Multivalency in heterogeneous glycoenvironments: Hetero-glycoclusters, -glycopolymers and -glycoassemblies†

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Despite efficiently imitating functional ligand presentations in terms of valency and density, most of the reported multivalent carbohydrate prototypes barely reflect the inherent heterogeneity of biological systems, therefore underestimating the potential contribution of synergistic or antagonistic effects to molecular recognition events. To address this question, the design of novel molecular and supramolecular entities displaying different saccharide motifs in a controlled manner is of critical importance. In this review we highlight the current efforts made to synthesize heteromultivalent glycosystems on different platforms (peptides, dendrimers, polymers, oligonucleotides, calixarenes, cyclodextrins, microarrays, vesicles) and to evaluate the influence of heterogeneity in carbohydrate-protein (lectin, antibody) recognition phenomena. Although the number of publications on this topic is limited as compared to the huge volume of reports on homomultivalent sugar displays, the current body of results has already unravelled the existence of new binding mechanisms that operate in heterogeneous environments whose exact biological significance remains to be unveiled.

1. Introduction

Molecular recognition phenomena occurring between carbohydrates and proteins are responsible for the initiation of critical events in many biological processes such as fertilization, cell-cell communication, host-pathogen interactions, immune response or cancer metastasis[1]. However, individual carbohydrates tend to bind weakly to their complementary proteins and stronger, biologically useful binding or enhanced inhibition is often ascribed to the interplay of multiple interactions by multivalent carbohydrates. Multivalency generally leads to greater affinity enhancements than predicted from the sum of the constitutive interactions. This phenomenon, first noted by Lee and co-workers[2] and referred as the "cluster" or "multivalent" glycoside effect[3], has found a wide range of application in biology and medicine.[4-8] Synthetic polyconjugates with well-defined structures have contributed to unravel the mechanisms at work,[9-12] leading eventually to useful tools for biotechnological[13,14] or therapeutic purposes.[15] Typically, these systems incorporate several copies of identical sugar motifs attached to an appropriate scaffold (molecular, dendritic, polymeric)[10,11,16-18] or self-assembled in supramolecular constructs (nanoparticles, vesicles, microarrays).[11,19]

It has been amply demonstrated that ligand multivalency increases protein-binding avidities dramatically. However, these models barely reflect the inherent heterogeneity of biological systems, therefore underestimating the potential contribution of synergistic or antagonistic effects to molecular recognition events. To investigate the significance of glycoheterogeneity in carbohydrate-protein binding, the development of efficient methodologies to build novel heteroglycoclusters (hGC) and heteroglycoassemblies (hGA) displaying different saccharide ligands in a controlled manner is mandatory (Figure 1). Developing tools to tune heterogeneity and mutivalency in artificial conjugates is of further interest to optimize carbohydrate binding to biomedically relevant receptor partners that, likewise, may be presented in combination in a given environment or be intrinsically heterotopic.

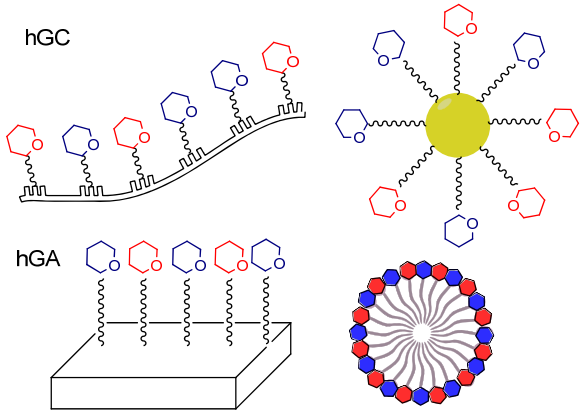

Figure 1. Schematic representation of heteroglycoclusters (hGC) and heteroglycoassemblies (hGA).

In this review we highlight the current efforts made to synthesize heteromultivalent glycoentities and to evaluate their protein-recognition properties. Most of the synthetic approaches put forward to access hGC and hGA are based on

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† Part of the multivalent scaffolds in glycosciences themed issue.

those previously reported for homoglycoclusters and homoglycoassemblies. Nevertheless, heterogeneity implies increased structural complexity and requires the development of more sophisticated strategies for the elaboration of suitable models. For the sake of clarity, the different heteroglycosystems described have been categorized by increasing ligand valency and density, which are the main parameters influencing their recognition behaviour. The aim is to provide a general view of the current state-of-art on these novel architectures and the information they provide on supplementary effects upon evaluation of their recognition abilities as compared to homoglycosylated constructs.

2. Low-density heteroglycoconjugates

The general prototype of multivalent sugar constructs comprises a core molecule serving as an oligovalent scaffold, a variable number of peripheral carbohydrate epitopes and suitable spacers to link the sugar moieties to the central core. When installing more than a glycotope in a given platform, two general arrangements can be considered, namely a mixed-up ("shuffled") distribution of the motifs or their clustering in a multidomain architecture. The choice of one or the other is intuitively expected to have strong consequences in the recognition properties of the ensemble.

(a) "Shuffled" heteroglycoconjugates

The preparation of molecular multiglycoligand architectures was first realized in the context of the synthesis of multiantigenic glycoconjugates for vaccine development. Thus, Danishefsky's group developped a general strategy consisting in the preparation of oligosaccharide tumor antigens from nonnatural glycoamino acids that could be assembled into unimolecular multivalent oligopeptides. The final heteroconjugates were armed for conjugation to a carrier protein.[20] Immunological evaluation of these constructs (e.g. **1**, Figure 2) for multiantigenic carbohydrate-based cancer vaccines demonstrated their potential to stimulate a multifaceted immune response.[21,22]

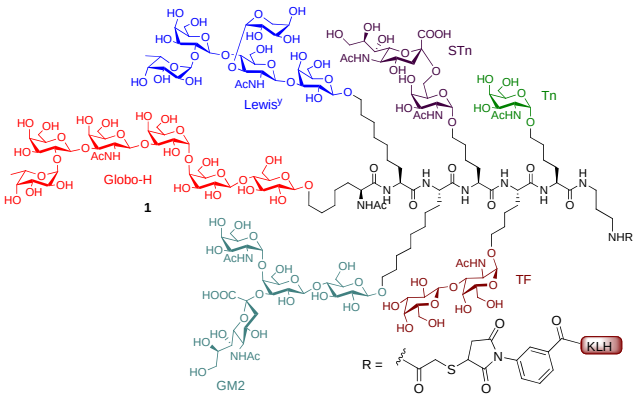


Figure 2. Multiepitope vaccine **1** designed by Danishefsky's group. TF: Thomsen-Friedenreich antigen; Tn: tumor-associated α-*N*-acetylgalatosaminyl epitope; STn: sialyl-α(2-6)Tn.[21,22]

In 2002, Lindhorst and coworkers proposed an approach based on the orthogonal derivatization of D-galactopyranose to attach different sugars (α-D-mannose = αMan, α-L-fucose = αFuc and β-lactose = βLact), thereby accessing novel "mixed"- or "hetero"-glycoclusters (e.g., **2**).[23] The different coating saccharides were sequentially incorporated, after activation of amine or carboxylic acid functional groups, through amide or thiourea ligation chemistries. Amide bond formation was also privileged for the construction of the heterodivalent glycoconjugate **3**, bearing a mannose trisaccharide and a monomeric mannosyl unit in separate branches (Figure 3).[24] Compound **3** was designed to explore whether or not the mannose-specific bacterial lectin found on type 1 fimbriae (FimH), for which the presence of a monovalent carbohydrate recognition domain has been characterized, possesses additional carbohydrate binding sites that might contribute to bacterial adhesion in a multi-epitope scenario. However, anti-adhesion assays showed no significant increase in the inhibition of type 1 fimbriae-mediated bacterial adhesion in comparison to the standard inhibitor methyl α-D-mannopyranoside.

