The Cluster Glycoside Effect

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1. Introduction

Myriad biological communication events achieve the requisite level of specificity by exploiting the rich structural and functional diversity of glycoconjugates. Although a wide range of important events in normal biology, such as immune function and fertilization, are controlled by protein−carbohydrate interaction, several disease states involve this recognition motif. Early events in the infectious cycles of many bacteria, viruses, mycoplasma, and parasites involve carbohydrate-mediated recognition of host by pathogen, as do early events in some metastatic processes. The ability to control these events with selective small molecule inhibitors offers enormous potential for the study of biology. On the other hand, most saccharide ligands bind to their protein receptors only weakly, seldom showing association constants beyond 10^6 M^{-1}; clearly the effective in vivo control of events mediated by protein−carbohydrate binding requires significantly greater affinity. Against this backdrop, the development of tight binding ligands for carbohydrate binding proteins continues apace throughout

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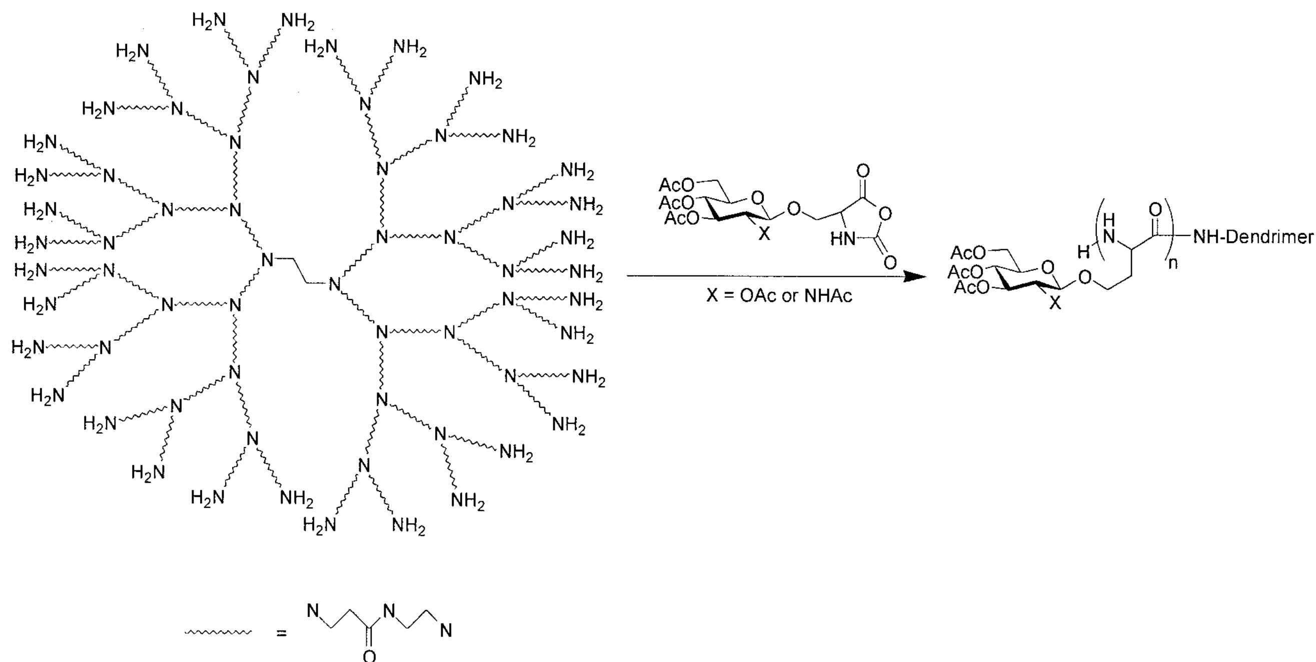

Figure 1. PAMAM dendrimer and incorporation of bifunctional saccharide binding domain.

the carbohydrate chemistry and biology community. Many among this group have taken a clue from nature; carbohydrate binding proteins are typically aggregated into higher-order oligomeric structures, existing as monodisperse entities with valencies to five or as aggregates of very high valency, such as viral particles. If nature has circumvented the tight binding limitation through multivalency, it seems reasonable that multivalent ligands should bind with high affinity as well. From this basis hundreds—if not thousands—of multimeric carbohydrates have been prepared and studied as ligands for a wide range of carbohydrate binding proteins. Most show some enhancement in activity compared to the corresponding monovalent ligand on a per mole of saccharide, or valence-corrected, basis. This phenomenon, noted by Lee and co-workers in 1995, is often referred to as the "cluster glycoside effect."[1]

