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PERSPECTIVE

Insights in the rational design of synthetic multivalent glycoconjugates as lectin ligands

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Much effort has been made during the last decade to design lectin inhibitors as therapeutics against viral and bacterial adhesion or to control biological functions. The chemical strategy adopted generally consists in the tethering of several binding epitopes on a common scaffold. The resulting multivalent glycoconjugates often display a much higher binding affinity for their targets compared to their monovalent counterparts, a phenomenon designed as the "cluster" or "multivalent effect". Hundreds of multimeric architectures have been designed so far and some of the compounds displayed impressive gains in binding affinity or *in vivo* efficiency. Progress in this area is, however, hampered by the difficulty to predict the potency of the new multimeric inhibitors. This review presents the recent efforts to probe the important structural features of the synthetic multivalent glycoconjugates for a tight binding with specific lectins. We hope that the reported examples will aid the reader to design efficient multivalent ligands in a more predictable way.

1. Introduction

Carbohydrate–protein interactions mediate a host of biological events and play a pivotal role in the binding of bacteria, viruses or toxins to cell membranes. Monovalent carbohydrates typically bind to their putative receptors termed lectins[1–4] with a low affinity, in the millimolar range. To circumvent this problem and to function as potent and specific effectors or inhibitors of biological processes, sugar residues are generally displayed in a multivalent fashion at the surface of cells. Multivalency generally leads to a greater affinity enhancement than predicted from the sum of the constitutive interactions. Several research groups have successfully exploited this key feature, and a tremendous number of synthetic multivalent ligands have been developped to modulate a wide range of biological processes. Most of the multimeric

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David Deniaud obtained his PhD in Organic Chemistry in 1996 from the University of Nantes, where he studied synthesis and properties of metalloporphyrines under the direction of Dr Bruno Bujoli. He completed postdoctoral research in catalysis with Dr Daniel Mansuy at the University of René Descartes, Paris and with Procter and Gamble Company. Currently, he is an Assistant Professor in the Chemistry Department at Nantes Faculty of Sciences. His major research interest includes the study of heteroatomic linkages, and the synthesis of polydentate ligands and study of their complexation properties with different metals for applications in nuclear medicine and oncology.

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ligands designed so far are synthetic inhibitors of lectins, and hundreds of glycomimetics with diverse chemical structures were published in the last decade. The affinity enhancement obtained with multivalent ligands compared to their monovalent references is often referred to as the multivalent or cluster effect.[5] Despite the abundant literature, rational design and predictive efficiency of a neoglycoconjugate toward a specific lectin remains challenging. This is largely due to the interplay of different multivalent binding events (see section 2), difficult to predict and depending both on the nature of the lectin and the glycoconjugate. Studying independantly the importance of valency, topological presentation of the epitope, size of spacer arm, and nature of the scaffold of glycoclusters is of primary importance. Ultimately, this could make possible a future rational design of multivalent ligands that would be optimized to generate a maximal cluster effect with a particular receptor.

The multivalent carbohydrate architectures, and their affinity with lectins, have been extensively reported in good reviews[6–14] and book chapters.[15–17] We invite the reader to refer to these papers for an overview of the biologically active multivalent glycoconjugates. The scope of this review is restricted to the current efforts made to evaluate how the constitutive elements (*i.e.* the scaffolds, the linkers' size and the epitopes' distribution) of synthetic multivalent glycoconjugates influence lectin binding. A short introduction will illustrate the carbohydrate–lectin binding mode that may operate during multivalent interactions, and the different classes of synthetic multivalent ligands designed so far. Then, selected examples emphasizing the important structural features of synthetic multivalent glycoconjugates for specific applications will be presented. The focus of all discussion will be based on the new insights provided with regard to the ligands' architecture.

2. Multivalent binding modes

Multivalency and cooperativity are two distinct phenomena which rely upon the formation of non-covalent bonds. Multivalent or cluster effects tend to be much stronger than the corresponding monovalent interactions.[18–21] A true cluster effect is observed if the binding potency value recorded with a multivalent construct having x tethered ligands (or epitopes) is more than x times greater than that of the corresponding monovalent ligand.[22] If this value, also termed the relative potency per ligand, is identical to the monomeric reference, the effect occurring is purely statistical and no real affinity gain is observed.

Cooperativity arises when the binding of one ligand influence the receptor's affinity towards subsequent ligands.[23] The interplay of individual interactions can lead to positive or negative cooperativity depending on whether one interaction favor or disfavor another. On a macromolecular level, cooperativity is a key feature in the assembly of organized oligomers, to switch between states and provide an "off" or "on" biological response. There is conflicting views and differing opinions about the proper assessment of cooperativity. Ercolani[24] published a critical examination of previous works by Pfeil and Lehn and by Taylor and Anderson on the cooperative self-assembly of helicates,[25] and porphyrin ladders,[26] respectively. The author reached opposite conclusions and demonstrated that the two self-assembly process occurred non-cooperatively.

In multivalent interactions, the receptor binding mechanisms presented in Fig. 1 may occur simultaneously or independantly, depending both on the structural features of the glycoconjugates and the nature of the lectin. Epitopes of a multivalent ligand may bind at a single site of its putative receptor, sliding and recapture of a second epitope increases the residence time and the binding affinity (Fig. 1A). The proximity of additional epitopes promotes the recapture mechanism. For oligomeric ligands, the so-called minicluster or heterocluster effect[27,28] is generally associated with a moderate gain of affinity with generally less than two orders of magnitude enhancements compared to the monovalent reference. However for polymeric glycoconjugates, the bind and slide process due to internal diffusion of the lectin along the polymeric chain can lead to much higher affinity systems, as shown by Brewer and coworkers[29] on mucin-type glycoproteins (Fig. 1B). A chelate binding mode may operate if the distance between binding

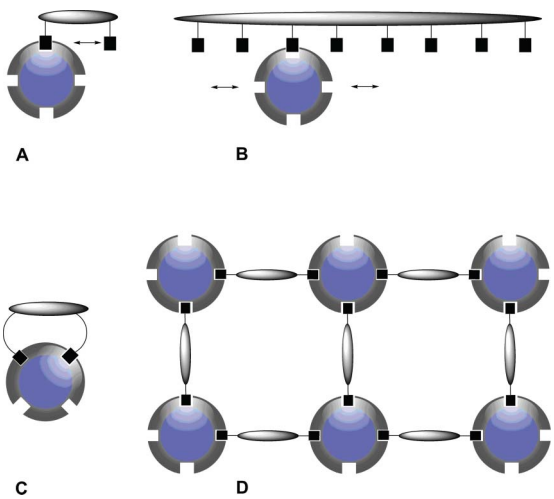


Fig. 1 Schematic representation of receptor binding mechanism for a multivalent glycocluster and a lectin with four Carbohydrate Recognition Domains (CRDs): A) Intrinsic affinity, a microcluster or heterocluster effect, B) Binding and sliding of the lectin along a polymeric chain, C) Chelate binding mode, D) Formation of cross-linked lattices.

Sébastien G. Gouin

Dr Sébastien G. Gouin was born in Le Mans (France) in 1976. He studied organic chemistry at the University of Nantes (France) where he received his PhD in 2003. After postdoctoral training with Prof. Paul V. Murphy at University College Dublin (Ireland), he was appointed as a CNRS researcher in the University of Amiens. His present research activities in the laboratory CEISAM in the University of Nantes, are focused on the synthesis of radiopharmaceuticals and multivalent ligands of lectins.

epitopes can span the distance between recognition sites of the lectin (Fig. 1C). Some of the largest multivalent stabilizations have been reported when a chelate effect is operating, with over million-fold affinity enhancement for pentameric toxins.[30,31] Such high multivalent effects are generally explained by low rotational and translational entropic costs paid by the additional binding events. Prior to interaction, the multivalent glycoconjugate and receptor are free to rotate and translate in the three space dimensions. Upon binding, the entropic penalty is paid by the first interaction making other binding events much more favorable. These high affinity enhancements also explained with the term of *effective concentration* (c_{eff}) which report the real ligand concentration in the close binding site proximity. After the first binding event, the effective ligand's concentration in the neighboring of a second protein binding site is altered by the tethered ligands. If the resulting c_{eff} is increased, an intramolecular binding is then favored and will likely proceed.[22]

Multivalent carbohydrate ligands may also cross-link multimeric lectins (Fig. 1D). For compounds bearing spacer arms of sufficient length, intermolecular binding may compete with the formation of intramolecular chelates. The multivalent entities will bind inter- instead of intramolecularly, if the conformational entropic penalty paid in the intramolecular chelate binding mode exceeds the gained translational and rotational entropy. Different type of ordered and homogeneous lectin-saccharide lattices may be obtained,[32] depending both on the nature and valency of the glycoconjugate and lectins. The potentially large aggregates that may be stabilized by lectin-lectin interactions can precipitate from the solution in particular conditions (*i.e.* ligands : lectins stoechiometry, temperature, pH and ionic strength of the solution). Less common binding mode not represented Fig. 1 may also occur. After interacting in a primary binding site, epitopes of heterovalent ligands may simultaneously contact other protein subsites and gain in binding energy.[33,34] Finally, steric stabilization may also occur with bulk multivalent ligands, preventing further access to competitive ligands.