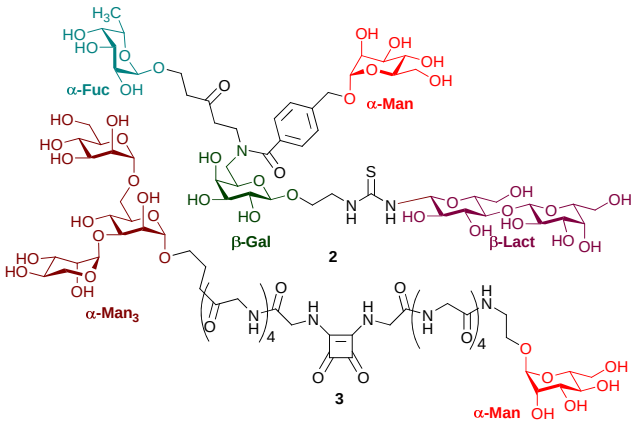
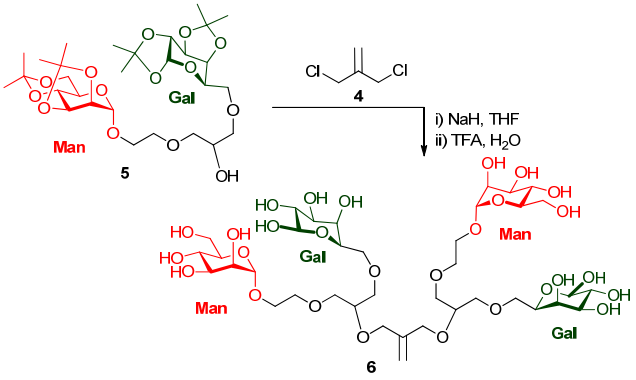


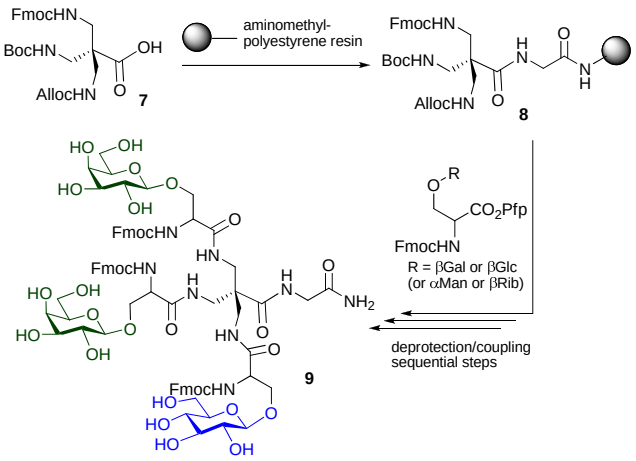
Figure 3. Examples of "mixed type" oligosaccharide mimetics based on carbohydrate (**2**) and peptide (**3**) scaffolds.[23,24]

The same research group has reported the preparation of polyether heteroglycodendrons by exploiting the nucleophilic addition of hydroxyl groups to methallyldichloride (**4**). The reaction proceeds sequentially, thereby allowing the incorporation of two successive sugar motifs, e.g. β-D-galactose (βGal) and αMan. The alkene functionality at the focal point can be then elaborated to produce hydroxyl-armed divalent dendrons (e.g. **5**) that can enter the cycle to produce second generation tetravalent compounds (e.g. **6**; Scheme 1). Unfortunately, the approach is limited to the use of ketal protecting groups and failed to afford higher generation of heteroglycodendrimers in acceptable yields and purity.[25]



Scheme 1. Preparation of heteroglycodendron **6**.[25]

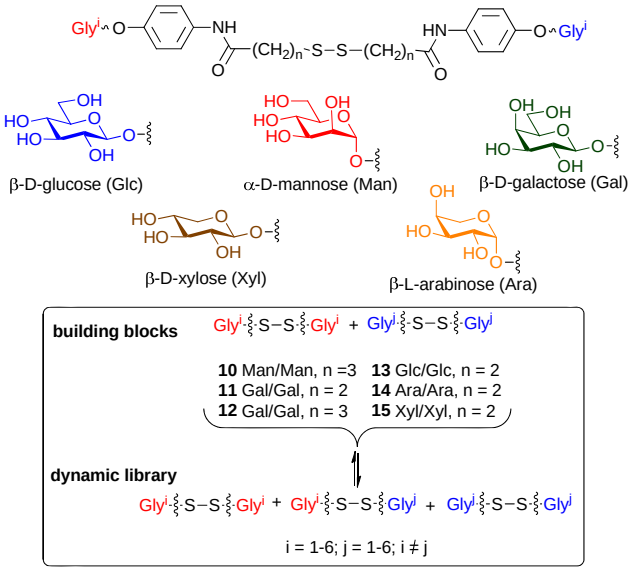
Katajisto, Lönnberg and coworkers combined the benefits of solid-phase synthesis and the efficiency of parallel synthesis for the generation of a short library of triantennary peptide heteroglycoclusters (Scheme 2).[26] The key building block is the α,α-bis(aminomethyl)-β-alanine derivative **7**, bearing conventional *N*'-Fmoc, *N*'-Boc and *N*'-Alloc protecting groups on the three amino functions and a free carboxylic acid group for the attachment to the solid support (→ **8**). The different glycotopes (βGlc, βGal, αMan, and β-D-ribopyranose) were incorporated sequentially by removal of the amino protections of the solid-supported amino acid core and subsequent coupling with the corresponding *O*-glycosylated, *N*-Fmoc-protected, pentafluorophenyl ester-activated serine derivatives (e.g., → **9**).



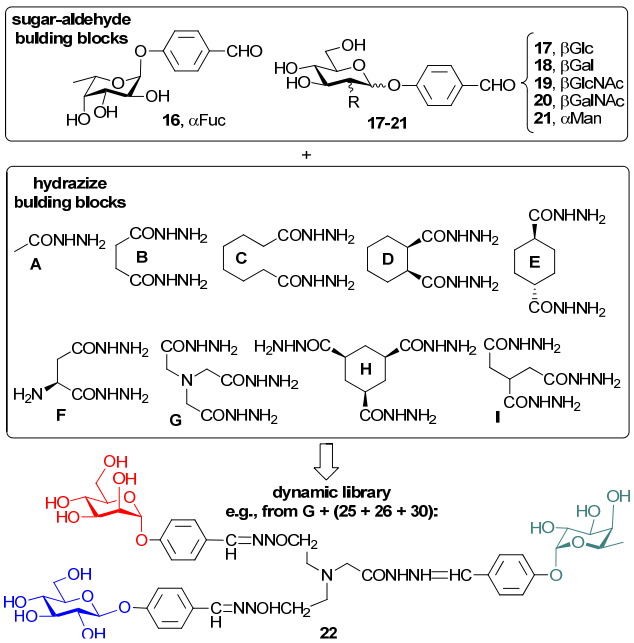
Scheme 2**.** Katajisto's strategy to generate a short library of triantennary peptide heteroglycoclusters (e.g., **9**).[26]

Lehn and coworkers proposed dynamic combinatorial chemistry (DCC) as a suitable strategy to investigate the affinity of lectins when faced to a multi-epitope pool.[27] The concept is based on the creation of reversible connections between suitable building blocks, leading to spontaneous assembly of all their possible combinations and allowing for the simple one-step generation of extended libraries. A dynamic library of bis-carbohydrate ligands **10-15** based on covalent disulfide bond formation between thiol-derivatized carbohydrates, including αMan, βGal, βGlc, β-L-arabinopyranosyl (βAra) and β-D-xylopyranosyl (βXyl) was thus generated (Scheme 3).[27] Screening of the library by adding concanavalin A (Con A), an αMan specific lectin, to the equilibrating mixture of library components did not evidenced significant differences in the binding affinity within the heterodimer series (Scheme 3).

A second family of dynamic carbohydrate libraries was generated from a pool of carbohydrate aldehydes (**16-21**) and di- or tritopic hydrazide components (**A-H**) through reversible acylhydrazone exchange (Scheme 4).[28] The library members can thus incorporate up to three different glycotopes (e.g. **22**) Deconvolution analysis of the di- and trivalent glycolibraries, using enzyme-linked lectin assay (ELLA), allowed the efficient identification of the best binder to Con A lectin, namely the trimannoside cluster having core **G**. Removal of the αMan building block fully abolished binding to the lectin, clearly indicating that this glycotope is required for molecular recognition. Much smaller effects were observed when other structural components were removed, preventing any conclusion about possible cooperative phenomena.

