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Carbohydrate Supramolecular Chemistry: Beyond the Multivalent Effect

Manuel González-Cuesta,* Carmen Ortiz Mellet** and José M. García Fernández*ab

It has been amply constated that sugar ligand multivalency increases lectin-binding avidities dramatically, thereby amplifying and modulating the capacity of carbohydrates to participate in supramolecular recognition processes involving transfer of biological information. The importance of this concept, the multivalent or glycoside cluster effect, in cell biology in general and in the glycoscences of particular in is reflected in the ever growing number of papers in the field. An impressive range of glycoarchitectures have been conceived to imitate the glycan coating of cells (the glycocalix) in order to target complementary lectin receptors. However, these models rarely address the heterogeneity and the fluidity of the densely glycosylated cell membrane. They also disregard the impact that high-density nanosized arrangements could have in the interactions with the whole spectrum of carbohydrate-interacting proteins, among which glycosidases are notable representatives. For many years it was tacitly assumed that: (i) efficient recognition by lectins generally requires high densities of the putative primary ligand and (ii) the mechanisms governing binding of a carbohydrate motif by a lectin or a glycosidase are totally disparate. Notwithstanding, an increasing amount of evidence consituted in the field. An impressive phasis in the potential risks and opportunities brought about functional viruses, the infecti

Introduction

The mutual recognition of different chemical entities is a fascinating phenomenon at the core of fundamental processes in life. The co-assembly of viral RNA and protein capsids to bring about functional viruses, the infection of a host cell, the immune response or the metastasis of tumors are examples of critical events determined by the reciprocal identification of specific biomolecular partners in a highly complex dynamic environment. Exquisite matching relationships are achieved through the complementary 3D disposition of functional hotspots in the intervening species, enabling the establishment of a network of noncovalent interactions that stabilizes a transient supramolecular body. The whole sequence can be seen as a decrypting process whereby precise encoded information is transferred, leading to the activation or deactivation of specific signaling pathways. For the communication flow to occur, a minimum time of contact between the participating ligand and receptor units is necessary, which in turn requires to reach a “biologically useful” binding affinity threshold. In some cases, this is warranted by the one-to-one complementation of the parties leading to a “lock-and-key” fitting mode. A typical example is the formation of antibody-antigen complexes. In other cases, however, the interactions engaged in the pairing episode are intrinsically too weak to reach effective ligand-host dissociation constants and require an amplification contrivance. In nature this is achieved by deploying several replicates of one or the two components so that they can interrelate in a cooperative manner. The system as a whole then behaves differently from anticipations based on the individual interactions acting in isolation, resulting in a net increase in the lifetime of the bound state. The accumulated strength resulting from individual affinities performing concurrently is known as avidity, whereas the general strategy is called multivalency.1,4 The recognition of carbohydrates by protein receptors (lectins) relies upon multivalency much more intensely than processes implying any other type of biomolecules. Sets of glycan

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recognition motifs (glycotopes) and cognate lectins possessing several carbohydrate recognition domains (CRDs) have typically to play concertedly in order to afford productive associations. In this manner, individually weak affinities are enhanced by orders of magnitude, an observation termed the multivalent or glycoside cluster effect. Since its enunciation in 1995, the multivalent effect has dominated carbohydrate supramolecular chemistry, delivering not only a general principle that has led to decisive advances in our understanding of glycobiology but also a versatile tactic to interfere in carbohydrate-mediated processes for fundamental studies or biomedical applications. The advent of “click” chemistry and the implementation of “precision macromolecular synthesis” concepts, together with the developments in nanotechnology and self-assembly, have further provided a fantastic toolbox to sculpt the topology of the glycoobjects and, eventually, incorporate refined functional properties. Collectively, this vast work has contributed to settle the assumption that a given glycostructure in multiple copies onto a suitable scaffold is a safe and efficacious way to target a complementary lectin, preserving or even enhancing binding selectivity. While the success of the above notion is attested by many showcases, a number of evidences question this one-dimensional picture. For instance, the recognition of glycans by complementary lectins has been found to be deeply dependent on density and architectural issues. The biological information encoded by carbohydrates, the “glycode”, does not seem to be written in the monosaccharide sequences forming the oligosaccharide chains, but in much more complex glycan patterns that are read by lectins behaving as pattern recognition proteins. Collectively, this vast work has contributed to settle the assumption that a given glycostructure in multiple copies onto a suitable scaffold is a safe and efficacious way to target a complementary lectin, preserving or even enhancing binding selectivity.

Carbohydrate-lectin interactions in heterogeneous environments: The heteromultivalent effect