3. Structure of the synthetic multivalent glycoconjugates

A large number of multivalent glycoconjugates with diverse scaffolds have been synthesized in the last decade. Such glycoclusters can be classified in two distinct groups. Glycoconjugates with controlled valency can be discriminated from polymeric materials and nanoparticles with a random distribution of epitopes. The first group generally displays less than 20 sugar units, although larger glycodendrimers with controlled valencies have been published.[35–38] A wide range of commercially available or synthetic scaffolds have been successfully used for designing discrete glycoconjugates. Relatively high degrees of freedom and adaptability towards the lectin binding sites can be obtained when epitopes are tethered by flexible linkers or scaffolds such as functionalized oligoethylene glycols (EG),[39,40] linear and ramified peptides[41] and other dendrimeric structures (Fig. 2).[42–46]

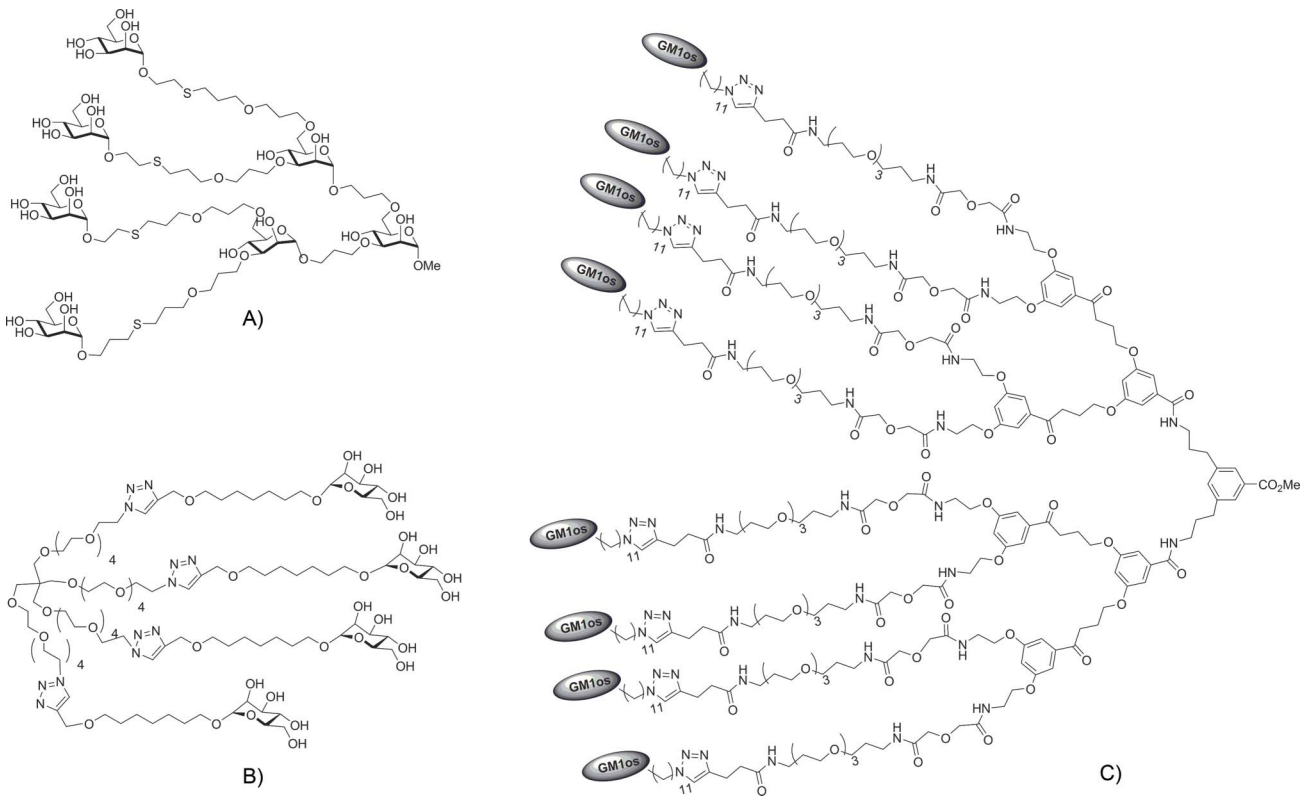


Fig. 2 Examples of mulivalent glycoclusters with a flexible core: A) A tetravalent mannoside ligand reported by Heidecke and Lindhorst,[46] obtained by an iterative synthesis from a bi-functional core glycoside. B) A tetravalent heptyl mannoside reported by Gouin *et al.*,[39a] based on a pentaerythritol skeleton that inhibits bacterial bladder cell binding in the nanomolar range. C) An octavalent GM1 ganglioside reported by Pieters and coworkers[42c] as a strong inhibitor of cholera toxin.

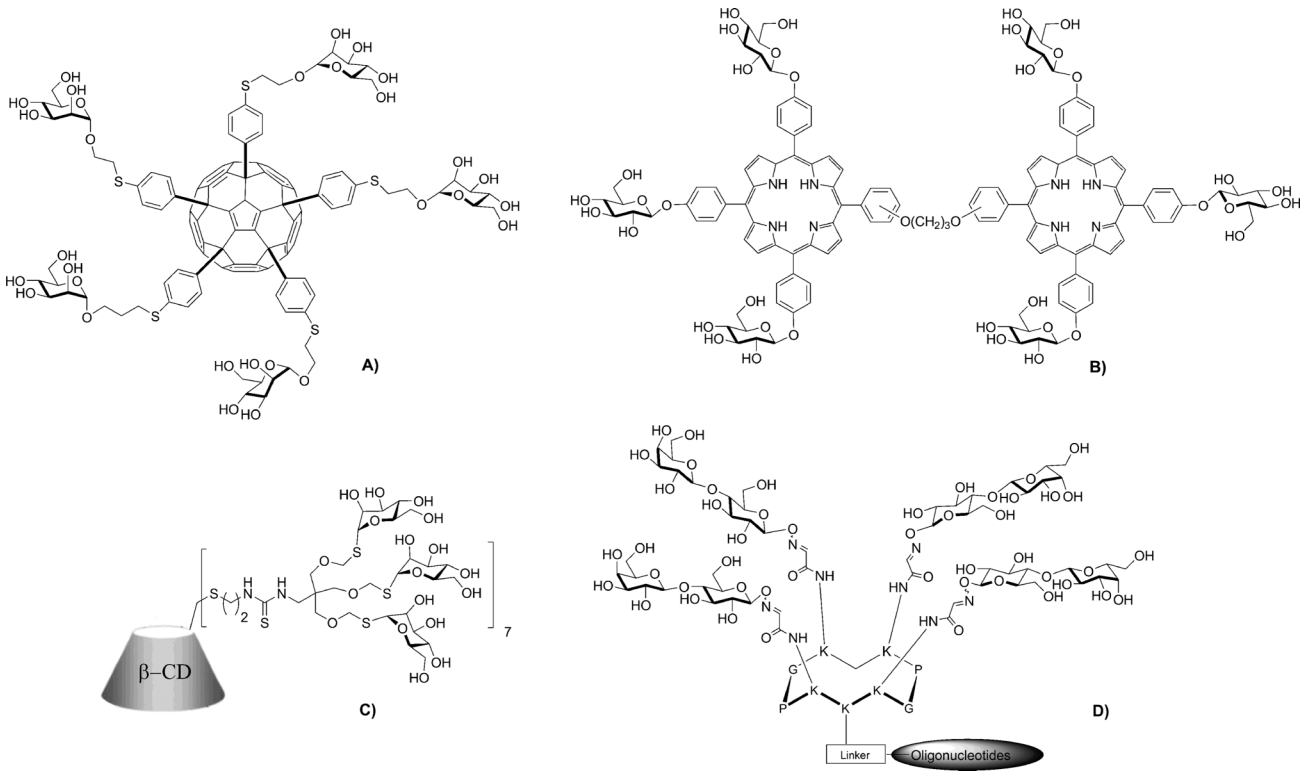


Fig. 3 Examples of mulivalent glycocluster with a rigid core: A) A fullerene pentavalent glycoconjugate and designed through sulfide connection in aqueous media (Nakamura and coworkers[48]) B) A glycosyl bis-porphyrin conjugates as a new photosensitizer for a potential application in photodynamic therapy (Krausz and coworkers[50]) C) Cyclodextrin-centered glycocluster where epitopes are displayed in triads, designed to probe secondary carbohydrate-lectin interactions (García Fernández and coworkers[56b]) D) A tetravalent glycopeptides-oligonucleotide conjugate based on a rigid cyclopeptide (Dumy and coworkers[61a]).