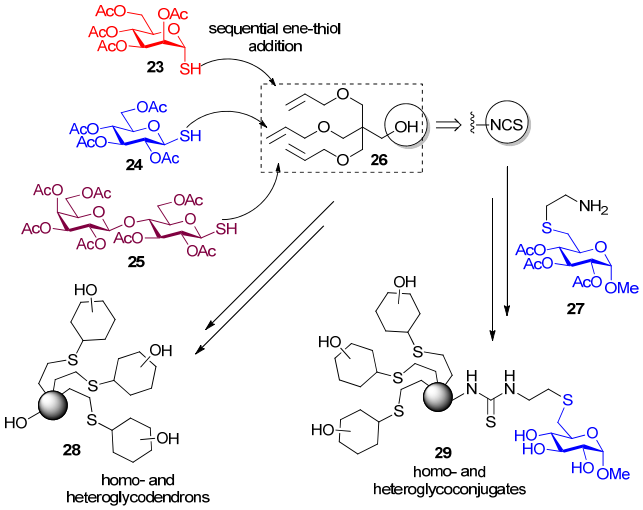


Scheme 3**.** Dynamic library generation using disulfide interchange.[27]



Scheme 4. Dynamic library generation using reversible acylhydrazone formation. Hydrazide compounds **A-I** are combined with six carbohydrate benzaldehydes, simultaneously.[28]

Jiménez Blanco, Benito and coworkers reported the synthesis of trivalent glycodendrons incorporating αMan/βGlc or αMan/βLac substituents (**28**).[29,30] The synthetic scheme exploited the radical addition of the corresponding per-*O*-acetylated 1-thiosugars (**23-25**) to a tri-*O*-allylated pentaerythritol derivative **26**. Reaction conditions were optimized to favour either single- double- or triple addition, which allows the incorporation of the different glycotopes in a sequential manner. In a second series of compounds, the resulting glycodendrons were armed with an isothiocyanate group and conjugated with the amine functionalized methyl α-D-mannopyranoside derivative **27** through the thiourea-forming reaction (→ **29**; Scheme 5). Evaluation of the binding abilities towards the mannose-specific lectin Con A and the β-galactose/lactose-specific lectin peanut agglutinin (PNA) by ELLA indicated that the presence of the second sugar was irrelevant regarding binding of the primary ligand to its complementary lectin receptor.

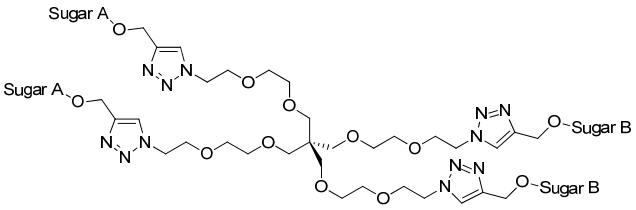


Scheme 5. Preparation of trivalent homo- and heteroglycoconjugates using thiol-ene and thiourea-forming reactions.[29,30]

The potential of the copper(I)-catalysed alkyne-azide cycloaddition (CuAAC), the archetypal "click"-type reaction,[31,32] to create heteromultivalent glycodisplays has been exploited by Santoyo-González and coworkers[33] to develop a modular synthesis of neoglycoconjugates incorporating two different monosaccarides among D-mannose, D-glucose and D-glucosamine onto a variety of scaffolds (methylene, ethylidene, erythritol, methyl-α-D-glucopyranoside, methyl-α-D-galactopyranoside and trehalose). Structural parameters such as the total and relative valencies, the anomeric configuration (α or β) of the coating sugars, the grafting pattern or the length of the spacers linking the peripheral glycotopes to the central core were systematically varied. The binding properties of all the library members towards Con A were evaluated by ELLA. By comparing the data for derivatives sharing the same scaffold (pentaerythritol; total valency 4) the authors observed a cooperative effect only in the case of heteroconjugates bearing αMan and αGlc. Thus, the relative potency per αMan unit was 1.5-fold higher for the $(\alpha Man)_2(\alpha Glc)_2$ derivative as compared to the $(\alpha Man)_4$ homoconjugate, even though αMan is a much better ligand for Con A than αGlc (Figure 4).

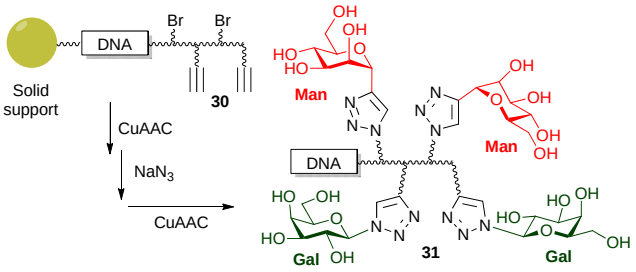
Morvan and coworkers[34] have implemented the CuAAC ligation strategy to access glycooligonucleotide conjugates exhibiting two αMan and two βGal residues (**31**, Scheme 6) intended to be incorporated in novel heteroglycoarrays for lectin affinity investigation upon DNA-directed immobilization. The methodology involves the use of two functionalized phosphoramidite derivatives, one bearing a bromoalkyl group as precursor of azide and another one that bears a clickable propargyl group. Both were incorporated into an oligonucleotide by phosphoramidite chemistry on a DNA synthesizer (**30**). After a first CuAAC cycle with a monosaccharide-azide derivative, the bromo groups are substituted by azide anion and a second CuAAC reaction with a different propargylated sugar was performed. Alternatively, 5'-bis-conjugation of oligonucleotides (→ **36**) was performed by combining amidative oxidation and CuAAC chemistries. In

that case, a propargylated phosphoramidite is incorporated in the oligonucleotide on the solid support (**32**) and then subjected to reaction with 3-bromopropylalanine (→ **33**). Sequential CuAAC (→ **34**), nucleophilic displacement of bromo by azide (→ **35**) and a second CuAAC allows installing αMan and βGlc motifs in the same phosphorous functionality (Scheme 7).[35]

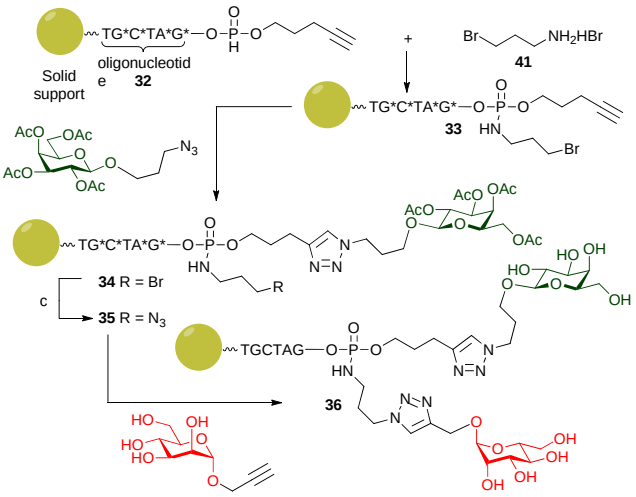


Sugar A	Sugar B	IC_{50} (mM)	Relative affinity	Rel.potency per aMan	Rel. potency per sugar
αMan	αGlc	0.21	4.28	2.14	1.07
αGlc	αGlc	0.48	1.87	---	0.47
αGlc	αGlc	0.16	5.62	1.40	1.40

Figure 4. Tetravalent "click" heteroglycoconjugates and their relative binding affinities towards Con A lectin.[33]

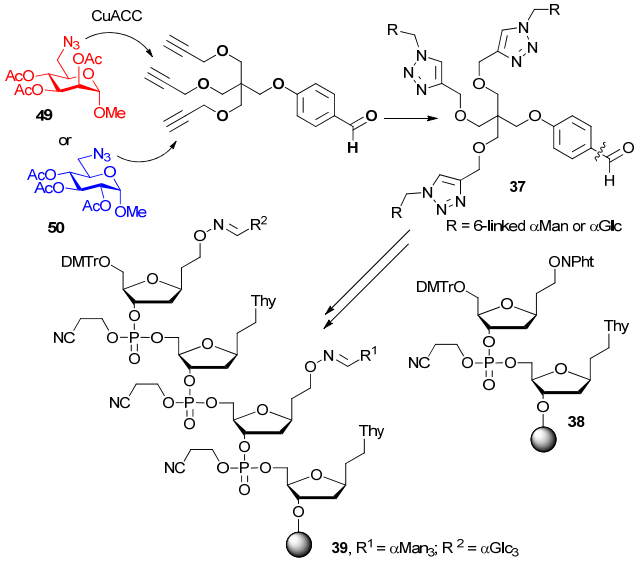


Scheme 6. General procedure of bi-click strategy to obtain Man/Gal-glycooligonucleotides.[35]



Scheme 7. General procedure of combined amidative oxidation-click strategy to obtain Man/Gal-glycooligonucleotides.

Karskela, Lönnberg and coworkers have extended the battery of oligonucleotide heteroglycoconjugates by preparing compounds having two dissimilar trivalent glycodendrons exposing either αMan or αGlc motifs (**37**).[36] In this case, the peracetylated methyl glycosides were first linked through their primary C-6 positions to a tripropargylated pentaerythritol core, armed with a benzaldehyde moiety, by CuACC. The aldehyde group of one of those glycodendrons was then engaged in oxime ligation with an oligonucleotide into which an aminooxy-modified building block had been incorporated on the DNA synthesizer (**38**). This cycle was repeated a second time with the next glycodendron to afford a $(Man)_3(Glc)_3$ conjugate (**39**, Scheme 8).[36] Unfortunately, no data on lectin binding properties of the oligonucleotide heteroglycoconjugates appear to be available up to date.