Imitating the densely glycosylated cell membrane to correctly evaluate glycan interactions and their biological consequences has been a constant motivation for research in the field of multivalency. Intuitively, the presence of a diverse ensemble of glycotopes in heteromultivalent glycosystems has the potential to enable simultaneous interactions with distant areas in the same lectin, e.g. subsites hosting different monosaccharide residues of an oligosaccharide ligand in the corresponding supramolecular complex, or with an arrangement of lectins with assorted specificities. The first vision was initially explored by Kobayashi and coworkers and systematized as the “carbohydrate module method”. Basically, it consists in deconstructing the glycan ligand whose lectin recognition properties are to be emulated into elemental motifs that are copolymerized together. Provided that the fragments in the side chain of the resulting synthetic heteroglycopolymers can act cooperatively with each other, enhanced affinities against the target lectin can be achieved (Figure 1).
The simplicity of the carbohydrate module method makes it very appealing, but the successful implementation remains challenging. It presupposes that the individual sugar moieties in the heteroglycopolymer will bind at the sites in the target lectin where they are found in the complex between the lectin and the full oligosaccharide ligand that inspired the design, giving rise to allosteric cooperativity. Yet, the effectiveness of this mechanism is sometimes questionable. Moreover, it is not always clear which modules in a natural glycan are essential for protein recognition: some are directly involved in binding and others are required for determining the right conformation. Underestimating the later can totally offset the enthalpic benefit of cooperativity due to the entropic penalty associated to linker flexibility. Recently, Zentel and coworkers and Tacke and coworkers proposed a more sophisticated approach for biomedical applications. The selectins are a family of cell adhesion molecules with an extracellular lectin domain that bind to fucosylated and sialylated glycoproteins and play a key role in the innate immune response. The common structure required for binding in several natural glycoproteins that behave as ligands of the selectins in the inflammatory cascade, but the monovalent tetrasaccharide itself has rather low affinities to all selectin members. The authors used a biocompatible poly(2-hydroxypropyl)methacrylamide (PHMA) backbone to multividentally and randomly present the fucose (Fuc), galactose (Gal) and neuraminic acid (NeuAc) substructures, bearing or not a sulfated tyramide sidechain to account for sulfation of the selectin glycoprotein ligands at tyrosine residues (Figure 2). Both heteroglycopolymers behaved as selectin binders in vitro in different cells. Additionally, the later heteroglycopolymer strongly bound to resident liver macrophages in vivo (mice) and meaningfully inhibited toxic liver injury and reduced the injury in a model of immune-mediated hepatitis.

Heteromultivalent prototypes having the crucial glycotopes prearranged in segregated domains are expected to be better suited to simultaneusly target lectins differing in their sugar selectivities. This concept has been probed by the groups of Roy and coworkers and Renaudet and coworkers with Janus (Figure 3A) and heterolayered hybrid glycodendrimer (Figure 3B) or regioselectively addressable functionalized template (RAFT) cyclopeptide scaffolded heteroglycoclusters (Figure 3C), respectively. Multiconjugates combining sugar head groups optimized for the Pseudomonas aeruginosa (P. aeruginosa) lectins LecA and LecB (namely Gal and Fuc) or LeCA and concanavalin A (ConA), a model mannose (Man)-binding lectin used throughout many fundamental studies, were synthesized and evaluated. Differently from the carbohydrate module approach, multidomain heterogeneous prototypes are intended to achieve multispecificity, which may be deliberate if the target lectins have different requirements in terms of optimal valency or architecture of their ligands. For instance, formation of the complex between the Janus heterovalent glycodendrimer in Figure 3A with LecB was much more complete than the one formed with LecA, likely due to a higher affinity of the fucoside residues towards LecB in comparison to that of the galactoside residues towards Lec A. Increasing the Gal glycotope valency or density, as in the heteromultivalents systems depicted in Figures 3B and 3C, led to increased LecB affinities. Considering that LecB is much less sensitive to multivalency than LecA, Nierengarten and coworkers proposed a more sophisticated design where ten Gal units are attached at the two rims of a pillar[5]arene platform and two Fuc residues are located as stoppers in a rotaxanated axel. The authors demonstrated that such “supermolecule” can indeed achieve high binding affinities towards the two lectins (Figure 3D).
LecA and LecB are particularly interesting targets from the biomedical point of view since both are involved in the recognition, adhesion and internalization of *P. aeruginosa* by airway epithelial cells. During the initial phase of infection, multiple types of glycans in the cell surfaces bind diverse types of lectins on the bacteria. This heterovalent network of carbohydrate-protein interactions is also critical for biofilm development and stabilization. Recently, Zhang and coworkers developed heteromultivalent nanotherapeutics inspired by this natural strengthening mechanism for the carbohydrate-lectin interactions between the bacteria and the cells. Gold nanorods (AuNRs) decorated with lactose (Lac) and Fuc homoglycopolymers specifically blocked LecA and LecB, promoted bacteria aggregation and inhibited biofilm formation. The near-infrared (NIR)-light-induced photothermal effect of AuNRs additionally endowed the system with bacteria killing properties (Figure 4A). The authors further extended this biomimetic approach to the co-assembly of polymers combining galactosylated and fucosylated blocks together with an acid-sensitive block that switched from hydrophobic to hydrophilic upon protonation. The resulting micelles with a heteroglycomultivalent shell specifically recognized *P. aeruginosa*, inhibited biofilm formation, protected native cells from bacterial infection and selectively released phototherapeutic agents included in their hydrophobic core under an acid microenvironment at the infection site (Figure 4B). Notably, the AuNRs and the co-assembled micelles coated with only the Lac, the Gal or the Fuc homopolymers showed much lower biofilm inhibition and bacteria killing properties efficacies, indicating the necessity of heteromultivalency to achieve a synergistic effect.