Alternatively, sugar presentation can be fine-tuned when these epitopes are directly grafted onto rigid architectures such as aryl clusters,[47] fullerenes,[48,49] or porphyrins scaffolds (Fig. 3A,B).[50] In particular, calixarenes that can be obtained in various blocked conformations,[51–55] or cyclodextrins that can be selectively modified on the upper and lower rim (Fig. 3C),[56–59] have been extensively investigated. Regioselectively addressable functionalized templates (RAFTs) are stable lysine-containing cyclopeptides providing two functional faces (Fig. 3D).[60] Two prolylglycine sequences induce β turns that constrain the backbone, allowing a specific presentation of the carbohydrate ligands at the upper face of the template.[61,62] The ligands' topology can also be varied with conformationally constrained linear molecules such as carbohydrates. We,[63] and others,[64–67] have designed rigid glycoclusters with specific epitopes' presentation by tethering the ligands to selected hydroxyls groups of the saccharides' scaffold.

Several polymeric glycoconjugates and nanoparticles with specific architectures have also been recently developed. Seeberger and coworkers[68] have grafted mannosides on a rigid poly(*p*-phenylene ethynylene) (PPE) core for the aggregation and fluorescent detection of bacteria in solution (Fig. 4A). Among the synthetic methods to generate water soluble polymers, the ring-opening metathesis polymerization has been successfully implemented by Kiessling and coworkers for the synthesis of glycopolymers with low polydispersity.[69] Compared to linear polymers, glyconanoparticles display different topological presentation with a globular shape. Most of the examples described, consist on the covalent linkage of various glycoconjugates to a metallic core (Fig. 4B).[70] Single or multiwalled carbon nanotubes (SWNTs and MWNTs) have potential biological applications for biosensing and imaging due to their unique electrochemical properties. Several recent studies describe the functionalization of carbon nanotubes with carbohydrates epitopes to generate water soluble system,[71] able to mimic cell surface mucins,[72] or to capture pathogens in solution such as *Escherichia coli* (*E. coli*),[73] or anthrax spores (Fig. 4C).[74] Aside from these covalently linked glycoclusters, strategies for the *in situ* self-assembly of monomeric synthons tethering sugar epitopes have been reported. A pioneering work by Sakai and Sasaki describes the metal-assisted association of carbohydrate functionalized bipyridine chelates.[75] The Fe^{II}(bipy-GalNAc) complexe formed was a more potent binder to *Vicia Villosa* B_4 lectin than the monovalent GalNAc references. The strategy was further successfully extended to different pyridine chelate structures or metallic cations such as copper,[76,77] or ruthenium[78,79] by other groups. Thoma *et al.*[80] have designed glycodendrimers with oligoaromatic core terminated by α-galactoside epitopes, which self-assemble into large architecture in water through intermolecular π–π interactions and hydrophobic contacts. The supramolecular aggregates were found to be potent inhibitors of the αGal–IgM interactions, both *in vitro* and *in vivo*. Recently, Müller and Brunsveld.[81] have reported a similar approach for multivalent targeting of bacteria with self-assembling polyvalent scaffolds (Fig. 4D). The method relies on the design of hydrophobic disc-shaped molecule decorated with sugars that can

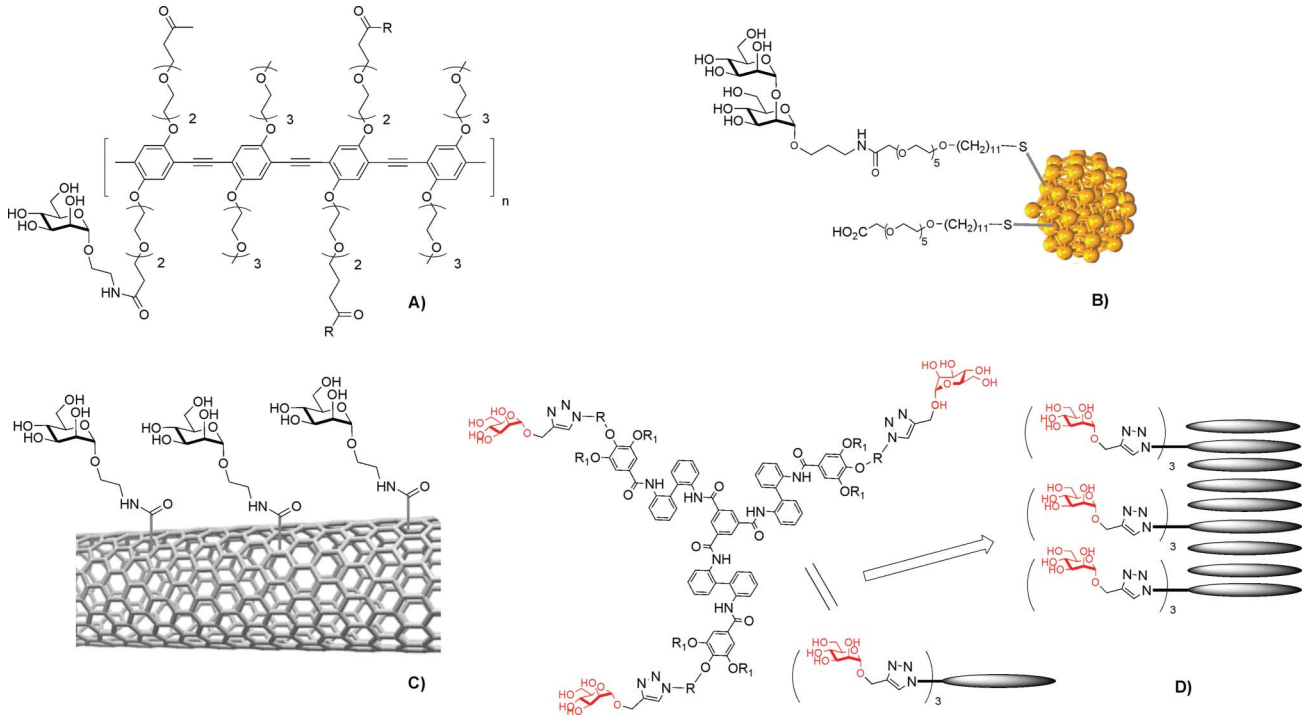


Fig. 4 Polymeric glycoconjugates and nanopartices: A) Carbohydrate-functionalized fluorescent polymer for the multivalent detection of *E. coli* in solution (Seeberger and coworkers[68]) B) Gold manno-glyconanoparticles as potential carbohydrate-based antiviral agents, showing inhibitory activity for the DC-SIGN binding to gp120 (Penadés and coworkers[70a]) C) Mannose-coated single-walled carbon nanotube that binds and aggregates with anthrax spores in the presence of Ca^{2+} (Sun and coworkers[73]) D) Hydrophobic disc-shaped molecules bearing mannosides that self-assemble in water into columnar supramolecular architectures (Müller and Brunsveld[81]).

assemble into columnar polymers in water. These supramolecular assemblies were able to aggregate bacteria although the valency corrected enhancement observed was moderate.

In the following sections, the intrinsic role of the constitutive elements of synthetic glycoclusters will be discussed through selected examples from literature.