Scheme 8. Oligonucleotide heteroglycoclusters.[36]

The group of Dumy and Renaudet proposed a combinatorial approach that allowed the rapid generation and screening of a structurally diverse library of tetravalent hGC combining various sugar motifs among αMan, α-galactosamine (αGalNAc), βLact and α-L-fucopyranoside (αFuc). They implemented the so-called template-assembled synthetic protein (TASP) concept by using the topological cyclodecapeptide scaffold **40**, bearing four aldehyde groups, as a regioselectively addressable functionalized template (RAFT), to couple multiple carbohydrate units in a parallel disposition through oxime-based ligation chemistry.[37] This strategy secures a quantitative coupling of biomolecules with a randomized and statistical distribution of each expected library species (Figure 5). Various hGC libraries combining up to four carbohydrates or carbohydrate and amino acid units were thus generated.[38] The composition and binding potency of each library was screened by HPLC with a Con A binding affinity column. Then, the libraries exhibiting the higher lectin affinities were subjected to separation by semipreparative HPLC to individually study the affinity of their components by surface plasmon resonance (SPR). The results indicated that the presence of hydrophobic residues, such as tyrosine, instead of a sugar in mannoside clusters improve the interaction with Con A. Competition studies suggested that the hydrophobic residue does no interact with the specific mannose-binding pocket, but with an independent binding site. The data evidenced the expected decrease in binding affinity when a mannose residue is replaced by any of

the other sugars. Nevertheless, they revealed differences in binding strength depending on the secondary glycotope nature; e.g. $(\alpha Man)_3(\alpha Fuc)$ binds Con A stronger than $(\alpha Man)_3(\beta Lac)$, and the later is a better ligand that a trimannoside in which the forth position is occupied by an aspartic acid residue. A quantitative determination of the binding affinity on a per α-Man basis was not carried out, however.

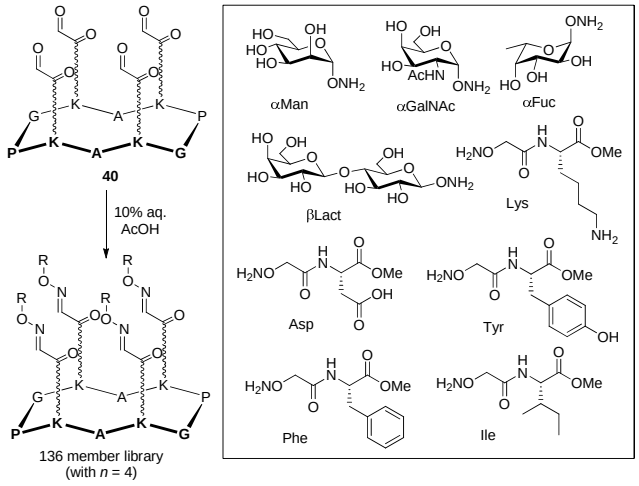


Figure 5. General strategy and structure of carbohydrates and aminoacids used for the generation of hGC libraries from cyclopeptide template **40** displaying four glyoxoaldehyde anchoring sites.[38]

The above combinatorial procedure leads to the formation of inseparable mixtures of regioisomers, which precludes their utilisation for further assays with relevant biological targets. The same group designed a novel synthetic protocol to prepare hGC keeping the same cyclopeptide template but in a regioselectively controlled manner.[39] The methodology consisted in the application of two successive chemoselective reactions, namely oxime ligation and CuAAC, to incorporate different carbohydrates onto cyclodecapeptides containing either two aldehyde and two azide or one aldehyde and three azide functionalities, respectively (Figure 6).[39]

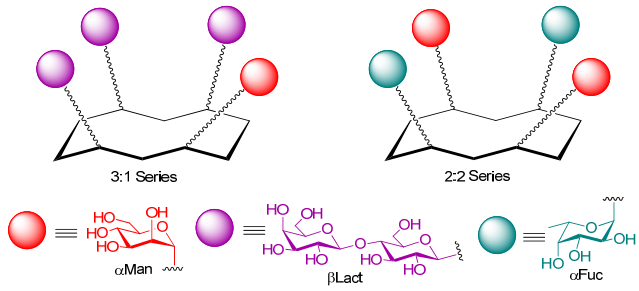


Figure 6. Schematic representation of cyclopeptide-scaffolded 1:3 and 2:2 βLac/αMan and αMan/αFuc heteroglycoclusters, respectively.

(b) Multidomain heteroglycoconjugates

Dondoni, Marra and coworkers[40] described a calix[4]arene glycoconjugate (**41**) in which two different sugars (βGlc and βGal) are installed at the upper and lower rims of the calix[4]arene macrocycle, respectively, *via* sequential CuAAC and photoinduced thiol-ene coupling (TEC).[41] In this heteroglycoconjugate prototype the two monosaccharides do not share the same space regions, but are instead homogeneously occupying opposite domains (Figure 7).

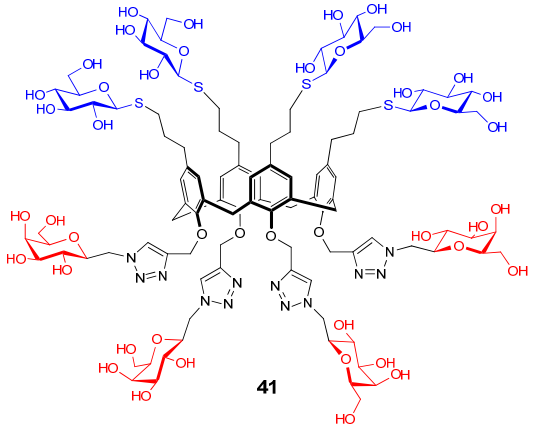


Figure 7. Tetravalent dual glucosylated and galactosylated calix[4]arene cluster **41**.[40]

In principle, heteromultivalent glycoconjugate prototypes consisting in spatially separated homoglycoclusters are likely to be incompatible with heterocooperativity upon binding to a given lectin. However, they might be well suited to cross-link two different lectins, each specific for one of the clusterized sugars. This hypotheses was explored by Roy and co-workers[42] in an attempt to develop efficient antiadhesion therapeutics against pathogenic *Pseudomonas areuginosa*. These bacteria express intracellular and outer membrane lectins, PA-IL (LecA) and PA-IIL (LecB), which are specific for D-Gal and L-Fuc residues, respectively. The authors demonstrated, using a turbidimetric assay, that the heterobifunctional "click" glycodendrimer **42**, possessing four αFuc and four βGal residues in opposite hemispheres (Figure 8), displayed fast cross-linking abilities with both PA-IL and PA-IIL simultaneously as planned. No interference in the binding of the βGal/ PA-IL and αFuc/ PA-IIL pairs due to the presence of the second sugar was observed.

The ensemble of the above commented results indicate that low-density heteroglycoclusters display a moderate glycoside cluster effect against lectin partners exclusively dependent on the valency of the "active" saccharide epitopes. If two sugar motifs exhibiting different affinity for the same lectin are presented together on the scaffold, the resulting heteroglycocluster may bind more efficiently than the respective homogeneous multivalent conjugates. However, no significant influence of "non-recognizable" residues in heteroglycoligand-protein recognition has been clearly established so far in that type of architecture. On the other hand, these molecularly well-defined multiglycoligand derivatives have a strong potential in the development of multiantigenic vaccines and multilectin-targeted inhibitors of bacterial infection.