In some cases, it was found that targeting different lectins simultaneously with heteromultivalent glycoconjugates could be achieved with no explicit phase separation of the distinct glycotopes. In a very instructive study, Jiang and coworkers prepared glycopolymeric micelles from α-Man- and β-Gal-modified aliphatic polysaccharides, individually as well as in admixture, or from a mixed α-Man/β-Gal-modified heteroglycopolymer analog. The purpose of the work was to investigate the interactions with the macrophage mannose receptor (MMR; CD206) and the macrophage galactose-binding lectin (MGL; CD301), both of which mediate clathrin-dependent endocytosis. Unexpectedly, the results showed that the nanoparticles built from the α-Man/β-Gal heteroglycopolymer were internalized by RAW 264.7 macrophages much more efficiently than not only the nanoparticles containing a single kind of monosaccharide, but also than the nanoparticles combining the α-Man- and β-Gal glycotopes in separate polymers, even though the relative α-Man/β-Gal ratio was identical (Figure 5). The same trend was
observed for the affinity towards the plant lectins ConA (α-Man specific) and peanut agglutinin (PNA, β-Gal specific) by isothermal titration microcalorimetry (ITC). The authors speculated that the unlike polymer chains tend to aggregate separately, forming individual domains at the surface of the nanoparticles that make it less favorable the establishment of concurrent interactions with MMR and MGL receptors at the cell membrane.

The carbohydrate module method and the multidomain approaches claim that the observed lectin binding capability enhancements in heteromultivalent systems arise from the synchronized action of two or more discrete supramolecular events that can be either confined in a reduced binding site or expand on a larger 3D contacting area. In 2005, García Fernández, Ortiz Mellet and coworkers reported that high-density heteroclusters alternating α-Man and β-Glc residues, built on a β-cyclodextrin (BCD) platform, exhibited ConA binding affinity enhancements that could not be rationalized on such grounds (Figure 6). In their initial work, horseradish peroxidase-labelled ConA (HRP-ConA) was used for enzyme-linked lectin assay (ELLA) and ITC determinations. Although at the neutral pH of the experiments ConA is a homotetramer, the presence of the high molecular weight HRP label prevents re-binding and sliding mechanisms to the multivalent effect (Figure 6). The overall result can be interpreted as the enhancement of the affinity of a lectin towards a primary glycoligand by the presence of a low affinity secondary ligand or, alternatively, as the activation of the recognition of a secondary ligand enabled by the presence of a low proportion of a primary ligand. The term “heteromultivalent or heterocluster effect” was coined to refer to this new facet of multivalency.

Figure 5. Schematic representation of the interactions of the macrophage mannose receptor (MMR; CD206) and the macrophage galactose-binding lectin (MGL; CD301) with nanoparticles self-assembled from α-Man (M) and β-Gal (G) glycopolymers or from heteroglycopolymers combining α-Man/β-Gal glycoconjugates and the consequences in the cell uptake rate as reported by Jiang and coworkers.56

Figure 6. Schematic representation of the re-binding and sliding mechanisms in the interaction of heteromultivalent α-Man/β-Glc BCD-centered heteroglycopolymers with the α-Man-specific lectin ConA as discussed by Garcia Fernandez and coworkers.85 Upon initial binding of the putative Man glycoligand, the secondary Glc residues can transiently bind to the carbohydrate recognition domains in the lectin and entropically favor both processes, enhancing the lifetime of the bound state.

The heteromultivalent effect as above expressed implies synergistic phenomena influencing not only glycoligand-lectin binding affinity but also selectivity, and shows in heteroglycoclusters merging cognate and non-cognate glycotopes without phase separation. Its occurrence has been further corroborated in several laboratories with model “shuffled” heterovalent systems built on a variety of scaffolds. Thus, García Fernández and Ortiz Mellet made full use of the opportunities offered by BCD for selective functionalization to generate heteroglycocluster diversity,88 Hartmann,90 Kikkeri93 and Chen94 synthesized heteroglycosylated sequence-controlled oligopeptides, oligoamides and polymers, respectively, and Liu and Deng95 engineered heteroglyco-gold nanoparticles. Importantly, the heteromultivalent effect was found to be strongly dependent on the total ligand density. As a general rule, it is not apparent in the case of low valency heteroglycoconjugates,87,96-101 but manifests in heavily glycosylated architectures.

Interestingly, lectins that exhibit similar affinities for the same putative carbohydrate motif can significantly differ in their response to heteromultivalency when this motif is displayed conjointly with additional weakly binding or nonbinding glycan. As a corollary, heterogeneous glycan patterns have the potential to enhance lectin discrimination capabilities by virtue of the heteromultivalent effect in a more finely tuned
manner than homogeneously glycosylated coatings do. This concept has been implemented by Gibson and coworkers\textsuperscript{102,103} and Kikkeri and coworkers\textsuperscript{104} for the development of analytical devices based on microarrays or gold nanoparticle glycotectonics. For instance, the Man-binding lectins ConA, human Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (CD-SIGN), mouse Specific Intracellular Adhesion molecule-3 Grabbing Non-Integrin homolog-related 3 C-type lectins (SIGNR3) and Macrophage inducible Ca\textsuperscript{2+}-dependent lectin receptor (Mincle) and the Gal-binding lectins PNA, Galanthus nivalis lectin (GNL), mouse Macrophage Galactose-type Lectin-1 (MGL1) and human galectins 1 (Gal-1) and 3 (Gal-3) showed fully different pattern recognitions when profiled against a battery of microarrays printed from homo and heterotrivalent dendrons combining Man, α(1–2)-mannonbiose, α(1–2)-mannotriose and Gal, as depicted in Figure 7.\textsuperscript{104}