4. Rigidity of the scaffold

Architecture rigidification is generally disadvantageous for optimized lectin binding, due to the restricted spatial presentation adopted by ligands that are unable to match the specific topology and spatial distances required. However, large benefits in term of affinity may occur when the ligand geometry is appropriate. Topological presentation of the sugar epitopes can be fine-tuned when using rigid scaffolds and linkers. Interestingly, several studies reported that glycoconjugates tethering conformationnaly constrained sugars have the potential to act as selective inhibitors, able to discriminate between lectins with closely related sequences. Pieters and coworkers[82] have reported the synthesis of rigid multivalent ligands containing lactose-2-aminothiazoline units. A tetravalent derivative (Fig. 5A) displayed a strong cluster effect for galectin-3, with a 4300-fold affinity enhancement relative to lactose. Evaluation of the glycocluster affinity toward the proto type galectin-1 and -5, differing in the binding sites presentation, led to an enhancement not exceeding a factor 143. The remarkable selectivity obtained highlights the feasibility to target galectin-type receptors selectively using rigid multivalent ligands with identical epitopes. Similar conclusions were also reported by Roy and coworkers with tri- and tetravalent glycoclusters obtained from cross-coupling of 2-propynyl lactosides (Fig. 5B).[83]

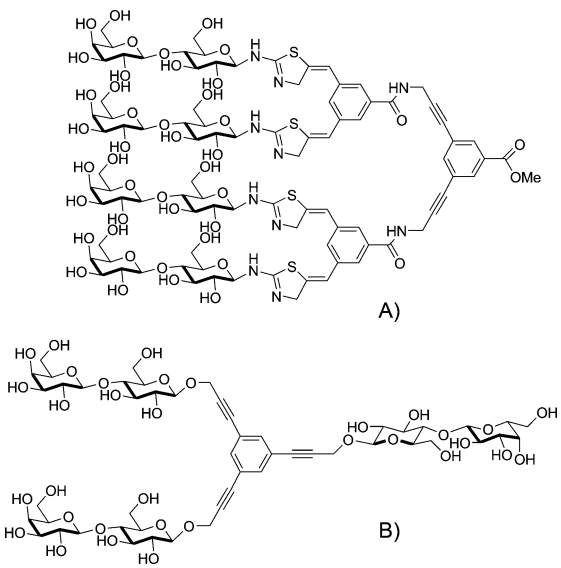


Fig. 5 Rigidified multivalent lactoside displaying selective inhibitory profiles for galectines A) Strong galectin-3 multivalent inhibitor showing a 4300-fold affinity enhancement compared to lactose (Pieters and coworkers[82]) B) Multivalent lactosides designed by tethering 2-propynyl lactosides with iodoarenes core using the Sonogashira cross-coupling reaction (Roy and coworkers[83]).

Calix[*n*]arenes are cyclic and relatively rigid platforms offering interesting opportunities to modulate shape and conformational flexibility of sugar ligands. In particular, calix[4]arenes may adopt four distinct structural conformations at room temperature identified as cone, partial cone, 1,2-alternate and 1,3-alternate. Locking theses conformations with alkyl groups grafted at the lower or upper rim provides scaffolds with identical chemical groups but displaying different ligands' topology. Ungaro and coworkers[84] designed a set of 14 calix[*n*]arenes functionalized at the upper rim with thiourea-linked galactose and lactose (Fig. 6). The biological evaluations evidenced that particular calix[4]arene conformations and shapes induce differences in inhibition profiles toward human galectins-1, -3 and -4 in cell assays. Thus, an appropriate selection of calixarenes conformations raises the possibility to target medically relevant galectins selectively.

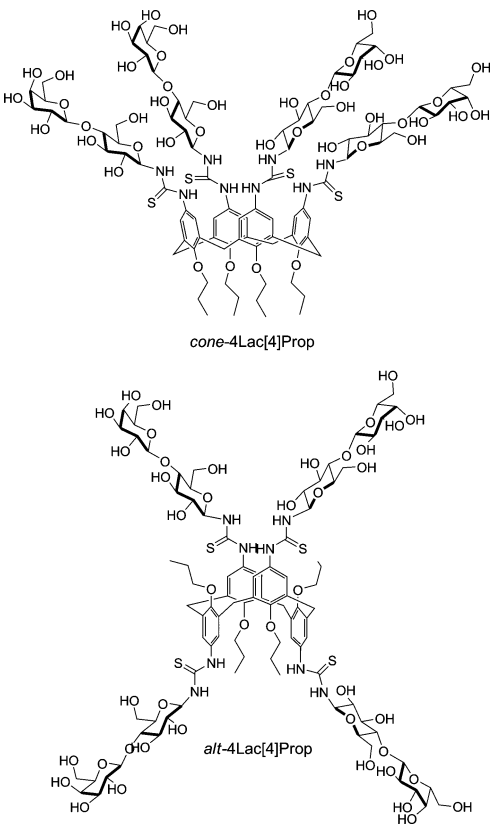


Fig. 6 Cone and alternate versions of calix[4]arenes bearing lactoside ligands and displaying different selectivity toward medically relevant galectins (Ungaro and coworkers[84]).

Specific inhibition profiles of isomeric calix[4]arenes toward bacterial lectins have also been recently reported by Vidal and coworkers.[55] Multi-galactosides were designed as potential anti-adhesive drugs of the bacterium *Pseudomonas aeruginosa*, a causative agent of lung infections. The multimers showed strong cluster effects for the galactose-binding lectin PA-IL during isothermal titration calorimetry (ITC) and Surface Plasmon Resonance (SPR) experiments. The 1,3-alternate tetravalent glycocluster was identified as the best PA-IL inhibitor to date, with a 800-fold increase of affinity compared to a monovalent galactoside reference. Despite the use of flexible linkers to tether the galactoside headgroups, the topologically distinct calixarene isomers displayed different dissociation constants by SPR.

Murphy and coworkers[64] have designed flexible and conformationnaly constrained bivalent mannosides and lactosides based on terephtalimides and *N*,*N*′-diglucosylterephtalimides scaffolds (Fig. 7A). Computational analysis revealed the lowest energy structures for the rigid subset of lactose-based glycoclusters **1**–**6**.[64c] Data obtained for the interligand relationship such as lactose orientations, interlactoside distance and terephtalimide torsion angles have shown that the glycoconjugates adopt discrete and specific interligand spacing and orientations depending both on the nature of the restricted scaffolds and of the anchoring point (Fig. 7B, 7C). Binding affinities of synthetic glycoconjugates for the plant toxin *Viscum album* agglutinin, galectin-3 and galectin-4 were evaluated by hemagglutination and cell surfaces binding assay. Inhibitory capacities toward the biological receptors were altered by the structural modifications introduced to the scaffold or by grafting the sugar headgroups to different locations of a common architecture.

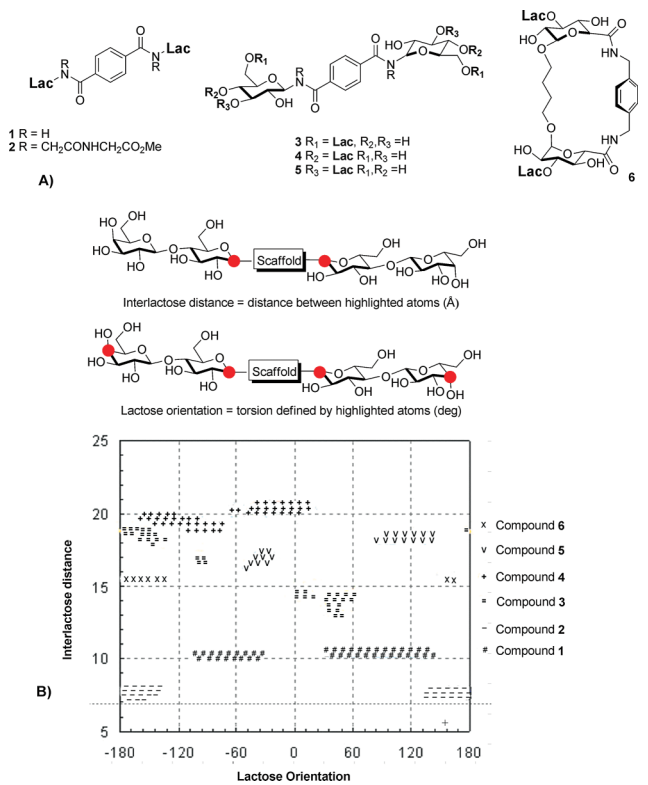


Fig. 7 A) Structure of divalent lactosides based on a terephtalimide or glycophane scaffold (Murphy and coworkers[64c]). B) Structural profiles of low-energy conformational isomers of **1**–**6**, each compound adopts a specific interlactose distance and lactose orientation depends on the nature of the scaffold.