The physical basis for these enhancements is less clear. The assays used to measure protein−carbohydrate interaction are complex processes that report on a range of events that occur during the course of the assay. Because thermodynamic parameters are state functions, all events that take place during the assay are coupled and reported as a single value. Here we review the cluster glycoside effect, considering first the construction of multivalent saccharides, next the various assays used to evaluate protein−carbohydrate binding, then the behavior of multivalent ligands in these assays, and finally the molecular basis for the observed activity. The cluster glycoside effect has been reviewed extensively, and we limit our review of the field here primarily to reports since 1996.[2−5] Our goal is to provide a framework in which to consider reported activities of multivalent oligosaccharide epitopes. We hope this framework will, in turn, provide the reader a molecular model of association that will aid the design of new molecules with predictable and defined activities.

2. Multivalent Glycoside Ligands

A wide range of multivalent saccharide ligands have been reported; most fit into a relatively small number of conceptual frameworks, specifically dendritic ligands, polymeric ligands constructed on either peptide or acrylamide backbones, and liposomes or other multivalent presentations created by self-assembly of amphiphilic carbohydrates. A smaller group of diverse ligands fall outside of these larger classifications. The field has been heavily reviewed, and here we describe the various groups only in enough detail to facilitate a discussion of mechanisms of action in future sections.[3,6−10]

2.1. Dendritic Multivalent Glycosides

Dendrimers, originally described independently by Newkome and Tomalia in 1985, are oligo- to polymeric structures prepared in a series of iterative steps.[11,12] The compounds can be prepared in either a convergent or a divergent sense, initiating synthesis at the periphery or the core of the macromolecule, respectively. Typically glycodendrimers display carbohydrates at the periphery of the macromolecule, and convergent approaches are better suited to the preparation of monodisperse homogeneous materials. In addition to monodispersity, dendritic structures offer the significant advantages of ease of preparation and nanoscopic dimensions, length scales intermediate between those characterizing glycoclusters and glycopolymers.

Dendrimers based on poly(amidoamine) cores, the so-called PAMAM backbone, form by far the largest group of dendritic multivalent saccharides (Figure 1).[13−15] This backbone offers the significant advantage of ease of preparation; indeed, PAMAM dendrimers of varying size are commercially available. On the other hand, the core is susceptible to base-induced

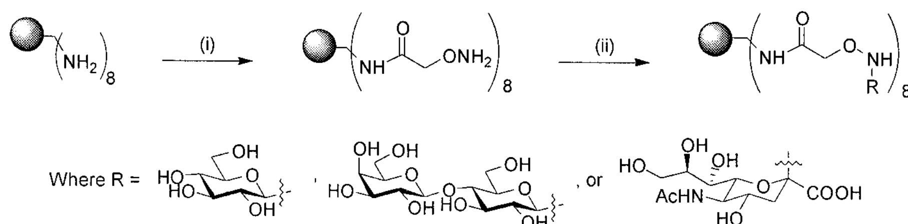

Figure 2. Incorporation of unfunctionalized carbohydrates to PAMAM dendrimers.

β-elimination during synthetic manipulation. Additionally, the synthesis of monodisperse compounds requires uniform incorporation of saccharide onto terminal amino residues following construction of the dendrimer scaffold in what amounts to a divergent synthetic approach. These couplings are typically conducted with glycosylated tethers terminated in isothiocyanates[16] or carboxylates.[17] Very high valent ligands have been constructed in this fashion. Roy and co-workers reported the preparation of PAMAM dendrimers containing 128 lactose epitopes[18] while Okada reported incorporation of GlcNAc into an eighth-generation PAMAM dendrimer, a scaffold containing over 1000 amine epitopes.[19] Okada and co-workers reported a synthetic strategy, termed radial growth polymerization, that produces a novel class of glycopolymers. Here, reaction of an amino-terminated PAMAM dendrimer with monomer containing both ligand and a masked amine couples a saccharide moiety and provides an additional nucleophile for subsequent addition. The coupling is highly efficient through two rounds of addition, at least by chromatographic and NMR evaluation.