The relevance of the heterocluster effect in cell biology was presumed since its formulation fifteen years ago upon model glycolcluster-lectin recognition studies.\textsuperscript{77,87} However, direct experimental evidence and theoretical support has been provided only very recently. The contribution by Wu and coworkers has been fundamental in this line. In their seminal work,\textsuperscript{111,112} the authors spotlighted unexplained data by Yanagisawa and coworkers\textsuperscript{113} signalling that binding of cholera toxin B subunit (CTB) homopentamers to mouse embryonic neural precursor cells does not correlate with GM1 ganglioside (Gal-β-(1–3)GalNAc-β-(1–4)[Neu5Ac-α-(2–3)]Gal-β-(1–4)-Glc-Ceramide) expression, even though GM1 is well known to be the primary ligand of the toxin. They also noted reports by Klassen and coworkers\textsuperscript{114,115} postulating cooperative effects in the binding of CTB to GM1. Most strikingly, the authors demonstrated that the GM2 ganglioside (GalNAc-β-(1–4)[Neu5Ac-α-(2–3)]Gal-β-(1–4)-Glc-Ceramide), which is a very weak ligand of the toxin, can contribute to CTB binding if present in a glycolipid mixture with a high affinity binder such as GM1 (Figure 9).\textsuperscript{112} The experimental observations cannot be explained on the basis of allosteric regulation, but they are undoubtedly dynamic in nature and associated to the simultaneous presence of the high and low affinity ligands over a certain expression level in a relatively large peripheral area. The fact that going from bulk to coating is a necessary requisite led the authors hypothesize that the heterocluster effect is mediated by a simple mechanism: reduction of dimensionality (RD). The basis of RD is that once the lectin has attached to a strong receptor in a fluid membrane, subsequent binding events are confined to a 2D surface. Surface binding rates can then be raised up to 10\textsuperscript{14} as compared with bulk binding rates, so that even a weak ligand, such as GM2 for CTB, can now participate in next binding events.

Tanaka and coworkers further demonstrated that glycoheterogeneity affects the fate of glycoated nanoconstructs in vivo by promoting differential interactions with protein receptor partners expressed in specific cells, tissues or organs.\textsuperscript{105–110} Thus, the arrangement of glycans relative to one another in a diverse series of structurally well-defined heteroglycoalbumins critically influenced pathway-selective excretion (urinary or gall bladder) or cell-specific targeting, thereby impacting biodistribution or tumor adhesion (Figure 8). Their results open new avenues for the development of glycan-based imaging or therapeutic tracers.

The ensemble of Man- and Gal binding lectins framed in green and yellow, respectively, were used to provide a proof of concept (see the text for acronym meaning).
Enzymatic substrate processing is generally considered to be preceded by a typical lock-and-key supramolecular recognition event that warrants enzyme specificity. In the case of a glycosidase (glycosyl hydrolase) the substrate is a glycoside that must reach the catalytic receptacle and fit the configurational and conformational requirements imposed by the architecture of the active site. In most cases, a rather strict complementarity between the glycone substitution pattern and the amino acids at the glycone hosting spot (so-called the -1 site) is achieved through an intricate network of hydrogen bonding, stacking and hydrophobic contacts. This matching relationship is mandatory for the glycoside to adopt the right conformation in the corresponding enzyme-substrate complex and the subsequent activation of the catalytic machinery.

Contributions to binding from the glycone moiety at the glycone (+1) binding site are commonly less vital, although the glycone nature can significantly affect the catalytic reaction rate or even impede enzymatic hydrolysis by steric and/or conformational biases, which can also be exploited in the design of enzyme regulators.127-129 The ambition of reproducing the biunivocal enzyme-substrate relationship has monopolized the search of glycosidase inhibitors for biological studies or biomedical applications. Not surprisingly, the iconic specimens are monomeric glycomimetics with hydroxylation profiles of structural complementarity with the glycone moiety of the natural substrate, among which the nitrogen-in-the-ring monosaccharide analogues of the iminosugar category are by far the most popular.130 Appropriately selected iminosugars, either from natural origin or chemically synthesized, enable altering the cellular glycosylation profile, interfering in the metabolism of glycoconjugates and carbohydrates, modifying the carbohydrate-dependent properties of glycoproteins or hindering the carbohydrate-mediated interaction of host cells with infective agents, among others.131 Defying the prevalent philosophy, Gouin and coworkers advanced in 2009 the visionary idea that glycosidases might be responsive to multivalent arrangements of inhibitory motifs (inhibitors) in the same manner as lectins having a single CRD available are sensitive to multivalent displays of carbohydrate ligands, providing that the kinetic and thermodynamic parameters of the individual binding phenomena are apt.132 The merit of their work is double: primarily, a potential analogy between carbohydrate/lectin and glycomimetic/glycosidase supramolecular binding mechanisms is invoked for the first time; secondly, by testing a trivalent conjugate of the iminosugar 1-deoxynojirimycin (DNJ), a piperidine analogue of D-glucopyranose, towards a panel of glycosyl hydrolases, they determined a 2.6-fold enhancement in the inhibitory potency towards Jack bean a-mannosidase (JbMan) in DNJ molar basis (Figure 10 A).