Lectins also discriminate between diastereomeric isomers as illustrated by Sakai *et al*.[85] The authors designed a 2,2′-bipyridine-modified 2-acetamido-2-deoxy-α-D-galactopyranose (bipy-GalNAc) that self-assemble in presence of Fe(II) to form trivalent bipy-GalNAc iron complexes (Fig. 8). Trivalent ligands are obtained as a mixture of four diastereomers Δ-*fac*, Λ-*fac*, Δ-*mer*, Λ-*mer* in dynamic equillibrum, that bind strongly to *Vicia*

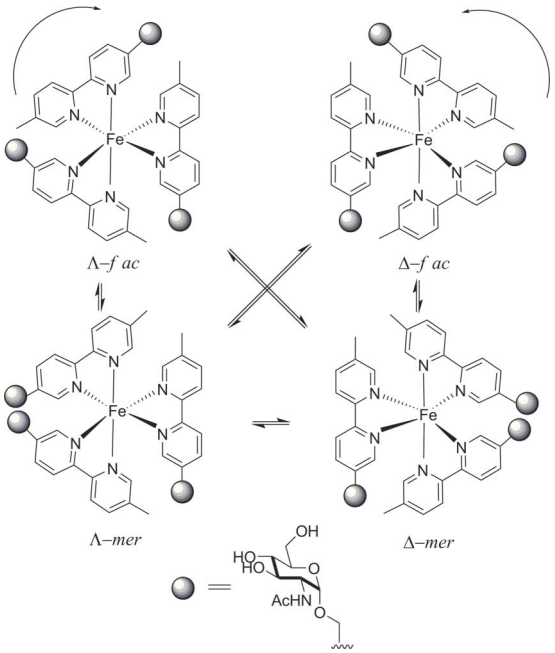


Fig. 8 Representation of the four diastereomers formed when a 2,2′-bipyridine modified GalNAc ligand is reacted with Fe(II) (Sakai *et al*.[85]) The dynamic equilibrium is shifted in presence of a B_4 lectin due to the preference of the receptor for a specific ligand's topology.

villosa B_4 lectin. Reverse-phase HPLC analysis of the complexes showed four peaks in a ratio of 29, 46, 10, 15, corresponding to the Δ-*fac*, Λ-*fac*, Δ-*mer* and Λ-*mer*, respectively. When the complexes are mixed with the B_4 lectin, the isomer ratio changed, and after 32 h, the Λ-*mer* proportion reached 85%. The dynamic shift in the isomerization ratio evidenced that GalNAc epitopes adopt a shape dictated by the topological preference of the lectin. Different relative binding constants to the B_4 lectin of 5, 1, 1, 18 were also calculated for the Δ-*fac*, Λ-*fac*, Δ-*mer* and Λ-*mer*, respectively. The *Glycine max* lectin, another GalNAc-specific lectin, displayed a complete opposite preference for the diastereomers with a relative affinity calculated to be 48, 2, 21 and 1. These last results suggest the feasibility to target lectins selectively with an appropriate selection of diasteromeric isomers.

Cross-linking and binding interactions between the plant lectins Concanavalin A (ConA) and Dioclea grandiflora DGL with a series of divalent mannosides possessing different backbone rigidities and distances between epitopes were investigated by Brewer and coworkers.[86] ITC results demonstrate that divalent ligands with inflexible skeleton **9** and **10** were less potent ligands than flexible derivatives **7** and **8** (Fig. 9). Differences in the kinetics of cross-linking and precipitation for the dimers with ConA and DGL were also evidenced. Electron microscopy revealed that differently organized cross-linked lattices are obtained, for the dimers possessing the shortest distances between the Man residues. Hence, the possibility to modulate the lectin binding affinities, kinetics of precipitation and patterns of cross-linked lattices is conceivable with simple specific templates.

An elegant methodology has been developed by Reymond and coworkers to design large libraries of multivalent ligands based on peptide dendrimer cores (see for example Fig. 10), for fucose-

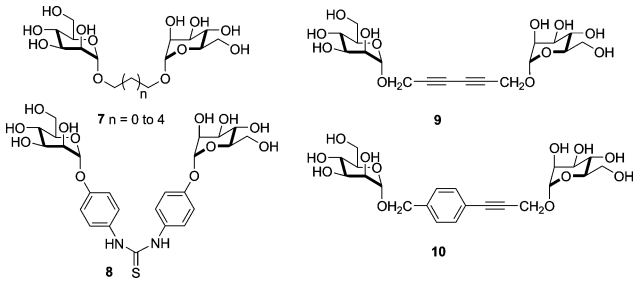
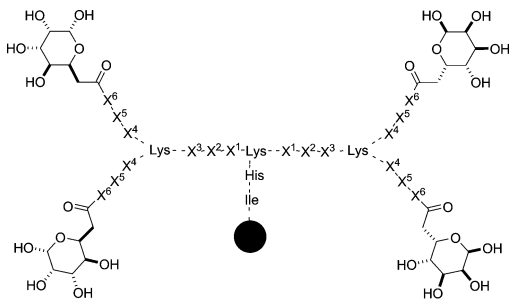


Fig. 9 Divalent mannosides with specific binding affinities for ConA and DGL (Brewer and coworkers[86]). Differently organized cross-linked lattices are formed depending on the scaffold.



X^1	X^2	X^3	X^4	X^5	X^6
Ala	Lys	Tyr	Leu	Arg	Lys
Thr	Arg	Phe	Val	Ala	Phe
Leu	Pro	Ser	Asp	Pro	Ser
Asp	Val	Glu	Tyr	Thr	Glu
Ile	Gly	His	Ile	His	Gly

Fig. 10 Combinatorial library containing 15 625 tetravalent C-fucosyl peptide dendrimers designed by SPPS (Reymond and coworkers[87d]).

specific lectins.[87] The approach to peptide dendrimer libraries is based on split-and-mix synthesis. The solid-phase peptide synthesis (SPPS) alternated α-amino-acids with branched lysines at different positions and was ended by the introduction of fucosyl building blocks. The resulting one-bead-one-compound libraries were screened with fucose-specific lectins such as biotinylated *Ulex europeaus* lectin (UEA-I) or rhodamine B labelled *P. aeruginosa lectin* LecB. Beads retaining fluorescence after washing with fucose were identified visually and separated. The peptide dendrimer sequences were determined by amino acid analysis. The positive hits were synthesized and lectin-dendrimer interactions were assessed by ELLA. Potent tetra- and octavalent dendrimers with up to 440-fold enhancement in potency over fucose were identified from a library containing 15 625 C-fucosyl dendrimers.[87d] Interestingly, ligands with identical valencies but different amino-acid sequences showed different inhibition potencies with UAE-I.[87a] This is highlighting that the affinity is dependant on the particular amino-acid sequence of the scaffold. These differences may be explained by specific contacts of the amino-acids with the lectin or/and by the particular orientation of the fucose ligands dictated by the peptide scaffold.

5. Spacer arm lengths

5.1 In the chelate binding mode

Spatial distances between binding epitopes of a synthetic multivalent ligand can be fine-tuned with an appropriate selection of linker arm length. This parameter may be critical for lectin affinity, particularly when a chelate binding mode is operating. Rigid linkers should be much more effective than flexible ones due to the severe theoretical loss in conformational entropy upon binding. Nevertheless, flexible linkers have been widely used with success to design glycoconjugates able to chelate proteins binding sites, with some of the largest affinity enhancements described so far. On the contrary, ligands tethered with rigid spacers remain uncommon due to synthetic hurdles and the necessity to exactly match their size with the distance separating the receptor binding domains.

A predictive method to optimize linker length of galabioside dimers interacting simultaneously in two CRDs located at the surface of Shiga-like toxin has been reported by Bundle and coworkers.[88] This approach involves calculation of the maximal local concentration of the pendant ligand at the second protein binding site, while the ligand tethered is already locked in the first one. Molecular dynamic simulations indicated the derivatives for which the pendant ligand is most highly populated at the additional binding site. A good correlation was observed with the experimentally measured affinity values. Interestingly, the authors have shown in a subsequent paper, that the effectiveness of the model is limited by protein dynamics.[89] The solution and crystal structure of the protein appeared to differ due to fast exchange between an axially symmetric form and a minor conformer related to that observed in the crystal. Thus, the binding of the dimer can not be interpreted in terms of a simple two-state model based on the crystal structure but was consistent with a sequential binding mode involving cooperative effects. This study is highlighting the limitation of using crystal structures for the design of multivalent ligands.