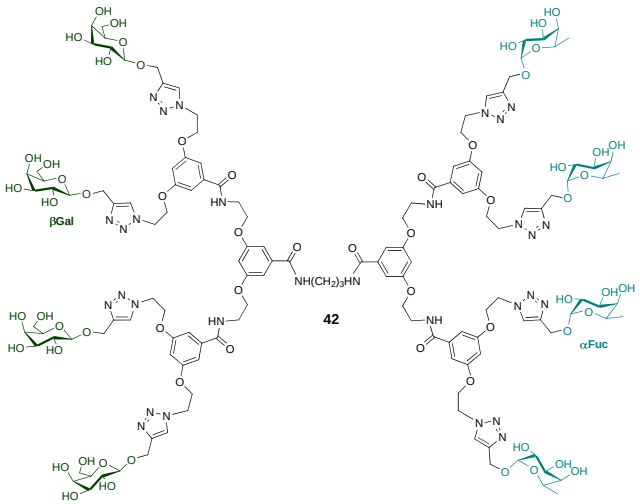


Figure 8. Octavalent αFuc/βGal heteroglycodendrimer **42**.[42]

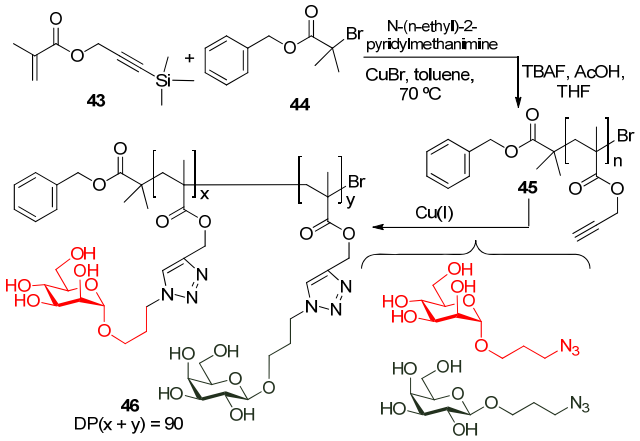
3. High-density heteroglycoconjugates

Carbohydrate-protein recognition in biological environments generally involves high density regions of the glycocalix. Varying the relative expression of the primary glycotope can then switch on or off a given process, e.g. inflammation.[43] Although increasing the valency of the primary recognition motif results in enhanced affinities for a complementary lectin even in low valency glycoconjugate models, it is conceivable that the observation of any supplementary effect due to the presence of other sugar motifs which themselves are not ligands for that lectin might require the involvement of heavily dense heteromultivalent glycodisplays. Heteroglycopolymers, hyperbranched heteroglycoclusters and heteroglycoassemblies have been designed for that purpose.

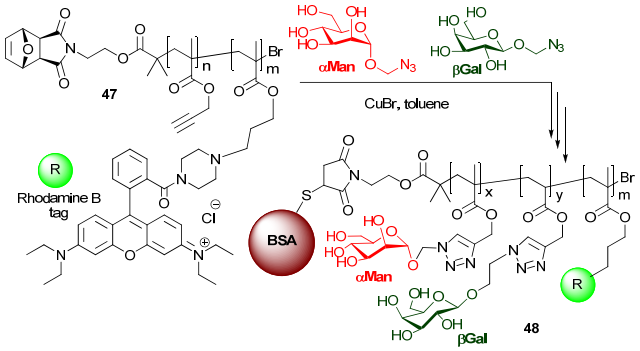
(a) Heteroglycopolymers.

Haddleton and coworkers[44] designed a synthetic protocol for the preparation of hetero-neoglycopolymers **46** based on "coclicking" two different sugar azides by CuAAC with a propargyl-functionalized polymer. The clickable polymeric scaffold was obtained by transition metal-mediated living radical polymerization (TMM LRP) of trimethylsilyl (TMS)-protected propargyl metacrylate precursors (e.g., **43**) and a benzyl α-bromoester (e.g., **44**) as polymerization initiator (→**45**). Small libraries of heteroglycopolymers bearing variable relative proportions of αMan and βGal motifs were prepared in this manner. The library components were assayed for their binding capacity to Con A by turbidimetry and quantitative precipitation. Interestingly, a 75:25 mannose:galactose ratio was found as efficient as the homomannosylated polymer in clusterizing the lectin, implying a 1.5-fold higher efficiency in a mannose molar basis. Although a saturation effect cannot be discarded, the results are consistent with the existence of synergic interactions involving the βGal residues (Scheme 9).[44]

The above heteroglycopolymers were further conjugated to bovine serum albumin (BSA). The coclicking strategy was extended in that case to incorporate a fluorescent probe (rhodamine B) in addition to the αMan and βGal motifs (**47** → **48**; Scheme 10).[45] The binding abilities of these BSA-neoglycopolymer hybrid materials towards the human dendritic cell associated lectin (DC-SIGN), known to bind mannoside residues, were evaluated by SPR.[46] The binding affinity and the relative affinity per mannose unit increased with the density of the αMan ligand, as expected. The experiments were designed to maintain constant the total sugar density varying the αMan:βGal ratio, which prevents the evaluation of the effect of the βGal moieties for compounds having identical αMan density, however.



Scheme 9. Synthesis of the heteroglycopolymer **46**.[44]



Scheme 10. Synthetic procedure to obtain a library of BSA-neoheteroglycopolymers containing a fluorescent tag (rhodamine B).[45]

In a work aiming at the generation of libraries of polymeric cholera toxin (CT) antagonists, the synthesis and activities of a series of heterobifunctional ligands conjugated to two polymer carriers (polyacrylamide and dextran) was described.[47] Since multivalent presentations of βGal residues have demonstrated to exhibit exceptional high activity towards the B subunit of CT (CTB), all the conjugates contained an invariable βGal fragment and variable non-galactose fragments incorporated by CuACC reaction. Considering that the principal ligand for CTB is the ganglioside GM_1 (Gal-β(1-3)-GalNAc-β(1-4)-[NeuNAc-α(2-3)]-Gal-β(1-4)-Glc-β(1-3)-ceramide) and that, in addition to the keystone βGal residue, the neuraminic acid moiety (NeuNAc) is an important fragment for affinity and selectivity, the corresponding heterobifunctional neoglycopolymers were first assayed (Figure 9). They actually showed partial inhibition in a competitive ELLA experiment,

while galactose-only progenitors showed no detectable activity. Nevertheless, the IC_{50} values were deceivingly high (0.5-0.8 mM), much higher than those obtained for non-carbohydrate fragments. The length of the linker is sufficient for both βGal and NeuAc to reach their respective positions in the GM_1 binding site on the surface of cholera toxin; however, entropy loss due to linker flexibility offsets the contribution from this additional interaction.

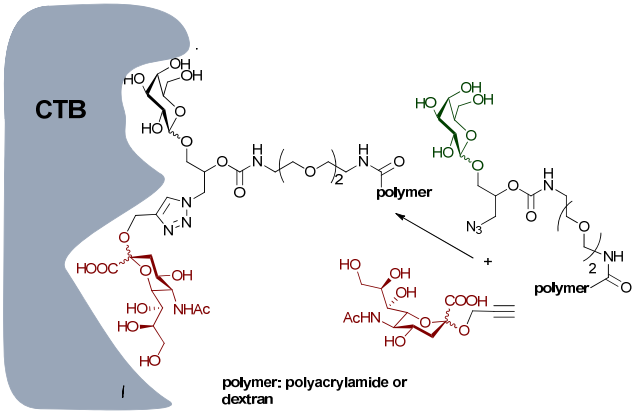


Figure 9. General design of a focused library of heterobifunctional polymers as CTB ligands.[47]

Kobayashi and Nishida designed a facile synthetic way to obtain acrylamide bi- and terpolymers as P- and L-selectin blockers by applying copolymerization synthetic strategies, involving vinyl monomers of α-L-fucoside and 3- or 6-sulfo- or 3,6-disulfo-β-D-galactoside as key carbohydrate modules to mimic sulfated sLe^x tetrasaccharide[48,49] (Figure 10). Binding assays showed that acrylamide bipolymers carrying only 3-sulfogalactoside did not display activity for any selectin while the fucosylated terpolymers showed potent activity to block both P- and L- selectin/sLe^x binding in an ELISA experiment at a concentration of few micrograms per milliliter.[48] The enhanced activity is ascribed to the cooperative binding effects of the fucoside and the sulfogalactoside residues. Deepening in this concept, this research group generalized the so-called "carbohydrate module method", which involves three steps: segmentation of a targeted oligosaccharide into smaller sugars, synthesis of the corresponding glycosylated monomers and the reassembly of oligosaccharide mimics by copolymerization of the modules.[49] The utility of the carbohydrate module method as a tool to assemble oligosaccharide mimics of high biological significance was further supported by the development of new heteroglycopolymers that combined αFuc, 3-sulfo-β-Gal and 6-sulfo-β-GlcNAc exhibiting a significant increase in binding affinity and selectivity towards L-selectin.