Although significant, the above multivalent enzyme inhibition (MEI) effect is modest and Gouin’s work would had probably passed overlooked if one year later Compain, Ortiz Mellet, and Nierengarten had not published that the inhibitory potency of DNJ towards JbMan was boosted up to 179-fold in DNJ molar basis when the inhibitope was exposed in 12 copies on a C60

**Multivalent glycosidase inhibition**

Through RD, heteromultivalency can significantly alter lectin binding properties, including avidities, selectivities and kinetics, offering a new strategy to design high-affinity carriers for targeted drug delivery.127-129 As a proof of concept, Wu and coworkers demonstrated that the retention of liposomes by the pathogenic bacteria Pseudomonas aeruginosa was significantly enhanced when the strong LeCl binder globotriaosylceramide (Gb3; Galα-(1-4)-Galβ-(1-4)-GlcCeramide) was combined with lactosyl-ceramide or Galβ-ceramide, which are much weaker LeCl ligands, as compared with liposomes with a homogenous display of Gb3.120 Heteromultivalent cooperative phenomena under RD also provide a rational for the fact that human intestinal epithelial cells or murine lung cells are efficiently infected by the bacteria in spite of expressing Gb3 at very low levels.121,122 Kinetic Monte Carlo (kMC) simulations supported the underpinning role of RD in the heterocluster effect.123 The main conclusions of the kMC computational study are in full agreement with the experimental observations, namely: (a) the density of the low affinity ligand is a critical parameter for the activation of the heterocluster effect and a threshold density is needed to enable its contribution to lectin binding, (b) a tiny amount of high affinity ligand is sufficient to accelerate lectin binding to low affinity ligands and (c) heteromultivalent binding phenomena can modulate lectin binding kinetics and lectin bound states via ligand exchange processes on the surface. Note that ligand exchange under RD is equivalent to the entropy-driven mechanism hypothesized for high-density nanosized heteroglycoclusters.77,87 In conjunction with results by other laboratories on biologically relevant lectins binding to heteromultivalent systems,124,126 the ensemble of evidences establishes that heteromultivalency plays a key role in lectin-glycan recognition and that cells probably utilize this mechanism to regulate the downstream of lectin functions.

**Figure 9.** Schematic representation of the reduction of dimensionality (RD) concept in the binding of cholera toxin B subunit (CTB) homopentamers to GM1 (high-affinity ligand) and GM2 (low-affinity ligand) ligands in the cell membrane as reported by Wu and coworkers.121,122 φ1 and φ2 are the binding rates of GM1 and GM2 to CTB in the bulk and in the surface, respectively. After a lectin has attached to a high-affinity ligand, a low-affinity ligand encounters the bound lectin, completing the heteromultivalent binding (φ2 > φ1). In a next step, the lectin can be stabilized by two low-affinity ligands (not shown) and the free high-affinity ligand can then accept another lectin from the solution phase.
fullerene scaffold (Figure 10 B). This was a counterintuitive aftermath as a promising tool for the design of potent glycosidase inhibitors and encouraged to visualize MEI as a variant of the glycoside cluster effect. MEI soon became a trending topic in the glycosciences that has been discussed in several reviews. \(^{49,134-139}\) A consistent body of work from the four mentioned laboratories as well as by the groups of Cardona, Moreno-Vargas, Blériot and Li, often in multicolaborative networks, confirmed the reactivity of JbMan and other glycosidases, such as α- and β-glucosidase, amyloglucosidase or the hexosaminidases, to multivalent displays of iminosugars. \(^{140-162}\) In addition to polyhydroxylated pyrimidine-type iminosugars like DNJ, iminosugars with other azaheterocyclic cores, e.g. pyrrolizidine, \(^{145,147}\) pyrrolidine\(^{157,158,160}\) or azepane\(^{156}\) derivatives, also proved competent to elicit MEI when conjugated to multi-armed platforms. A strong dependency of the inhibition potency enhancement elicited by multivalency from the total valency and architectural parameters was documented. Yet, only in the case of JbMan a parallelism with the behavior generally observed in carbohydrate-protein interactions could be supported throughout the different studies.

\[
\begin{align*}
K_i &= 270 \mu M \\
K_i &= 35 \mu M \\
K_i &= 0.15 \mu M \\
K_i &= 0.001 \mu M
\end{align*}
\]

\[\text{DNJ} / \text{n} = 2.6 \]

\[\text{JbMan} / \text{n} = 179 \]

\[\text{C} / \text{n} = 2147 \]