Whitesides and coworkers[90] also studied the influence of flexible linker length when a multivalent ligand is interacting with a multivalent protein. As a chelate binding mode model system, a ligand was covalently tethered to the surface of the monovalent human carbonic anhydrase (HCA) by EGs of different lengths (Fig. 11).

Comparison of the experimental dissociation constants with theoretical estimations from polymer theory suggest that the linker acts as a random coil polymer. ITC experiments also highlighted that the spacer plays an exclusive entropic role in the thermodynamics of the system, corroborating previous observations by the authors showing that EG spacers prevent non-specific interactions with proteins.[91] Interestingly, the linker displays a significant conformational mobility, even after that the ligand is bound to the active site of the protein. Effective molarities (M_{eff}), a term related to the effective concentration of the ligand in the close proximity of the binding site, were calculated from dissociation constants. Values of M_{eff} were the lowest when the EG length is too short for an effective binding, reached a maximum when the ligand bound without strains ($n = 2$), and only decreased by a small factor (~ 8 from $n = 2$ to $n = 20$ EG units) when the tether length pasts this optimal value. These results are particularly

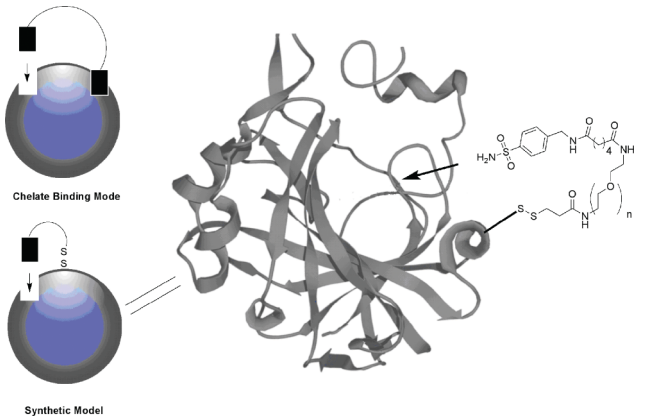


Fig. 11 Experimental model designed to evaluate effective molarities M_{eff} for the intramolecular binding of a benezenesulfonamide ligand covalently attached to a mutated HCA II by EGs of different lengths (Whitesides and coworkers[90]).

valuable for designing multivalent ligands of multimeric proteins. Indeed, the low decrease of M_{eff} with increased EG length suggests that connecting ligands with flexible spacers of greater length than the distance between binding sites of the receptor is an effective strategy.

Long spacer arm length should be selected to design multivalent ligands able to span the distance between recognition domains of a receptor, the average length of flexible linkers in solution being much shorter than in the extended conformation. This is well illustrated by pioneering work by Kramen and Karpen[92] showing that the most effective divalent ligands of a series of proteins containing four nucleotide binding sites, are tethered with long PEG spacers containing one or two thousand of monomeric units. Using PEG chains of different lengths, the authors designed potent and selective inhibitors, able to discriminate between the tested proteins with different binding site distances. An estimate of these distances was also provided, using published values of specific effective PEGs length in water,[93] and assuming that their polymer length is proportional to the square root of the number of monomers.

Other striking examples dealing with the importance of spacer arm lengths in the chelate binding mode have been reported by Fan *et al.*[31a] (Fig. 12). High affinity pentavalent ligands were designed to block a heat-labile enterotoxin (LT), member of the AB_5 family of bacterial toxins for which some of the highest multivalent enhancements have been described so far.[30,31,94] The pentavalent derivative **12** with the longest spacer arm ($n = 4$) showed an IC_{50} for the toxin that is 10^5-fold better than galactose. In comparison, compound **11** with a shorter linker ($n = 1$) was only 240 fold more potent. When the tethers of **11** are considered in their extended conformation, the biting distance between the galactosides is theorically sufficient to embrace the toxin binding sites. However, the calculated linkers' *effective* length was significantly shorter and did not match this distance. These experimental results clearly reflect that the spacer average length and not the extended conformation, should be considered for designing an efficient multivalent ligand able to chelate receptor with multiple binding sites. The authors also showed that the binding affinity for CTB_5 cholera toxin decreased, when the spacers' effective length was

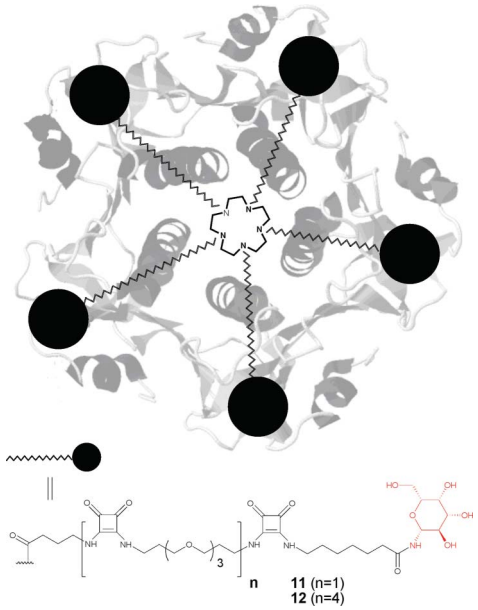


Fig. 12 Schematic representation of a synthetic pentavalent galactoside bound to the Heat-Labile enterotoxin (LT) (Fan *et al.*[31a]).

longer than the distance between the CRDs.[95] It should be noted that most of the multimeric inhibitors of the pentameric toxins are presented as chelators of the binding sites. However, Turnbull and coworkers have shown that particular aggregation mechanisms may also occur with synthetic divalent and tetravalent gangliosides and cholera or *E. coli* heat-labile toxins.[96]

5.2 Instrinsic affinities and cross-linking

The importance of the linker arm length is less intuitive when a multivalent ligand interacts at a single receptor binding site (Fig. 1A) or when an aggregative process is occuring (Fig. 1D). A careful selection of the binding assay can provide insights on particular binding events. Enzyme Linked Lectin Assay (ELLA) measures the ability of a soluble saccharide to inhibit the association between a labeled lectin and a ligand immobilized on the well. Previous reports indicate that the horseradish peroxidase (HRP) label used in ELLA is supposed to prevent aggregative process and to promote 1 : 1 ligand : lectin stoichiometries.[97,98] Thus, ELLA can be considered as a model to study intrinsic affinities devoided from aggregative process or cross-linking. We recently used this feature to study the intrinsic affinity of glycoclusters bearing one to four lactoside epitopes tethered with EGs of different length (Fig. 13A).[99] Virtually identical binding affinities were observed for the different linker length series. Such low EG tether size effects have been also observed by Roy and coworkers[100] during ELLA experiments performed with glycoclusters bearing the αFuc(1,4)GlcNAc disaccharide and the PA-IIL lectin. We performed some molecular dynamic simulations on the whole set of compounds. Results revealed that glycoconjugates adopt a pseudo "globular" tri-dimensional structure with a random distribution of lactosides (Fig. 13B). Initial structures for MD simulations were constructed where EG units adopt an elongated *anti* conformation. During MD equilibration, the measured distance between two lactosides quickly drops from 40 to less than

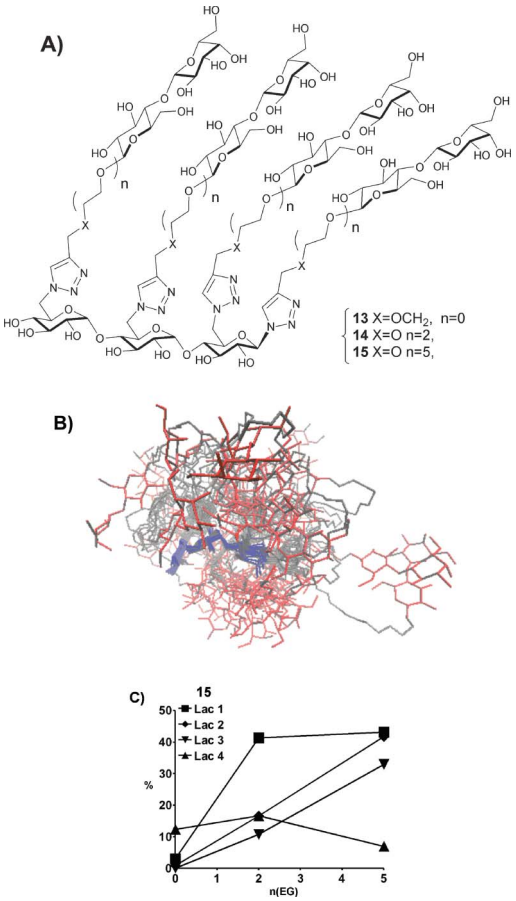


Fig. 13 A) Structure of the tetravalent click lactosides **13–15** based on a carbohydrate scaffold (Gouin *et al.*[99]). B) Superimposition of MD snapshots over the scaffold heavy atoms for the glycoclusters **15**. MD snapshots taken every 4 ns over 50 ns MD simulations. Glucose scaffolds, lactoside epitopes and organic spacers are represented by blue, red and tan colors, respectively. Hydrogen atoms are omitted for clarity. C) Percentages of the time a lactose epitope of **15** is considered "free" and solvated during 50 ns MD simulations. For each lactose epitope, the time percentage a lactose is considered free *versus* the length of the organic linker it is connected to is plotted (*n* represents the number of EG units constituting a spacer arm; $n = 0$, 2 or 5). Lactose 4 is the epitope connected to the glucose anomeric carbon of the scaffold.