More recently, Lambert and co-workers reported functionalization of the PAMAM core by coupling amino moieties to levulinic acid, generating ketone-terminated dendrimers.[20] This modification reverses the polarity of the core, creating an electrophilic reactive periphery. Uncatalyzed addition of peptides terminated in aminooxyacetic acid moieties produces a stable oxime linkage. Alternatively, incorporation of an oxime nucleophile through addition of aminooxyacetic acid facilitated direct incorporation of carbohydrate residues, presumably through formation of a stable hemiaminal (Figure 2). Although this methodology offers the advantages of simplicity and the use of unprotected carbohydrates, chromatographic evaluation of the products suggests significant heterogeneity.

In most cases dendritic ligands are evaluated chromatographically and spectroscopically, typically using NMR and electrospray and/or FAB or MALDI ionization mass spectroscopy. The various gel permeation chromatographic techniques provide information regarding average molecular weights and polydispersity, while spectroscopic studies confirm the existence of specific products. The evaluation of purity of species with molecular weights in the thousands is extraordinarily difficult. Impurities will be observed spectroscopically only if individual impurities are populated to a significant extent, likely greater than 1%. A more likely scenario during the divergent construction of dendritic ligands is the weak population of a large number of closely related impurities. The effect of these impurities on binding studies, beyond simply diminishing the concentration of the target ligand below that of the nominal concentration, is indeterminate.

Closely related to the PAMAM dendrimers are ligands based on the poly(propylene imine) backbone (Figure 3). In contrast to the PAMAM ligands, these compounds are stable to β-elimination. Fraser Stoddart and co-workers have incorporated up to 64 lactose moieties into the preformed dendrimer core, adding saccharide ligands as the *N*-hydroxysuccinamide esters.[21,22] Thompson and Schengrund have used the same core to display the GM1 pentasaccharide in the preparation of multivalent ligands for the *Escherichia coli* heat-labile toxin.[23] The iminobis(propylamine) core has also been used for glycodendrimer synthesis. Again, these oligomers provide the important advantage of base stability, a crucial consideration during oligosaccharide synthesis. Roy and co-workers demonstrated the suitability of this backbone for solid-phase synthesis.[24]

Matsuoka and Kuzuhara reported a novel carbosilane core structurally related to the poly(propylene imine) backbone.[25-27] In this work a saccharide epitope tethered to an alkyl thiol was used to displace a halide leaving group from a bromopropylsilane core (Figure 4). Radical addition of thiotoluene across the vinyl functionality of an allyl glycoside was followed by cleavage of the benzyl thioether in a Birch reduction. This latter step can be conducted in the presence of a halide-terminated carbosilane core, providing a one-pot coupling reaction. The approach has been used to decorate small cores with a range of mono- and oligosaccharides. The silane core offers a number of interesting advantages over the PAMAM, poly(propyl imine), and related cores, including tetravalent branch points, great chemical stability, and charge neutrality of the core in aqueous solution.

Divergent synthetic approaches allow coupling of valuable saccharide residues to the dendritic core in the final step of a synthesis. On the other hand, polydispersity arising from incomplete reaction and the intractability of analytical problems present complications during the interpretation of binding data. An attractive solution to these concerns is the convergent synthetic approach, where the limitations of divergent syntheses, particularly with regard to analysis, are largely obviated. In divergent syntheses, differences in fully and partially coupled products become smaller—and thus more difficult to detect—at successive generations. In contrast, these same distinctions are magnified at successive generation in convergent approaches.

Several convergent glycodendrimer syntheses based on aromatic cores, a core first described by Neenan and Miller, have been reported.[28,29] Fraser Stoddart