JbMan and other α-mannosidases of the glycosyl hydrolase (GH) family GH38 exhibit a rather open and accessible active site and often are multimeric in their functional form, a scenario that is reminiscent of that encountered in lectins. Thus, JbMan itself is a dimer of heterodimers, each of the heterodimers possessing a catalytic receptacle. It seemed therefore logical to assume that it can share with lectins the same mechanisms leading to high avidities when faced to clustered ligand partners, that is, the sliding and rebinding processes associated to high local epitope density and the crosslinking and chelation phenomena characteristic of multipoint interactions. \(^{43}\) Indeed, experimental evidences for co-aggregation events involving multivalent iminosugar constructs and JbMan have been obtained by atom force microscopy (AFM), transmission electron microscopy (TEM), mass spectrometry and analytical ultracentrifugation measurements. \(^{143,152,157}\) Recently, Compain and coworkers further obtained the first high-resolution crystal structures of apo JbMan and of its complex with a 36-valent DNJ-cyclopeptoid conjugate that displayed the largest inhibition enhancement observed for this enzyme so far (Figure 10 C and D). \(^{169}\) The data clearly demonstrated the interplay of bridging and chelation processes in the formation of a 2:1 JbMan-multivalent inhibitor sandwich-type complex, with all four glycone sites occupied by DNJ subunits. Differently from JbMan, other glycosidases reported to experience MEI are monomeric and/or have buried catalytic sites, which is hardly compatible with the carbohydrate-lectin affinity enhancement-like mechanisms sustained for the former. Notably, in some cases multivalency switched on, rather than enhanced, the inhibitory capacity of a glycomimetic towards a glycosidase while simultaneously up- or down-regulating the inhibition activity against others. To reconcile the experimental observations, García Fernández, Nierengarten, Ortiz Mellet and coworkers launched the hypothesis that multimerization of a glycomimetic onto a nanometric scaffold elicits binding modes to the glycosidases that can be radically different from the binding mode of the monomer. \(^{164}\) The authors capitalized on the unique stereoelectronic and chemical properties of the so-called sp²-iminosugar glycomimetics, \(^{165}\) in which the underlying amine group of iminosugars is replaced into a trigonal planar pseudoaamido-type nitrogen (N-carbonyl, N-thiocarbonyl, N-imino group), with substantial sp²-hybridation nature (e.g., as in carbamate, \(^{166}\) thiocarbamate, \(^{167,169}\) urea, \(^{170,172}\) thiourea, \(^{173,180}\) isourea, \(^{180-187}\) isothiourea, \(^{187-195}\) guanidine, \(^{196,197}\) sulfamide or thiohydantoin) \(^{199}\) functionalities), to access stable O-, S-, N- and C-pseudoglycosides. \(^{200-211}\) Through click-type multiconjugation strategies, they then succeeded at preparing homogeneous multivalent glycoside analogues onto a fullerene C60 scaffold that exhibited dual recognition capabilities towards lectins and glycosidases. \(^{164}\) A competitive lectin-glycosidase binding assay was next implemented that enabled mapping the enzyme regions interacting with the high-valuation sp²-iminosugar conjugates. Briefly, it consists at determining the effects of reference inhibitors of the enzyme known to bind at either the glycone, the glycone and aglycone or surface binding sites in the steady-state partition of the multivalent inhibitor between the lectin, labelled with horseradish peroxidase (HRP; reporter enzyme), and the
glycosidase. If the reference inhibitor and the multivalent ligand under study compete for the same site in the glycosidase, the capability of the enzyme to sequester the ligand will lessen in the presence of the former (Figure 11). By using this technique, JbMan/multivalent enzyme inhibitor complex formation was found to involve substantial implication of the glycone site, which is in agreement with the reported crystal structure. In sharp contrast, in the case of the α-glycosidase from *Saccharomices cerevisiae* (yeast maltase; GH13) the multivalent derivatives bind at low affinity non-glycone binding sites of the enzyme, leading to inhibition by a "recognition and blockage" mechanism. This operational model likely applies also to the isomaltase (*Saccharomices cerevisiae*), β-glycosidase (bovine liver), and α-galactosidase (green coffee beans) enzymes, all of them belonging to glycosyl hydrolase families known to possess relatively deep catalytic sites (GH13, GH1, and GH27, respectively).

![Figure 11. Schematic representation of the lectin-glycosidase competitive binding assay developed by Ortiz Mellet, García Fernández and coworkers to map the binding mode involved in multivalent enzyme inhibition (MEI). The assay construction further implies a non-labelled lectin fixed to the surface of microplate wells, thereby enabling quantification of the effect of the reference glycosidase inhibitors in the lectin crosslinking capabilities of the probed compound. The data can be directly correlated with the displacement of the equilibrium between the lectin-multivalent inhibitor complex and the glycosidase-multivalent inhibitor complex. Note that the choice of the reference inhibitors depends on the glycosidase under study. The structures depicted here correspond to examples of α-glycosidase inhibitors.](image)

The monovalent/multivalent shift in preferred binding mode supported by the above data provides an immediate explanation for the multivalency-dependent "on-off" switching effect observed for some inhibitory–glycosidase pairs. It may happen, for instance, that a monovalent inhibito does not match the glycone site but is able to bind at aglycone and/or surface sites, then benefitting from multivalency when exposed in multiplex manner and eventually impairing the access of the substrate to the catalytic locus. Alternatively, it is conceivable that upon binding at secondary surface binding sites the multivalent ligand could stabilize enzyme conformations facilitating, instead of hampering, substrate processing, in the same manner as some polysaccharides act as enhancers of the amylases. This reasoning provides a reliable explanation to the apparently contradictory observation by Gouin and coworkers on dextran-based DNJ and 1-deoxymannojirimycin (DMJ) polymers, with valencies ranging from 20 to 900, behaving as activators of several glycosidases (two galactosidases, a fucosidase and a bacterial mannoside phosphorlylase).