The same concept was applied to the synthesis of galacto-trehalose (GT) acrylamide polymers. The binding abilities of such polymers were evaluated against BSI-B_4 lectin (*Bandeiraea simplicifolia*), which is specific to α-galactoside-carrying oligosaccharides including Gb3 ceramide and human blood B determinants.[51] The results supported that both α,α- and α,β-GT-polymers have binding activity towards this lectin, whereas homoglycopolymers bearing βGal or βGlcNAc, used as negative references, did not show any binding. The binding activity increased with the αGal density in the polymer and could be integrated as the result of multivalent binding and/or carbohydrate cluster effects. The effect of βGlc and βGlcNAc residues added as second sugars in the heteroglycopolymers was next examined. Unexpectedly, a terpolymer carrying both α,α-GT and βGlcNAc was found to exhibit the strongest affinity to this lectin. In contrast, βGlc residues brought about no positive effect in any of the polymers and it was even detrimental in the case of the α,β-GT polymer. A similar trend was observed for Shiga toxin-1, another αGal specific binding protein. Thus, heteroglycopolymer **49** carrying both α,α-GT and βGlcNAc (Figure 11) along the polymer chain displayed significantly higher detoxifying activity than the α,α-GT homopolymer. The authors invoked a "module effect" to explain the notable role of βGlcNAc for enhancing interactions with these carbohydrate binding proteins in a supplementary way, but they admited that it is hard to imagine such a molecular packing geometry in which the α,α-GT and GlcNAc residues are fused along a polymer chain to make a Gb3 ceramide mimetic. The exact mechanism by which GlcNAc assists the carbohydrate-protein interaction in this particular systems remain, thus, misterious.

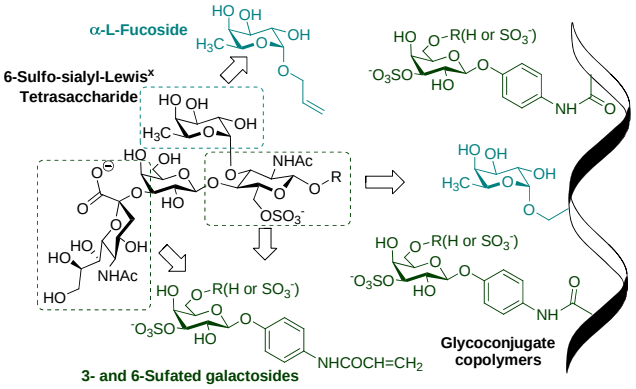


Figure 1.:Artificial selectin blockers: heterocopolymers carrying 6-sulfo sialyl Lewis^x tetrasaccharide as key carbohydrate modules.[48]

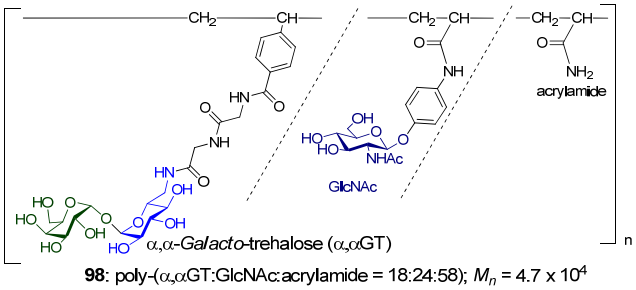


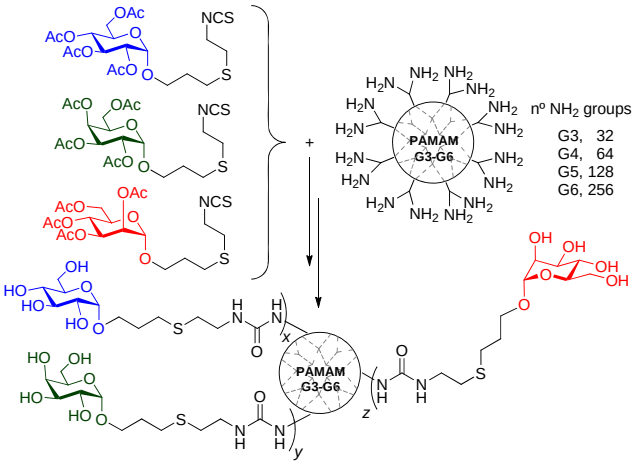
Figure 11. Structure of polyvalent α,α-GT and β-GlcNAc acrylamide polymer **49** mimicking globosyl 3 (Gb3).[51]

Wolfenden and Cloninger[52,53] functionalized poly(amidoamine) (PAMAM) dendrimers of generations G3, G4, G5 and G6 with αMan, αGlc and αGal glycotopes (Scheme 11) to investigate Whitesides' relationship between association constants for monovalent and multivalent associations given in eq. (1), where *N* is the number of

receptor-ligand interactions and α is the cooperativity factor.[54]

$$K_N^{poly} = \left(K^{mono}\right)^{\alpha N} \quad (1)$$

Based on size and architectural considerations, they assumed a divalent interaction (N = 2) between tetrameric Con A and αMan-containing PAMAM glycodendrimers, with a positive cooperativity factor α = 1. Considering that monomeric αMan is recognized by the lectin with a 4-fold higher affinity than αGlc, a 16-fold decrease would be expected when going from 50% αMan loaded PAMAM homoglycodendrimers to heteroanalogues keeping the same total carbohydrate loading but with an 1:1 αMan:αGlc composition. The experimental values obtained from hemagglutination experiments were very close to the theoretical ones for the G4 and G5 glycodendrimers, which led the author to conclude that the activity of heteroglycoligands can be modulated for those systems in a predictable manner. However, significantly lower decreases in binding affinity were observed for the sixth-generation αMan:αGlc dendrimer and, especially, in the case of αGlc:αGal conjugates. It was advanced that in systems having either higher flexibility or lower affinity, proximity/statistical effects are more important to binding. Thus, dendrimers with more glucose residues appear to compensate for the steric downfall of full functionalization by relying more on proximity enhancements than mannose functionalized dendrimers do.[53] Nevertheless, the existence of synergistic interactions involving the second sugar that compensate, in part, the diminution of the primary ligand concentration cannot be discarded.



Scheme 11. Mannose/glucose/galactose-coated PAMAM dendrimers.[52,53]

(b) Heteroglycoclusters

The use of polymeric scaffolds to build highly dense glycoarchitectures implies an intrinsic polydispersity and lack of conformational control that hampers a rigorous evaluation of the influence of architectural parameters in the binding affinity to protein receptors, a fact that is exacerbated for multiligand-coated derivatives. In order to get a deeper insight on the issues related to heteromultivalency, Ortiz Mellet, Defaye and García Fernández designed a series of hyperbranched heteroglycoclusters that comply with the requirements for polyvalency, high density and monodispersity. An efficient procedure based on multiple coupling of isothiocyanate-armed glycoligands or heteroglycodendrons to a per(C-6)cysteaminyl cyclomaltoheptaose (βCD) derivative[16,55] proved to be very efficient for the construction of 7-, 14- and 21-antennary heteroglycoclusters[56] having all the branches oriented toward the same espace region, thereby acting as surrogates of heavily glycosylated patches at the cell membrane. A critical advantage of the methodology is that it allows sampling compounds with varied, yet perfectly defined densities of the constitutive sugars (αMan and βGlc or βLact; Figure 12). The effect of the valency and density of the receptor-binding elements in a homogeneous compared to a heterogeneous environment was then explored by ELLA and isothermal titration microcalorimetry (ITC) measurements. The results reflected unexpectedly high Con A-binding affinities for the mixed-type αMan/βGlc and αMan/βLac heteroglycoclusters in comparison with homoglycoclusters with identical mannose valency.[29,30,56] The authors hypothesized the existence of a "heterocluster effect" that cannot be explained in terms of a difference in effective epitope concentration.