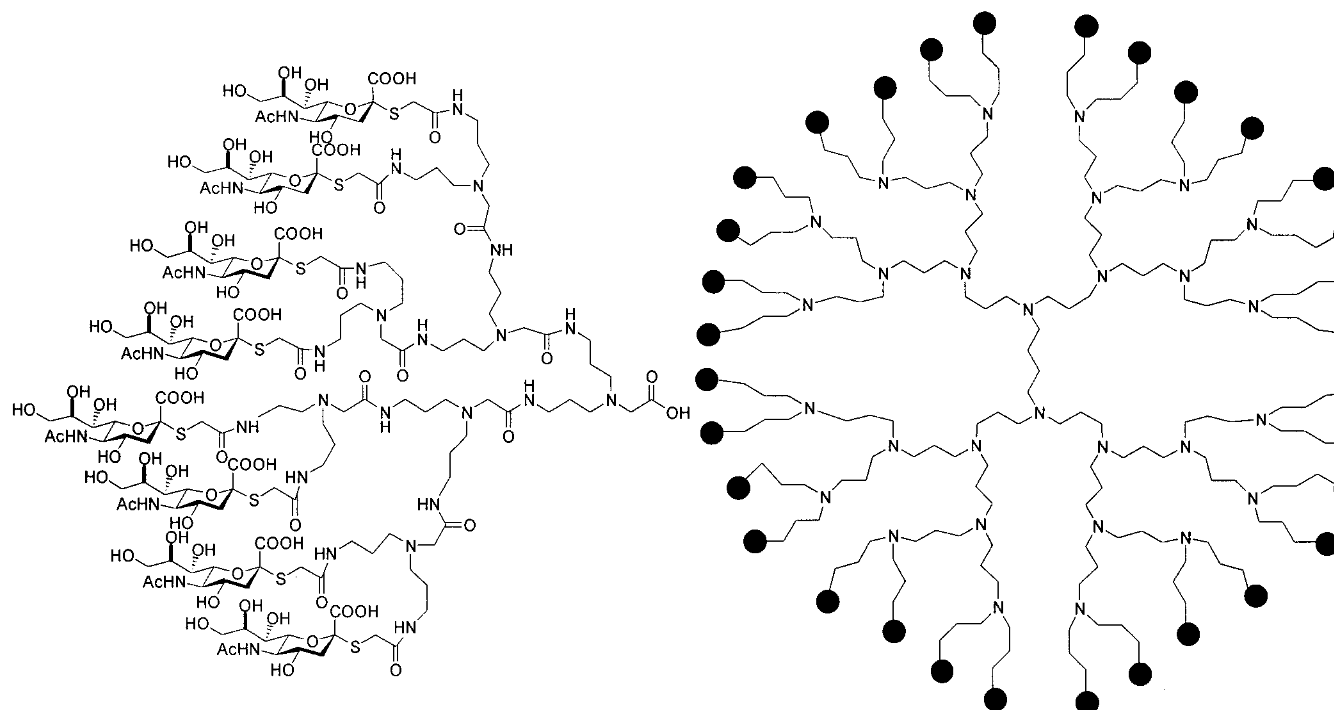

Figure 3. Left: sialic acid-laden dendron on an aminobis(polypropylamine) backbone. Right: a fourth-generation poly(propylene imine) dendritic backbone.

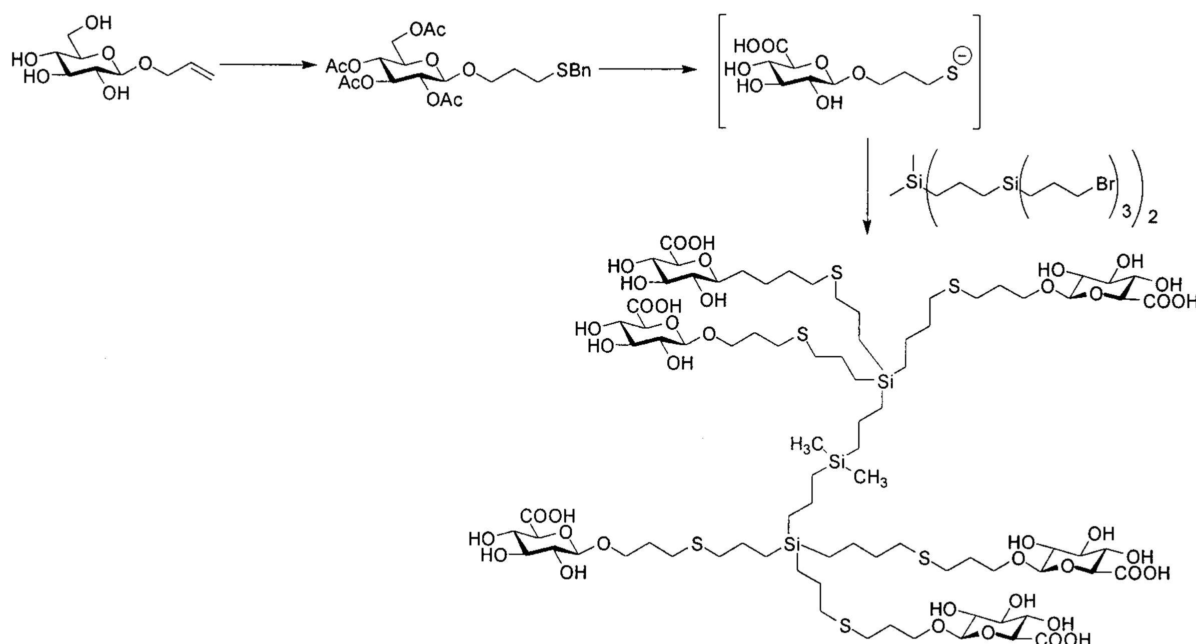

Figure 4. One-pot construction of carbosilane glycodendrimers.