As already demonstrated for classical monovalent inhibitors, MEI offers the possibility of developing glycosidase ligands that stabilize the proper folding of disease-causative misfolded mutant forms of the enzyme, a therapeutic paradigm termed pharmacological chaperone therapy. Compain and coworkers also reported a beneficial effect of multivalent DNJ derivatives in cystic fibrosis by rescuing the misfolded mutant cystic fibrosis transmembrane conductance regulator (CFTR) protein from endoplasmic reticulum-associated degradation (ERAD) in patient cells. The results are compatible with a calnexin-dependent mechanism of action, but not with the inhibition of the ER α-glycosidases I and II as it is the case for monovalent DNJ derivatives.

Nguyen and coworkers used computational studies to implement a non-imosugars-based strategy for MEI-mediated selective inhibition of heparanase, a β-endoglucuronidase whose main function is cleaving the internal β-(1–4) glycosidic bond between glucuronic acid (GlcA) and N-sulfated glucosamine (GlcNS) along heparan sulfate (HS) chains in proteoglycans in the extracellular matrix. The authors first identified the disulfated (at the N¹- and 6’-O-positions) disaccharide GlcNS(6S)α-1-(1-4)GlcA as the preferred unit for supramolecular binding at the -2 and -1 glycone binding sites. They next designed glycopolymers exposing this component for maximal heparanase inhibition and minimal antioxidative activity, from which a 12-valent representative was determined to be the most potent heparanase inhibitor with a picomolar inhibitory concentration (meaning an over 1000-fold enhancement as compared with the monovalent control) and tight binding characteristics. This is notable, because heparanase is a monomorphic enzyme with a hindered active site. While the molecular basis for MEI are not discussed, it is interesting to speculate that the dissociation rate of the disaccharide motif, which is indeed the same present in the reaction product of enzymatic hydrolysis of HS, must be fast enough to allow rebinding processes to operate, as far as the spacers and ligand densities are adjusted to prevent steric clashes. The main motivation for this research is that HS degradation by heparanase in the extracellular matrix has been correlated with tumor angiogenesis and metastasis. Rewardingly, the authors found that the optimal glycopolymers showed potent antimetastatic effect against 4T1 mammary carcinoma cells and inhibited experimental metastasis into the lungs in vivo (Figure 12). MEI has also...
been invoked by Nishimura and coworkers to account for the efficiency of nanosomes exposing β-linked N-acetylgalactosamine (GlcNAc) suicide substrates at inhibiting the lysosomal β-hexosaminidases and triggering apoptosis in cancer cells as well as in a mouse model, although the underlying molecular mechanism remains unclear.\(^{224}\)

Multivalent enzyme inhibition for a given inhibitory motif/ enzyme pair can be significantly altered by the presence of a second partner, leading to positive or negative synergies, what represents an extension of the heterocluster effect.

The authors found that α-O-glucosides and also α-O-mannosides, when conjugated on ND particles, were not only resistant towards the hydrolytic action of the corresponding matching glycosidases, but acquired the ability to inhibit them. Moreover, the glycoated NDs further became inhibitors of mismatching enzymes for which they do not serve as substrates even when in their monovalent form. A step ahead, it was established that glycosidase inhibition was sensitive to heterogeneous arrangements of the Glc and Man motifs in the same manner as binding affinity to ConA lectin was. For instance, homoglucosylated-NDs (Glc-NDs) and homomannosylated-NDs (Man-NDs) inhibited S. cerevisiae α-glucosidase with inhibition constant (Ki) values of 22 and 9.4 µM, respectively, whereas Glc/Man-NDs (Glc:Man 1:1) with identical total saccharide loadings were about 5-fold more potent inhibitors of this enzyme (Ki 1.9 µM). Conversely, inhibition of E. coli β-galactosidase by Glc-NDs (K_i 1.9 µM) and Man-NDs (K_i 13.2 µM) was thwarted when both glycotopes were exposed together in Glc/Man-NDs (K_i 268 µM). In other words, the supramolecular events underlining multivalent enzyme inhibition for a given inhibitory motif/ enzyme pair can be significantly altered by the presence of a second partner, leading to positive or negative synergies, what represents an extension of the heterocluster effect.

Additional evidence for enzyme inhibition by multivalent glycosystems has been provided using glycofullerenes\(^{226}\) and glycocyclodextrin conjugates.\(^{227,228}\) Integrated mechanistic studies exploiting ELLA and lectin/glycosidase competitive assays were conducted for homogeneous constructs as well as for mixed glycoside/ glycomimetic (sp^2-iminosugar) heterovalent displays.\(^{226,227}\) Collectively, the ensemble of results substantiates the vision that multivalent presentations of a glycotope or an inhibito can promote binding modes to glycosidases that share significant analogies to those governing carbohydrate-lectin supramolecular interactions. Multivalent displays can thus simultaneously act on lectins and glycosidases in a multimodal manner. Moreover, a given glycotope or inhibito moiety may elicit different responses depending on the presence or absence of a second glyco(mimetic) motif, even if a priory irrelevant towards the lectin /glycosidase target, supporting a unified framework for the glycoside cluster effect, the heteromultivalent effect and multivalent enzyme inhibition: the generalized multivalent effect.\(^{44}\) By changing total and partial valencies and adjusting the overall topology of the (hetero)multivalent construct, “on” or “off” statuses for a range of lectins and glycosidases can be triggered, markedly altering the selectivity profile encountered for monovalent derivatives.

