6a] cyclodextrins,[6b] dendrimers,[6c] cyclopeptide,[6d] etc.). While the biological potential of homoglycoclusters is undeniable,[7] such structures do not reflect the promiscuous display of the cell surface glycocalix. For this reason, the synthesis of a new generation of glycoclusters containing various ligands (heteroglycoclusters, hGC) appears to be an excellent tool for exploration of the potential effects of a heterogenic cluster display in protein-binding processes. In this paper, we report for the first time a robust procedure to generate mixture-based libraries of hGC and to investigate their binding properties with a lectin.

Presumably because of synthetic difficulties, only a few reports have described the synthesis of hGC so far. For example, the preparation of a cyclodextrin scaffold grafted with combinations of mannose/lactose and mannose/glucose and binding studies with Concanavalin A (ConA) have led to speculation about new biological mechanisms that involve synergetic interactions between different sugars.[8] In addition, trivalent carbohydrate-based ligands have been generated and selected from dynamic combinatorial libraries with the same lectin.[9] In addition to these studies, we propose herein a combinatorial approach that allows rapid generation and screening of the first structurally diverse library of hGC that combine various biomolecules. On the basis of the TASP concept (template assembled synthetic protein),[10] we have selected a topological cyclopeptide scaffold **1** to assemble multiple recognition units in a defined spatial orientation[11] and an oxime-based strategy to secure a quantitative assembly of biomolecules with various functionalities (Figure 1).[12] Furthermore, because carbohydrate−protein interactions occur, in principle, by formation of a hydrogen-bond network and hydrophobic contacts,[3] we reasoned that varying the nature of aminooxy-modified carbohydrates (αMan **2**, αGalNAc **3**, βLac **4**, and αFuc **5**) or amino acid building blocks (positively charged Lys **6**, negatively charged Asp **7**, and hydrophobic Tyr **8**, Phe **9**, and Ile **10**) at the surface of the scaffold would provide molecular diversity that might give evidence for secondary contacts with protein.

The aminooxy carbohydrates **2**−**5** were prepared from the corresponding fluoride glycosyl donors as reported previously.[13] The incorporation of aminooxy acetic acid linker to the α-amino function of lysine aspartic acid, tyrosine, phenylalanine, and isoleucine residues was realized using the newly described ethoxyethylidene protecting group.[14] The aminooxy-modified amino acids **6**−**10** were obtained in 41−87% yield after final deprotection and purification from the corresponding methyl ester amino acids protected with Boc (for **6**) or tBu (for **7** and **8**).

By contrast with imine or hydrazone linkages, which are suitable to generate combinatorial libraries under thermodynamic control,[15] it was shown that oxime bond is hardly reversible under ambient conditions. As a result, we assumed that the generation of libraries from template **1** and a mixture of several aminooxy building blocks should theoretically ensure the randomized and statistical distribution of each expected library species in a comparable amount.[16] To confirm this hypothesis, a small mixture-based library was prepared first using a 12-fold excess of an equimolar solution of aminooxy mannose **2** and fucose **5** in aqueous acetic acid (library **C**, Table 1). After 1 h, total conversion was observed by RP-HPLC analysis. When the symmetry and the four glyoxo-aldehyde functions displayed by the template are considered, 136 species might be obtained (with 4 ligands), differing by the composition and the distribution of each building block on the template. In this case, we observed a clean reaction mixture showing, in both RP-HPLC and mass spectrometry analyses, five peaks confirming the presence of the five expected compounds containing up to four mannose, fucose, or both moieties (Figure 2).

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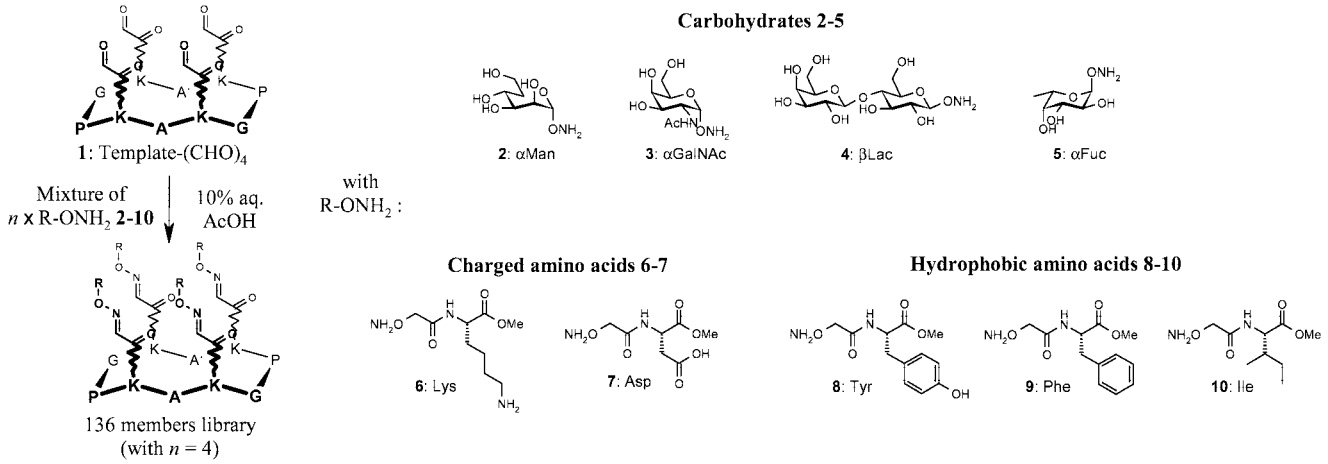