and co-workers have reported convergent syntheses of several polyvalent dendritic compounds (Figure 5). In some instances, the compounds prepared are of interest for their materials properties in addition to their activities as ligands for carbohydrate binding proteins. Thus, carbohydrate-centered ligands are terminated with a total of 12 glucose residues, although the microscopic environment of each is somewhat different.[30] A related group of ligands, again derived from a convergent synthetic route and centered on an aromatic core, present terminal glucose residues in nominally equivalent microenvironments. These ligands demonstrate the power of the convergent approach, and dendrimers displaying up to 36 glucose epitopes have been prepared.[31,32] The diminished use of protecting groups continues as a trend in oligosaccharide synthesis, and the ease of amide coupling during dendrimer assembly facilitates the use of unprotected saccharides.[33] Patch and co-workers utilized a glycal epoxide ring opening strategy to create trivalent ligands based on a 1,3,5-benzenetrimethanol core.[34]

The rational design of multivalent ligands requires predictable placement of the saccharide epitopes. As the dendrimer core is modified, both with respect to size and to composition, the position of saccharide

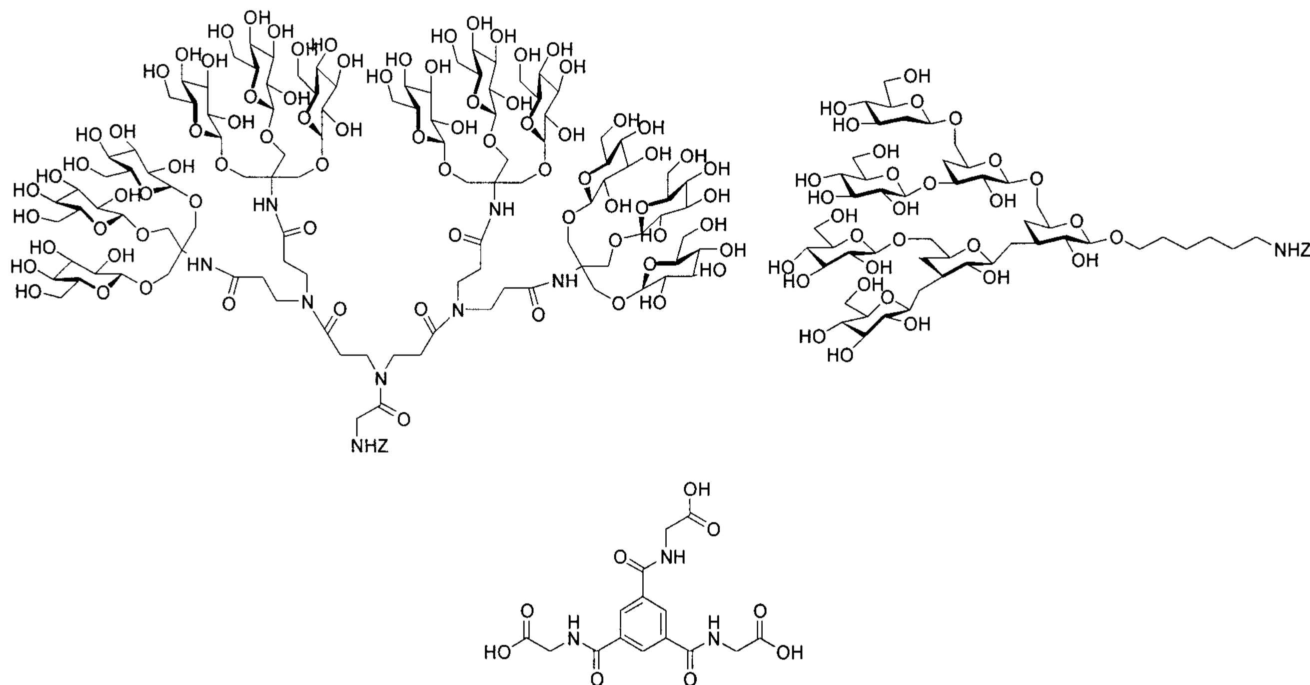

Figure 5. Fraser Stoddart aromatic dendrimer synthesis. Left: 12-mer wedge on amide linkers. Right: 4-mer wedge on trisaccharide linker. In both cases, coupling to trivalent aromatic core (bottom) with DCC produces the complete dendrimer of valence 36 or 12, respectively.