15 Å. This collapse generates a discrepancy between the theoretical tether length in its extended conformations and its effective length in solution. We also evaluated the tendency of each lactoside epitope to remain "free" and surrounded by solvent molecules (Fig. 13C). For most of the epitopes considered, an increasing arm length doesn't lead to significantly higher percentages of free lactosides. Altogether, these results explain the similar affinity values observed by ELLA for the glycoconjugates series with different spacer arm lengths. It seems therefore that the spacer arm length does not significantly affect the binding affinity due to the similar spatial distribution of the lactosides.

Cross-linking abilities were also evaluated by a sandwich assay. Different inhibition profiles were observed, the synthetic glycoclusters bearing the longest spacer arm being the most potent cross-linkers. Apparently, the weak and non-specific interaction between constitutive elements of the glycocluster permit an

extension of the tether that allows the epitopes to reach out binding site of additional lectin(s). Results published by Usui and coworkers[101] on spacer *N*-linked double-headed glycosides with wheat germ agglutinin also pointed out that spacing and flexibility of linkers affect the structure and precipitation capacities of cross-linked complexes. Altogether, these results suggest that a careful selection of spacer length should be considered depending on the process we intent to interfere with.

Several adhesive organelles such as pili or fimbriae can be expressed by *E. coli* for attachment to the host cells. Lee *et al.* reported the binding affinity of mannose-terminated dendrimers and neoglycoproteins for the FimH adhesin, situated at the fibrillum tip of the bacteria.[102] The presence of an α-oriented aglycon next to the mannosyl oxygen, and of long spacer arms were both presented as important factors for an effective affinity with the adhesins. The authors suggest that the sub-nanomolar potency observed for the most potent multi-mannosides that have long Man to Man span (>20 nm), might be due to their ability to cross-link several FimH displayed on different fimbriae tips.

The asialoglycoprotein receptors (ASGP-R) that recognize terminal galactoses and internalize their ligands by endocytosis, are specifically located on the mammalian liver cell. The existence of dimers and trimers species of human ASGP-R makes it interesting receptor for multivalent *galacto*-targeted delivery of drugs and genes to liver.[103] Biessen *et al.*[104] described the synthesis of a set of tri-antennary galactosides **16–18** tethered by flexible linkers with maximal theoretical distances ranging from 4 to 20 Å (Fig. 14). Inhibition constants were shown to be highly dependent on the distance between the vicinal galactosides. Elongation of the spacer from 4 to 20 Å led to a 2000-fold increase in the binding affinity for ASGP-R.

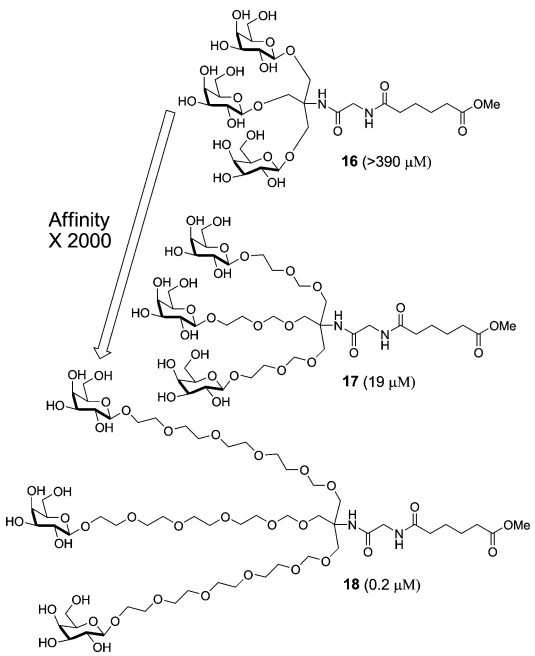


Fig. 14 Trivalent galactosides with high affinity for the hepatic asialoglycoprotein receptor (Biessen *et al.*[104]).

6. Binding epitopes

6.1 Number of epitopes

It is now well established that a higher number of epitopes doesn't necessary lead to a higher binding potency of the resulting glycoclusters. As examples, Stoddart and Roy have reported independantly the biological evaluations of mannopyranoside-containing dendrimers.[105,106] A plateau of inhibition was observed in both case for dendrimers with valency in the middle of the series.

Several efforts have been made to predict and quantify the enhancement expected from the multivalent presentation of binding epitopes. Lees and coworkers[107] described a general solution for the binding enhancement (BE) of a multivalent ligand interacting intramolecularly with a multimeric receptor, that is $BE = F[sK_a(10^{-2})]^{(n-1)}$, where n is the smaller of the number of binding sites on the receptor or valency of the ligand. The term F is a system specific statistical factor, K_a the affinity constant of the monomeric ligand with the receptor, and $s = 30/$(distance between the binding sites in Å). A modified trisaccharide ligand for a Shiga toxin was co-polymerized with acrylamide and evaluated with a cell-based assay. The inhibition constant measured for the polymer was more than 5×10^3 fold greater than the value for the monovalent trisaccharide reference. This enhancement value compared relatively well with the one predicted by the equation ($>10^4$). The theoretical method is however limited by the following assumptions, i) the linker is flexible, of optimal length and do not interact with the receptor, ii) binding sites are equivalent and no cooperative binding occurs, iii) the affinity enhancement is only due to intramolecular binding.

A different approach has been developed by Kitov and Bundle to predict the affinity enhancements in chelate binding modes.[108] The thermodynamic model presented is an adaptation of a precedent method to evaluate the increased affinity upon multivalent interactions.[19,109] The authors included a term called "avidity entropy", a combinatorial factor reflecting the probability of association and dissociation of the ligands. This term represents the statistical probability of binding and grows rapidely with ligand valency. The model suggest that extra ligand branches, that are unable to directly bind to the receptor, are nevertheless beneficial as they increase the probability of interaction. The binding affinity of an octavalent ligand for the SLT was accuratly predicted with the model ($K_{avidity}$ predicted $= 1.26 \times 10^9$ *vs.* 1.4×10^9 measured).

Cloninger and coworkers[110] studied the variation of the affinity enhancement for ConA when different percentages of mannosides and glucosides are grafted onto dendrimeric PAMAM backbones (Fig. 15). The goal of the research was to study the relationship between monovalent and multivalent associations using Whitesides' model $K_N^{poly} = (K^{mono})^{\alpha N}$. The cooperativity factor α was assumed equal to 1, meaning that the interaction with ConA

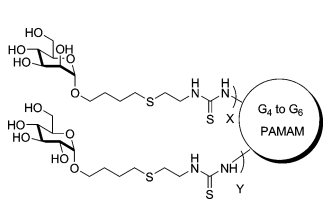


Fig. 15 Mannose/glucose functionalized dendrimers to evaluate the predictability of multivalent associations (Wolfenden and Cloninger[110d]).

was hypothesized to be non-cooperative. The number of receptor-ligand interactions N was assumed to be 2, as the globular shape of the dendrimers precludes a trivalent or tetravalent interaction with ConA. Although $N = 1$ was possible, the value was discarded by previously published assay.[111] Exchanging mannose to glucose, which is a four times weaker ligand, would cause a 4^2- or 16-fold reduction in affinity. Glucose functionalized and mannose functionalized G(4) and G(5) dendrimers displayed a difference in relative activity close to 16, respectively 14.7 and 15.5. The authors also showed that dendrimers with various mannoses to glucoses ratios exhibited the affinity changes predicted by the equation. These results were obtained for a 50% of sugar loading onto the dendrimers. Above this value, the dendrimers were less effective ligands to ConA,[112] and deviation from the 16-fold affinity predicted was observed.[113] These experimental results are of particular interest, as they clearly show that multivalent affinity can be predicted based on monovalent association constants. Mixing different ligands onto a scaffold provides a way to attenuate the binding potency in a predictable way.