Figure 1. General strategy and structure of carbohydrates (**2**−**5**) and aminoacids (**6**−**10**) used for the generation of heterocluster libraries from cyclodecapeptide template **1** displaying four glyoxoaldehyde anchoring sites.

Table 1. Composition of Heteroglycocluster Libraries[a]

Library	Ligands	Multivalent display on the template (number of regioisomers)	Total[b]
A	●+◆	●●●●(1)+●●●●◆(2)+●●●◆◆(4)+●◆◆◆◆(2)+◆◆◆◆◆(1)	10
B	●+●	●●●●(1)+●●●●●(2)+●●●●●(4)+●●●●●(2)+●●●●●(1)	10
C	●+■	●●●●(1)+●●●■(2)+●●■■(4)+●■■■(2)+■■■■(1)	10
D	◆+●	◆◆◆◆(1)+◆◆◆●(2)+◆◆●●(4)+◆●●●(2)+●●●●(1)	10
E	◆+■	◆◆◆◆(1)+◆◆◆■(2)+◆◆■■(4)+◆■■■(2)+■■■■(1)	10
F	●+■	●●●●(1)+●●●■(2)+●●■■(4)+●■■■(2)+■■■■(1)	10
G	●+◆+●	Libraries A+B+D	45
H	●+◆+■	Libraries A+C+E	45
I	●+●+■	Libraries B+C+F	45
J	◆+●+■	Libraries D+E+F	45
K	●+◆+●+■	Libraries A+B+D+E+F+G+H+I+J	136
L	●+▼	●●●●(1)+●●●▼(2)+●●▼▼(4)+●▼▼▼(2)+▼▼▼▼(1)	10
M	●+▲	●●●●(1)+●●●▲(2)+●●▲▲(4)+●▲▲▲(2)+▲▲▲▲(1)	10
N	●+⬡	●●●●(1)+●●●⬡(2)+●●⬡⬡(4)+●⬡⬡⬡(2)+⬡⬡⬡⬡(1)	10
O	●+◺	●●●●(1)+●●●◺(2)+●●◺◺(4)+●◺◺◺(2)+◺◺◺◺(1)	10
P	●+⬟	●●●●(1)+●●●⬟(2)+●●⬟⬟(4)+●⬟⬟⬟(2)+⬟⬟⬟⬟(1)	10

[a] ● = αMan **2**; ◆ = αGalNAc **3**; ● = βLac **4**; ■ = αFuc **5**; ▼ = Lys **6**; ▲ = Asp **7**; ⬡ = Tyr **8**; ◺ = Phe **9**; ⬟ = Ile **10**.

[b] The total number of species in each library comprise all the possible positional isomers (regioisomers).

The excess of unreacted aminooxy building blocks was finally removed from the crude mixture by incubation with a Toyopearl resin functionalized with aldehydes; the solution was then filtered and lyophilized. Unlike preparative HPLC, this method offers the advantage of entire recovery of each library member. Various hGC libraries combining either up to four carbohydrates or carbohydrate and amino acid (Table 1, library **A**−**K**) have been generated using the same strategy. In each case, we observed, by mass spectrometry, the presence of signals corresponding to the molecular weight, salt adducts, or fragments of the predicted library members (see Supporting Information). Notably, the principal 136-member library **K** comprises nine sublibraries of two or three sugars (libraries **A**−**J**) in addition to the 12 regioisomers displaying four sugar moieties.

As a proof of concept for this randomized, mixture-based combinatorial library, we next envisioned the screening with a model lectin. For this purpose, we investigated the binding of seven mixtures of five mannose-based hGC (**B**, **C**, **L**, **M**, **N**, **O**, **P**) with concanavalinA (ConA) bound to an agarose gel. In this assay, each library was eluted over the affinity

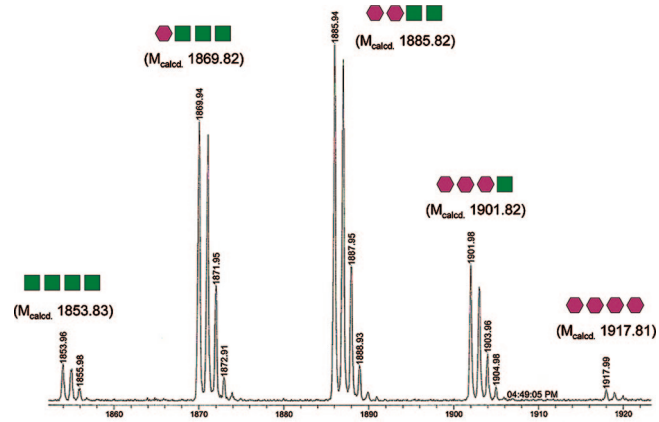

Figure 2. Mass spectrum (MALDI-TOF) of the library **C**.