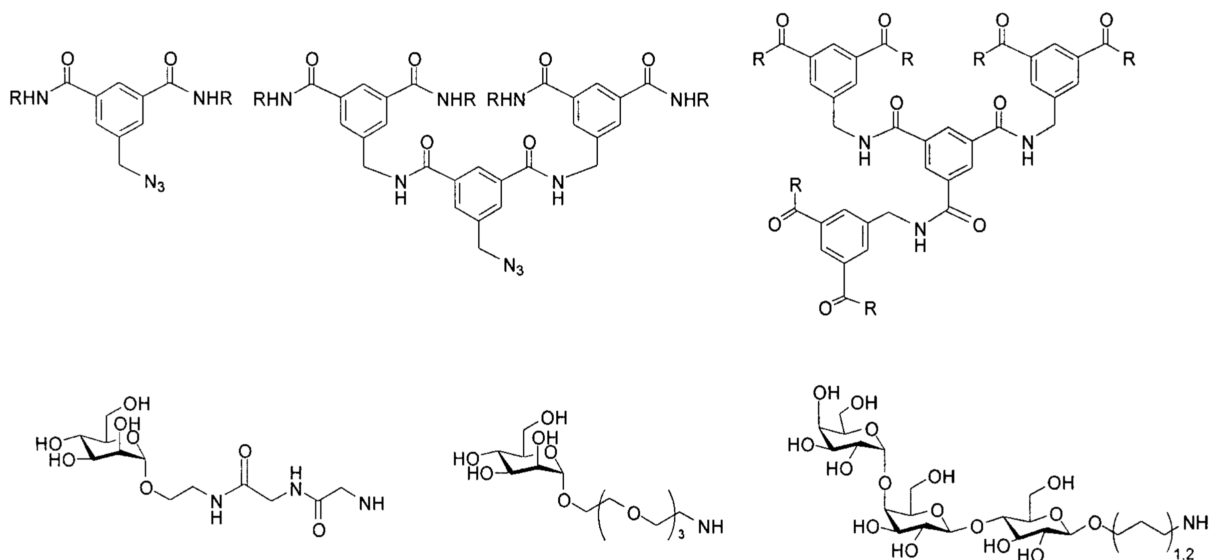

Figure 6. Toone aromatic glycodendrimer synthesis. Top: bi-, tetra-, and hexavalent cores. Bottom: representative carbohydrate termini.

epitopes in space and the flexibility of the external glycan layer become more difficult to predict. At low generation—certainly below fourth generation (G4) and likely beyond this level—dendrimeric cores show considerable flexibility and no discrete higher-order structure.[35−38] Somewhere beyond this point, at a size dependent on the specific composition, dendrimers collapse onto themselves and develop a compact core. This collapse may lead to highly ordered structures or trap microheterogeneity at the level of surface composition.

Toone and co-workers utilized a core essentially identical to that described by Neenan and Miller, replacing the original ester linkages with more robust amides (Figure 6).[39−41] Hindsgaul and co-workers have utilized a similar approach.[42] A key attribute of this strategy is the flexibility it provides. The dendron 3-dimethyl azidomethyl-1,3-benzenedicarboxylate can be differentiated by pig liver esterase, generating an asymmetric core. Propagating this differentiation allows construction of a range of patterned dendrimer surfaces containing two or more surface functionalities, where the dimensions and spacing between epitopes is adjusted by modification of the linker. On the other hand, couplings become inefficient at higher generation, presumably reflective of secondary structure and steric encumbrance of the reactive groups. Additionally, aqueous solubilities diminish at later generations; this phenomenon is typical of polyamide compounds. Several ligands