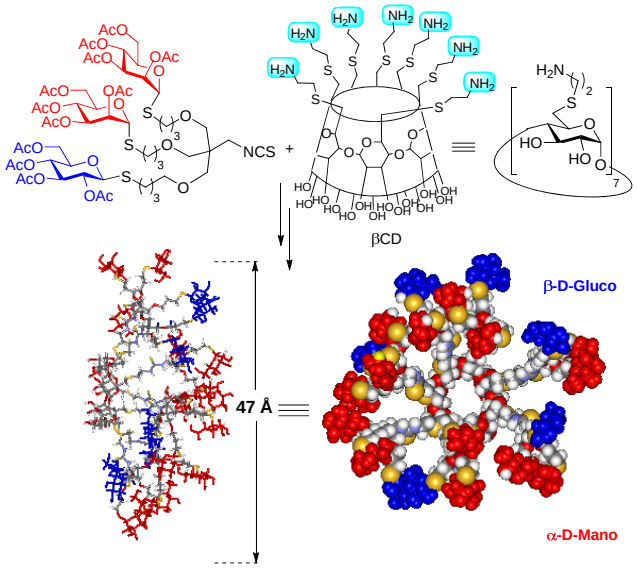


Figure 12. Synthesis of $(Man)_{14}(Glc)_7$ βCD-centred heteroglycocluster. $(Man)_{21}$, $(Man)_7(Glc)_{14}$, $(Glc)_{21}$, $(Man)_7(Lac)_{14}$, $(Man)_{14}(Lac)_7$ and $(Lac)_{14}$ hyperbrached conjugates were also synthesized and evaluated for their lectin binding abilities.[56]

To confirm the above results, the binding properties of high- and low-density homo-and heteroglycoclusters with αMan and βGlc residues towards Con A lectin were assessed by using a range of competitive and non-competitive binding assays including ELLA, ITC and surface plasmon resonance (SPR).[29] In all cases, highly-dense glycoclusters displayed a substantial amplification of the binding strength compared to low-density counterparts as expected from the glycoside cluster effect. Whereas in the low-density heteroglycoclusters the presence of the second sugar was irrelevant for binding, in highly-dense displays the binding efficiency on an αMan molar basis was significantly higher in the presence of the

"non-ligand" βGlc. The results were rationalized assuming the existence of secondary interactions involving the "non-active" βGlc residues in the presence of a certain density of the "active" αMan ligand, that is, a synergistic heterocluster effect. The thermodynamic data further suggested that such heterocluster effect has an entropic origin, which is compatible with a more efficient sliding of the heteroglycocluster over the binding site in the lectin promoted by the presence of the secondary epitope.

In a further work, αMan/βLact βCD-scaffolded heteroglycoclusters were evaluated by ELLA, two-site-ELLA and turbidity assays against Con A and PNA lectins to investigate their ability to interact with a single binding site in the protein, their capacity to cross-link two lectin molecules and their potential to induce the formation of three-dimensional aggregates, respectively (Figure 13).[30] It was observed that recognition of the primary sugar by its complementary lectin was enhanced in the presence of the second sugar. Thus, the $(\alpha Man)_7(\beta Lac)_{14}$ heteroglycocluster was more efficient at cross-linking Con A than the $(\alpha Man)_7$ homogeneous derivative. Similarly, $(\alpha Man)_{14}(\beta Lac)_7$ proved a better PNA cross-linking ligand than $(\beta Lac)_7$. The turbidity experiments evidenced that the supplementary binding enhancement due to the secondary glycotope is sugar-specific, since the formation of the three-dimensional lattices was affected by the presence of a high concentration of the corresponding monovalent sugar in the solution. Overall, the results supported that the proposed "heterocluster effect" was not a curiosity restricted to Con A but can also influence the binding mechanisms of other lectins.

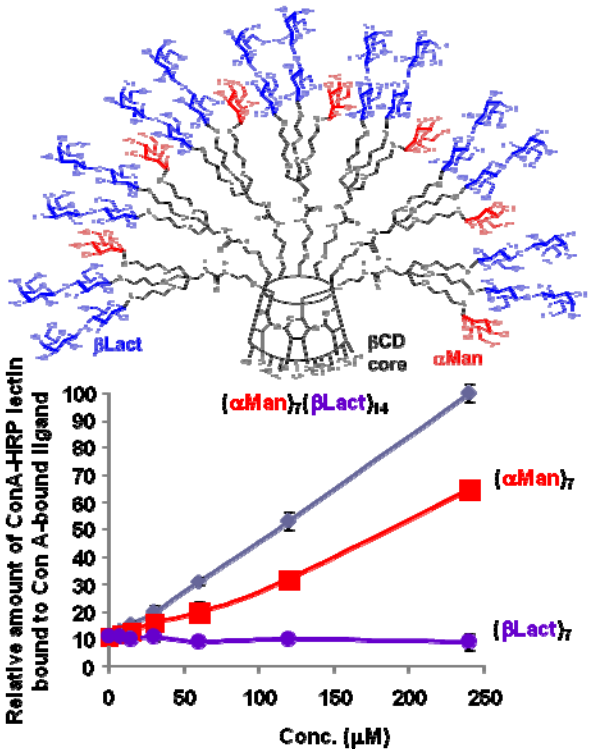


Figure 13. Relative cross-linking efficiencies of αMan/βLact CD-centred heteroglycoclusters against the mannose specific lectin Con A at different concentrations in comparison with αMan and βLact homoglycoclusters.[30]

(c) Heteroglycoassemblies

In a seminal work, Horan, Isobe and Kahne[57] prepared carbohydrate-derivatized self-assembled monolayers (SAMs) incorporating two different disaccharide ligans, namely βGal(1-3)βGalNAc and αGal(1-3)αGalN^{i}Pent (^{i}Pent = isopentanoyl), to investigate the influence of relative ligand density in the binding affinity towards *Bauhinia purpurea* (BP) lectin (Figure 14). By using SPR, they encountered that the binding selectivity of BP lectin for the carbohydrate ligands depended on their surface density in the mixed SAMs, even though binding is polyvalent at all densities investigated. The results suggest that secondary interactions contribute significantly to protein avidity. The authors speculated that as the density of the carbohydrate ligands increases, interactions between the carbohydrates may affect the individual binding interactions with the protein. Alternatively, protein–protein interactions may be established at high carbohydrate surface densities. These protein–protein interactions may well differ for different carbohydrate ligands. Regardless of their precise nature, secondary interactions could have a significant effect on protein binding, with the result that the binding selectivity switches at high surface densities.

The above observation raises the possibility that cell-surface carbohydrates may be involved in the regulation of biological pathways in a more complex manner than previously considered from our knowledge on homomultivalent carbohydrate-protein recognition. However, the transcendence of Kahne's work remained someway ignored by the scientific community. Thus, even though an intense research has been devoted to the development of carbohydrate microarrays,[58] not further examples of carbohydrate-lectin recognition studies using immobilized heteroglycodisplays are found in the literature. Very recently Wong, Wu and coworkers reported heterogeneous glycan arrays to study anti-SSEA3 (stage-specific embryonic antigen 3, Gb5) antibody interactions in a density-dependent manner.[59] Six heterogeneous glycan arrays (SSEA4/Gb5, Globo H/Gb5, Gb4/Gb5, Gb2/Gb5, Bb2/Gb5; 1:1 mole ratio by mixing the glycan with Gb5) and two homoglycan arrays (Gb5, linker amino-1-pentanol/Gb5 with 1:1 molar ratio) having all of them the same amount of Gb5 were prepared for measurement of anti-Gb5 antibody binding by fluoresce spectroscopy (Figure 14). The heteroglycan array SSEA4/Gb5 displayed the highest fluorescence intensity, which was likely a result of the cross-reactivity and multiligand effects which were caused by one antibody binding with two different structures of glycans simultaneously. Unexpectedly, the Gb3/Gb5 array also demonstrated an unexpected high avidity for anti-Gb5 antibody, which is in contradiction with the so-called "glycan shield" mechanism in which the presence of high density neighbouring glycans with the antigen hampers antibody recognition.

In the same work, the authors attached two different high-mannose glycans, namely Man_4 and Man_9, to an AB_2-type second generation dendrimeric scaffold at different ratios. This oligomannose dendrons, armed with a terminal amino group at the focal point, were then printed onto a *N*-hydroxysuccinimide-activated glass slide to form an array of

conjugates with various densities that were evaluated for their binding efficienty towards the broadly HIV-1-neutralizing monoclonal antibody 2G12. The $(Man_4)_5(Man_9)_4$ heteroglycodendron (Figure 15) exhibited the strongest affinity to 2G12, about 2.5-fold stronger as compared with the corresponding Man_9 or Man_4 homoglycodentrons.[59] These studies demonstrate that not only carbohydrate-lectin, but also carbohydrate-antibody binding is affected by the density and structures of neighbouring glycans.