This assumption may be however limited to ideal models, and that would be a misleading interpretation to consider these observations as general rules to predict the binding affinity of a neoglycoconjugate in a complex biological system. As an illustrative example, André *et al.* described the binding affinity of a set of persubstitued β-cyclodextrin glycoclusters bearing galactoside, lactoside or *N*-acetyllactosamine epitopes in lectin-mediated haemagglutination and in a solid-phase assay. Inhibitory capacity and IC_{50} values measured with plant and mammalian lectins showed very different multivalent enhancements depending on the target, with unpredictable inhibitory profiles based on the binding values of the galactose and lactose references.[114]

6.2 Density of epitopes

Modulation of the glycocluster epitopes' density has been shown to influence lectin binding significantly. Lehn and coworkers[115] reported an efficient method for the identification of the important structural feature for an efficient binding to ConA. A dynamic combinatorial carbohydrate library was built from a pool of six monosaccharide aldehydes and nine mono, di- or trivalent hydrazide cores that self-assemble through reversible acylhydrazone formation (Fig. 16A). To identify the active building blocks, a deconvolution protocol relying on the removal of single building blocks from the library was adopted. The fifteen sub-libraries obtained and the complete library were evaluated toward ConA by ELLA. An increased ConA activity for a given sub-library would indicate that the omitted synthon contributes significantly to the inhibitory potency. In contrary, a decreased activity would mean that the synthon hampered the effect of the more active compound. The aldehyde sugar and hydrazide core identified as the most important building blocks by the deconvolution process, were reacted to generate the trivalent mannoside **19** (Fig. 16B). The compound showed potent affinity for ConA with an IC_{50} of 22 μM. Authors highlighted that the binding enhancement was comparable to the natural trimannoside ligand. Later on, García-Fernández and coworkers[28] observed similar behaviour with β-cyclodextrins bearing multivalent mannoside ligands as drug delivery systems. No true cluster effect were observed for inhibition of ConA-yeast mannan when comparing mono- and divalent or

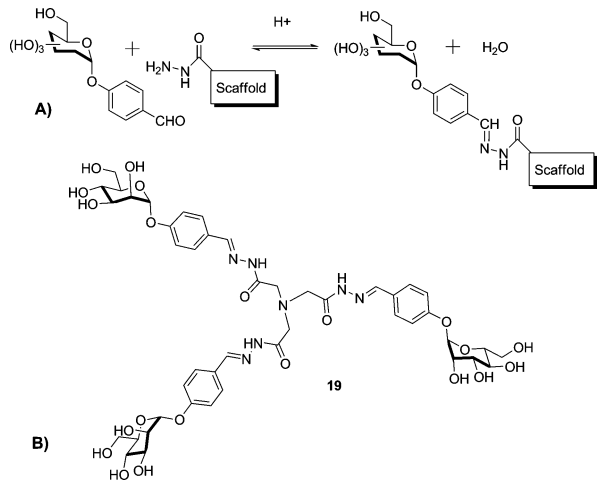


Fig. 16 A) Reversible acylhydrazone reaction used to generate a dynamic library from a pool of carbohydrate aldehyde and hydrazide scaffolds (Lehn and coworkers[115]). B) Tritopic mannoside **19** identified from the library showing an IC_{50}-value of 22 μM with the lectin ConA.

tri- and tetravalent derivatives. A significant amplification of lectin binding was, however observed for compounds containing triads of mannopyranosyl ligands (Fig. 3C). The authors concluded that the increased intrinsic affinity was mainly due to a ligand density effect.

Shiga toxins (Stx) called Vero toxin produced by strains in the gut causes diarrhea and hemorrhagic colitis in human. The toxin can cross the epithelium and pass into the circulation to cause systemic vascular damages manifested as hemolytic uremic syndromes in humans. Stx, from the AB_5 toxins group, binds to the cell surface receptor globotriaosyl ceramide through the pentameric B subunits. A pool of synthetic globotriaose-coated carbosilane dendrimers, referred to as SUPER TWIG, have been developped by Nishikawa *et al.*[116] to inhibit the Stx-cell binding. Some of the dendrimers bound to Stx *in vitro* with low dissociation constants, in the μM range, and inhibited toxin incorporation in the target cells (Fig. 17). More strikingly, when the SUPER TWIG **20** was intravenously co-administrated with a dose of the more toxic Stx2 to mice, it completely suppressed the lethal effect. The compound was also effective to protect mice from death after an oral infection with Stx producing O157 : H7.[116a] These spectacular results provide i) a unique medical perspective to eliminate Stx bacterial toxins from the body ii) a clear-cut proof of concept for the *in vivo* efficiency of anti-adhesive drugs, even when administrated after the establishment of the infection. Authors proposed a dual mechanism of action to explain the high *in vivo* activity of the SUPER TWIGs **20** and **21**. The multimers prevent efficiently the Stx-cell interaction by embracing different binding site of the toxin, and induce its active uptake and degradation by macrophages in the reticuloendothelium. The optimal structures required for the multimeric neutralizer to function in the circulation were also further identified.[116b] Among the requirements, terminal globotriaose epitopes with spacers should be tethered to the same terminal silicon atoms to be clustered in high density, conferring a dumbbell-shaped structure to the potent SUPER TWIGs (1)6 and (2)18. These particular epitope density and topology allow an efficient binding to the Stx,

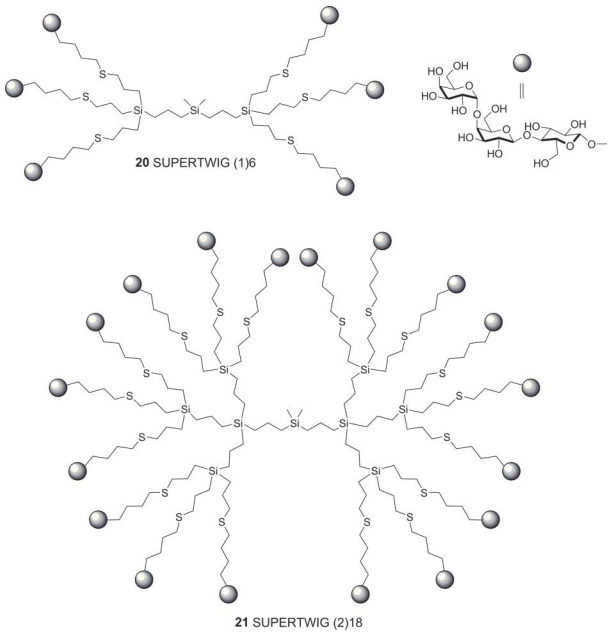


Fig. 17 SUPERTWIGs (1)6 and (2)18 with an optimized dumbbell shape structure (Nishikawa *et al.*[116b]). The compounds are efficient neutralizers of the Shiga toxin *in vivo*.

while providing an adequate exposition of the hydrophobic core for recognition of the complex by macrophages.

7. Conclusion and Perspectives

This review has described the recent efforts to evaluate how the topological aspects of synthetic multivalent glycoconjugates influence lectin binding. Multivalent interactions are highly complex binding events depending both on the nature of the multivalent ligands and their targets. Nevertheless, much progress has been accomplished during the last decade to evaluate the independent role of the constitutive elements of the multivalent glycoconjugates. It would be a naive expectation to consider that increasing the number of tethered epitopes would necessary lead to a real cluster effect. Examples cited here, highlight that several aspects of the ligand's topology have to be implemented depending on the target or the process we want to interfere with. Epitope's density and presentation are critical for a tight binding and its tailoring offers opportunity to modulate specific biological activities *in vivo*. The effective linker arm length in solution controls the epitope's biting distance and should be carefully adjusted when a multimeric lectin or toxin has to be inhibited or when receptors aggregation on a surface have to be promoted. Interestingly, glycoconjugates with similar headgroups but different scaffold's rigidity have been shown to discriminate between lectins with closely related sequences, or to modulate kinetics of precipitation and patterns of cross-linked lectin-saccharide lattices. While valuable theoretical models have been developed to predict the affinity enhancement observed during multivalent interactions, applications are currently limited by several assumptions and restricted to specific targets. Thus, research opportunities remain abundant in the chemical front, and new multivalent probes are required to build still more efficient synthetic glycoclusters rationally.

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