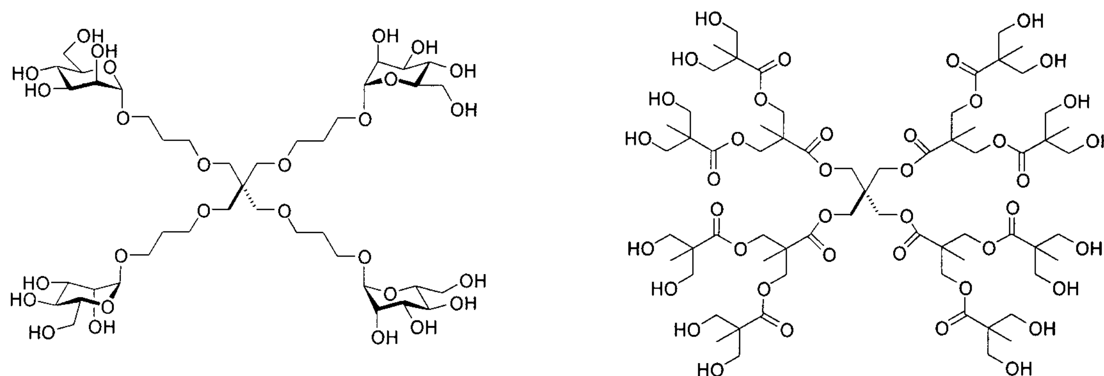

Figure 7. Ley (left) and Parquette (right) pentaerythritol dendrimers.

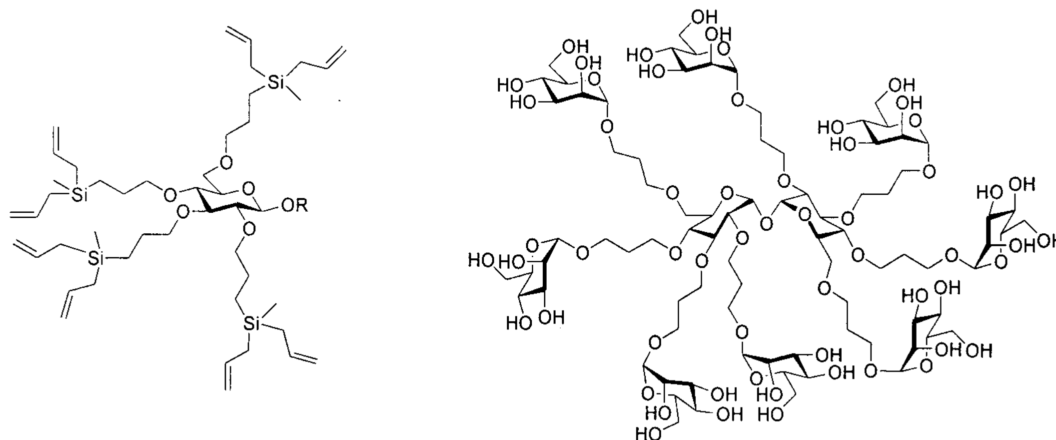

Figure 8. Left: carbosilane dendrimer surrounding a gluose core. Right: octavalent dendrimer based on a α,α-trehalose core.

have been prepared displaying mono- and oligosaccharide epitopes linked to the core through alkyl, oligoethyleneglycol, and peptide spacers.

Ley, Parquette, and Binder independently described syntheses of dendritic ligands based on pentaerythritol cores (Figure 7).[43–45] Ley and co-workers coupled mono- and oligosaccharides to the tetravalent core through propylene glycol linkers, while Parquette utilized a hyperbranced derivative of pentaerythritol as a high molecular weight support on which to conduct oligosaccharide synthesis. Although the construction of high-valency ligands was not the goal of this work, modification of the linker domain would be straightforward, and the work can fairly be regarded as a multivalent dendritic saccharide synthesis.

A range of other novel cores has been used in the construction of multivalent dendritic saccharides. Lindhorst and co-workers prepared multivalent ligands extending from a central glucose core. In these approaches, glucose is functionalized with a variety of reactive moieties, including carbosilanes, hydroxyl, and amino groups (Figure 8).[46–49] Coupling of dendritic fragments to the central core proceeds in the usual fashion. These cores have the additional advantage of differential reactivity at various branch points, in principle facilitating the construction of unsymmetrical or patterned surfaces.