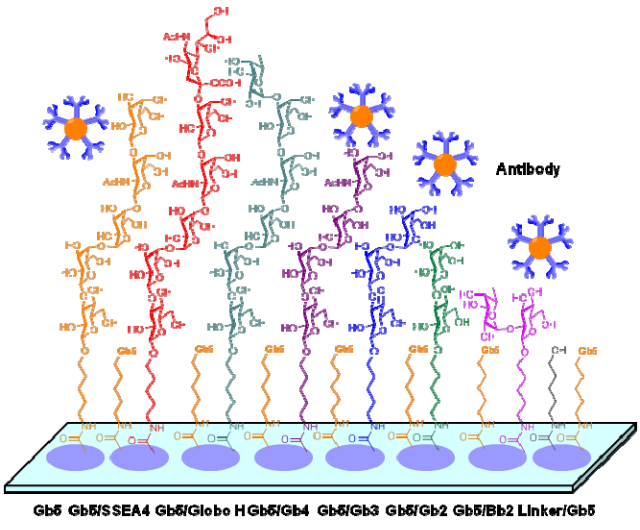


Figure 14. Gb5-containing heteroglycoarrays prepared by Wong and coworkers to study anti-Gb5 antibody interactions.[59]

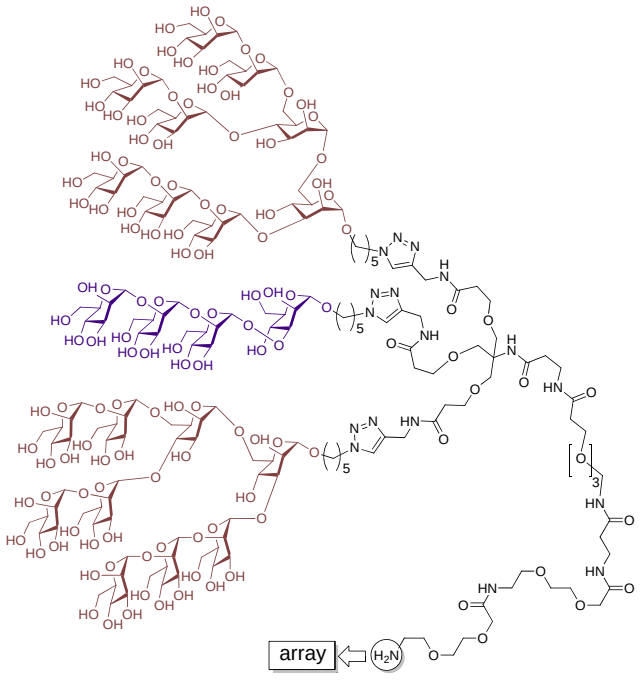


Figure 15. Amine-armed high mannose oligosaccharide heteroglycodendron used by Wong and co-workers for glycan array preparation to assay 2G12 monoclonal antibody binding affinity.[59]

To study the influence of sugar ligand density on lectin-carbohydrate recognition, Ravoo's group has recently designed artificial glycocalix mimics from an amphiphilic β-cyclodextrin derivative that self-assembles into unilamellar bilayer vesicles. The surface of such vesicles was decorated with maltose- and lactose-adamantane conjugates through host-guest interactions (Figure 16). The multivalent interaction of these βCD vesicles (CDV)-maltose-lactose ternary systems with Con A and PNA lectins was investigated by using ITC, dynamic light scattering (DLS), UV-visible spectroscopy, and cryogenic transmission electron mycroscopy (cryo-TEM). The results demonstrated that lectin agglutination is reversible and does not disrupt the vesicle monolayer. The association strongly depends on the surface coverage of carbohydrate on the vesicles; only highly-dense sugar-coated vesicles induced either PNA (for lactose) and Con A (for maltose) agglutination.[60]

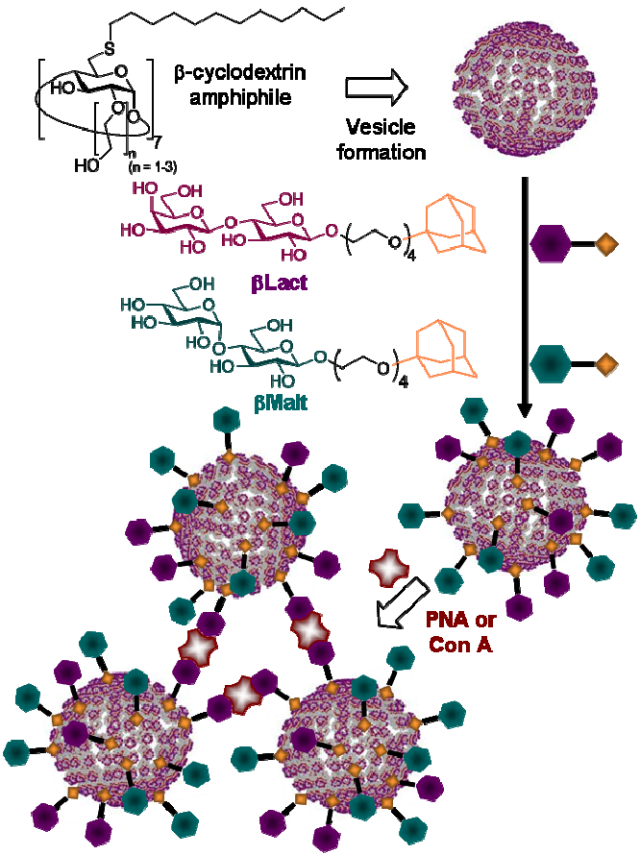


Figure 16. Schematic representation of αMan/βLact supramolecularly coated cyclodextrin vesicle (CDV) agglutination induced by Con A or PNA lectins.[60]

The kinetics of the orthogonal multivalent interfacial interactions with the lectins were studied by time-dependent measurements of the optical density at 400 nm.[60] It was found that the initial rate of aggregation scales linearly with the lectin as well as with the CDV concentration and that each lectin requires a characteristically critical density of "active" carbohydrate at the vesicle surface. Their differential kinetic behaviour, with a faster aggregation for Con A in comparison with PNA, is ascribed to a receptor-induced local clustering of the "active" carbohydrate ligand in order to maximize the interaction. Since aggregation speed enhances with the effective binding site separation at the lectins, these model systems have been proposed to estimate the binding site

distance in multivalent receptors.

The potential of heteroglycocoated CDVs as self-assembled glycocalix mimics to assess non-linear effects ascribable to cooperative interactions involving different glycotopes remains to be exploited. Nevertheless, these constructs have the merit to reproduce not only the heterogeneity but also the fluidity of the glycolipid membrane. Comparing data from rigid and soft multivalent glycosystems may provide precious information on static versus dynamic supplementary effects derived from multiligand presentation, which otherwise would pass unnoted.

4. Conclusions and Perspectives.

Synthetic multivalent glyco-constructs have become powerful tools for the elucidation of the structural features ruling carbohydrate recognition events and the design of novel glycodrugs. Most of the bibliography published in this topic is focussed on systems incorporating multiple copies of identical sugar structural motifs on an appropriate scaffold (molecular, dendritic, polymeric or supramolecular). Only recently the design and synthesis of multivalent heteroglycosystems that resemble more accurately the cell membrane environment has reached a significant body of results. The ensemble of data demonstrates that glycohetereogeneity have an impact in carbohydrate-protein and carbohydrate-antibody recognition events that is dramatically depending on the total and relative densities of the exposed glycotopes. Different terms have been proposed to refer to this concept, which someway stem from the hypotheses advanced by the authors to rationalize the experimental observations, such as "heterocluster effect" or "carbohydrate module effect". Regardless of their precise nature, a main conclusion is that secondary interactions could have a significant effect on protein binding. Although neighbouring effects seem to operate only at high density of the ligands, the efforts made in the synthesis of low-density heteroglycoconjugates are not wasted. First, most of the approaches can be translated to high-density displays. Second, they allow a high degree of structural and conformational control that, in addition to the classical application in multiantigenic vaccine generation, may be exploited in multilectin interaction processes.

The findings obtained with high-density heteroglycoconjugates suggest that cell-surface carbohydrates may be involved in the regulation of biological pathways in a more complex manner than the "on-off" switch model, associated to high density-low density expression of a single recognition element, that has been considered previously. In the same way as genes or proteins can regulate the activity of other genes or proteins, recognition phenomena involving carbohydrate ligands might be up- or down-regulated by the changes in the expression of secondary sugar motifs. Actually, the work collected in this review strongly suggests that carbohydrate expression levels in heterogeneous environments can modulate far more complicated response patterns, i.e. switching not just from an "off" to an "on" state, but from one "on" to another "on" state. It may be also viewed as a saving mechanism; a relatively low expression level of a putative sugar ligand may be activated by expressing a second, "les costly" epitope. Similarly, neighbouring effects affect antibody interactions in a density-dependent manner, which should be applicable to better mimic complex epitope presentation.

Diversity-oriented methodologies that allow the efficient control of the composition and the geometry of mixed-type glycoconjugates will undoubtedly help to understand the basis of supplementary phenomena in heterogeneous scenarios, which may have implications in the design of synthetic ligands for therapeutically relevant carbohydrate receptors. The real scope of heteromultivalency in biological systems remains nowadays uncertain. Yet, the current data introduce a new and intriguing variable in the still underexplored field of multivalency that with no doubt will concentrate many efforts in the near future.

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