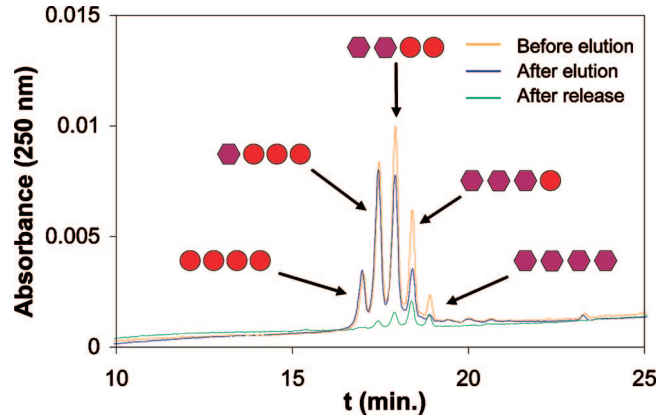

Figure 3. Study of the binding of library **B** with ConA (mannose/lactose) by HPLC analysis.

column. Because each species can be easily assigned by HPLC analysis, we identified the best ligands by comparison of the chromatograms before and after elution (see Supporting Information and Figure 3). The binding potency of each library member was thus calculated, after correction using the tetramannosyl ligand as internal reference, by measurement of the corresponding HPLC peak area. As shown with library **B**, in which mannose and lactose ligands were mixed, we first observed that the binding is improved, as expected, when the number of mannose residue increases. However, we discovered a significant difference of interaction depending on the combination and the nature of the building block

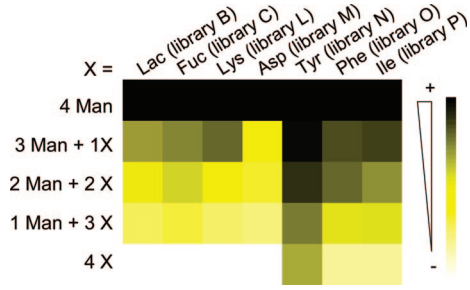

Figure 4. Presentation of the mapping of ConA binding properties.

associated with the mannose moiety. This result was illustrated using a color gradient depicting the binding properties of each hGC to ConA (Figure 4). This representation clearly shows that the association of ConA-specific mannose residue, either with unspecific carbohydrates (e.g., lactose and fucose) or positively and negatively charged amino acid does not affect the binding. On the other hand, the display of mannose with hydrophobic residue, especially tyrosine, seems to improve the interaction with ConA, even though the mannose residue is not present. In addition, the species bound to ConA were released using a concentrated solution of methyl mannopyranoside (0.5 M), except for libraries **N**−**P**. This suggests that hydrophobic residues interact, not in the mannose-specific pocket but in an independent binding site (Figure 3). Similar observations have been reported with peptides containing a tyrosine-rich consensus sequence.[17]

To validate the results of the screening and investigate whether tyrosine participates in binding, the five compounds of libraries **B** and **N** were isolated by semipreparative HPLC for SPR assays (see Supporting Information). To compare the affinity of each isolated hGC to Con A, the lectin was immobilized on the sensor chip. The dissociation constant (K_D) between the hGC and the lectin was determined by steady-state analysis. As suggested earlier, we first observed for both libraries that the affinity increases with the number of mannose. Second, by comparison of species displaying an identical amount of mannose, we confirmed that the presence of hydrophobic residue improves the affinity to the lectin because K_D values are systematically lower when mannose is associated with tyrosine (50, 145, and 320 μM for 3 Man/1 Tyr, 2 Man/2 Tyr, 1 Man/3 Tyr) than with lactose (106, 163, and 531 μM for 3 Man/1 Lac, 2 Man/2 Lac, 1 Man/3 Lac). In additon, the exchange of one mannose residue by one tyrosine has no significant effect on the binding ($K_D = 50\mu$M) in comparison with the tetramannosyl cluster ($K_D = 44\ \mu$M), while the replacement with a lactose leads to a 2-fold decrease ($K_D = 106\ \mu$M).

In conclusion, we have developed an expedient strategy for generation and purification of randomized mixture-based hGC library displaying up to four carbohydrates and amino acids. To emphasize the potential applications of this new generation of glycoclusters and demonstrate the proof of this concept, we have explored by affinity chromatography the contact surface of ConA with a mannose-based library. We have thus selected ligands and shown by SPR that tyrosine influences the binding. This approach might find broad interest in glycomic or proteomic research, going from the discovery of new selective ligands or glycomimetics, to the profiling of the glycopatterns of proteins.

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Supporting Information Available. Detailed description of synthetic procedure, affinity chromatography method, SPR experiments, and HPLC and MS analysis of hGC libraries. This material is available free of charge via the Internet at http://pubs.acs.org.

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