The shift in the perception of multivalency, from a natural and safe strategy to achieve useful responses in carbohydrate-mediated supramolecular processes to a multichannel switcher with the potential to act on a range of receptor/enzyme recognition events, depicts a much more complex scenario than classically assumed. On the one hand, the generalized multivalent effect calls for a careful evaluation of the potential risks derived from multivalency-associated “biological messiness”, expanding from carbohydrate receptors to glycoprocessing enzymes. On the other hand, the new evidences also inform the possibility of taking advantage of multivalency to finely shaping the supramolecular...
properties of carbohydrates in an intrinsically multifactorial biological context. It is conceivable, for instance to purposely tailoring glycoligands to specifically interact with biomedically relevant lectin/glycosidase subsets, opening new channels in multitargeted drug design. García Fernández, Renaudet and Ortiz Mellet have recently implemented this notion in the development of mannosyl-coated glycoclusters with the ability to simultaneously and distinctly target the macrophage mannose receptor and lysosomal storage disorder-associated lysosomal glycosidases. After screening a series of structurally diverse candidates, the authors encountered that the MMR avidity, as determined by a modified ELLA protocol, was essentially dependent on the total Man valency. On the contrary, glycosidase selectivity was strongly reliant on the overall glycoarchitecture topology: a heptavalent β-cyclodextrin conjugate (Figure 14, upper pathway) behaved as a selective inhibitor of β-glucocerebrosidase (IC50 0.1 μM) whereas an hexadecavalent RAFT cyclopeptide derivative (Figure 14, lower pathway) turned to be a highly selective inhibitor of lysosomal α-mannosidase. Since binding to the MMR inherently elicits macropage uptake, these compounds epitomize the first examples of intrinsically site-specific, self-deliverable glycosidase regulators.

Conclusions and future outlooks

Pioneering work in 1999 by Kahne and coworkers already described that lectin specificity could be altered in heterovalent as compared with homovalent glycosurfaces. Ten years later, Penadés and coworkers reported that lactoside substituents became resistant to the action of β-galactosidase when multivalently exposed at the periphery of micelles or gold nanoparticles while keeping the ability to bind the galactose-specific agglutinin from V. album. The authors speculated that β-galactosidase recognition probably occurred, but that binding of a first enzyme molecule to the multivalent conjugate reduces the accessibility of other lactose residues to new enzyme molecules. These early manifestations of the heteromultivalent effect and multivalent enzyme inhibition have been largely comforted by an increasing number of publications highlighting the relevance of those phenomena, not only in model systems but also in the biological context. The last years have witnessed crucial advances in our understanding of multivalency-induced carbohydrate recognition promiscuity under the new generalized multivalent effect paradigm. Yet, much work is still needed to unveil the precise mechanisms at play; this is critical both to prevent potential risks of and to program specific activities.

The increasing awareness of the multilateral character of (hetero)multivalency, with additional reports expanding the range of MEI responsive glycosidases and extending the generalized multivalent effect to the glycosyltransferase enzyme category and anti-carbohydrate antigen antibodies, will likely fuel research in carbohydrate supramolecular chemistry with a new perspective. The current embodiments exploiting the heteromultivalent effect to reach optimal cell targeting in vivo and multivalent inhibitors to regulate the activity of disease-associated enzymes, e.g. in the context of LSDs, or cancer, pave the way for future developments. The possibility of combining enhanced glycoreceptor capabilities and specific glycosidase inhibitory properties in designing multitargeted glycodrug prototypes is particularly appealing in this sense. Glycoclusters targeting the MMR and LSD-causative glycosidase pairs are representative showcases, but many other therapies could benefit from this concept. Thus, GlcNAc-coated nanoparticles having a hard metal core such as quantum dots or gold nanoparticles and a soft shell, namely nanos (NSs), were recently found to be efficiently internalized by human hepatocarcinoma HepG2 cells in vitro, probably through lectin-mediated endocytosis. NSs bearing a multivalent presentation of a GlcNAc-based suicide inhibitor additionally inhibited very competently lysosomal β-hexosaminidase, which is a promising target that induces a lysosomal membrane permeabilization (LMP)-mediated cell death pathway. This can be seen as an example of self-deliverable anticancer therapeutic nanomedicine benefiting from multivalent enzyme inhibition, a notion that deserves to be explored.

Advancing the fundamental knowledge in the generalized multivalent effect will also shed light on other supramolecular events essential for cell life, e.g. the role of glycoheterogeneity in the packing processes involving polysaccharides at the glyocalix and the extracellular matrix. It is pertinent noting that many of the expressions of the generalized multivalent effect have a dimensional character, that is, they imply nanometric entities and relay on surface contacts, a biomimetic mechanism. It is then conceivable that other non-carbohydrate or glycomimetic motifs, or even the own nanoparticle shape and surface nature, could actively influence the supramolecular chemistry of (hetero)multivalent
glycoligands towards both lectins and enzymes. Multifunctional molecular nanoparticles designed to simultaneously bind nucleic acids and lectins represent a particularly interesting case of study in this sense.\textsuperscript{243,247} Further outlooks includes the conception of hybrid (hetero)glycomaterials to interrogate the compositional and functional complexity of the cell surface, which at its turn has the potential to lead to unanticipated advances in precision glycananotechnology.\textsuperscript{52,249}

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