Several groups have prepared multivalent ligands based on a cyclodextrin core. This core provides the additional advantage of transport activity; hydrophobic species loaded into the central pore might be targeted to various sights by a saccharide recognition domain. Several coupling strategies have been employed to couple carbohydrates or glycoclusters to the cyclodextrin core(Figure 9). Cyclodextrin modified as the C6 amine has been coupled using isothiocyante[50–52] or carboxylate electrophiles.[53] Both mono- and peraminated cyclodextrins have been utilized, in principle leading to ordered high-valence compounds. Dipolar cycloaddition strategies have been utilized for coupling to the central core.[54] Nishimura and coworkers coupled thiolated saccharides to a per(6-deoxy-6-iodo) cyclodextrin;[55] similar approaches have been reported by Santoyo-González.[56] Finally, Fraser Stoddart reported a photochemically induced addition of thioglycosides to allyl-substituted cyclodextrins.[57]

Several syntheses of dendritic ligands based on peptide backbones have been reported. Roy and coworkers reported dendritic ligands based on poly-L-lysine, while a Novartis group led by Thoma created multivalent ligands using a polyaspartic acid backbone.[58,59]

2.2. Other Low-Valent Glycoclusters

Several multivalent saccharide ligands not properly classified as dendrimers were reported in the past 5 years. Burke, Kiessling, and co-workers clustered three mannosyl residues around a macrocyclic core producing a rigid trivalent ligand.[60] Wittmann and Seeberger created a glycoarray similar in structure to the macrocycle-derived species through solid-

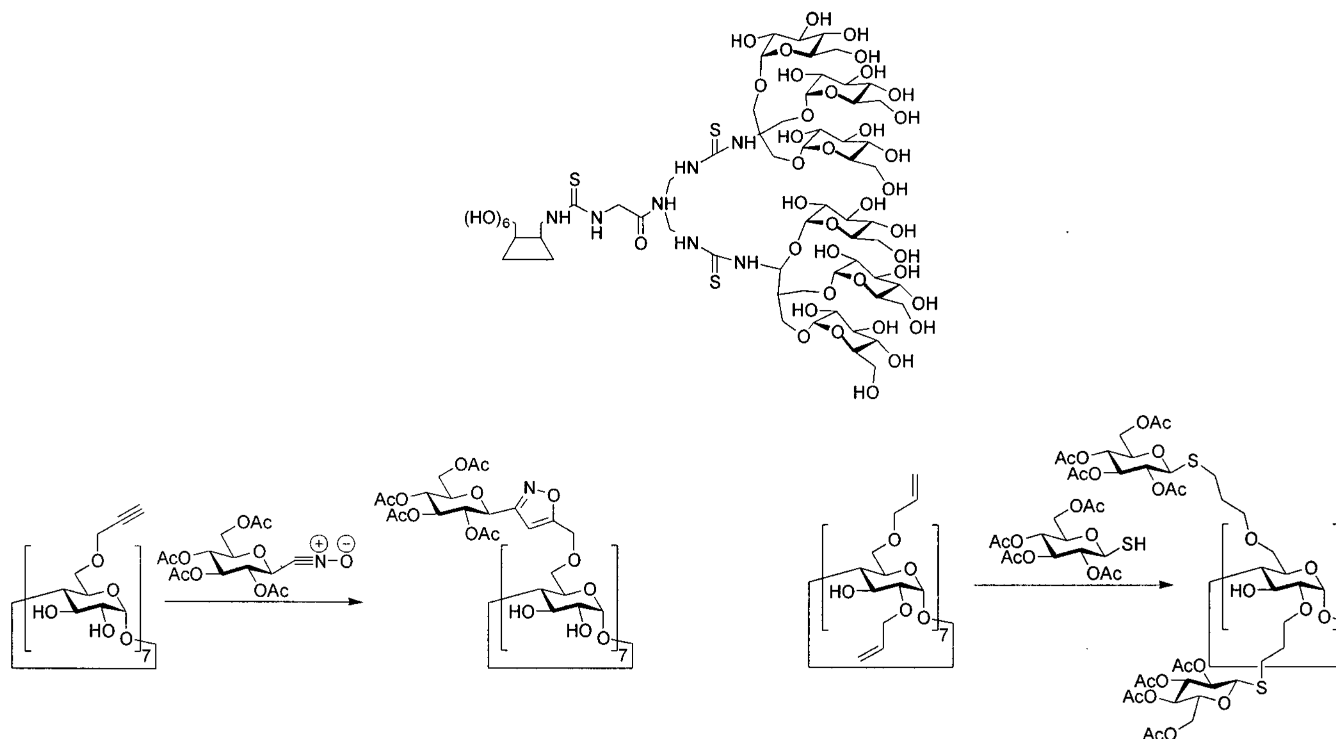

Figure 9. Cyclodextrin-based glycodendrimers. Top: β-cycldextrin incorporating a hexavalent dendron. Bottom left: dipolar cycloaddition strategy for cyclodextrin addition. Bottom right: photochemically initiated radical addition to allylated cyclodextrins.

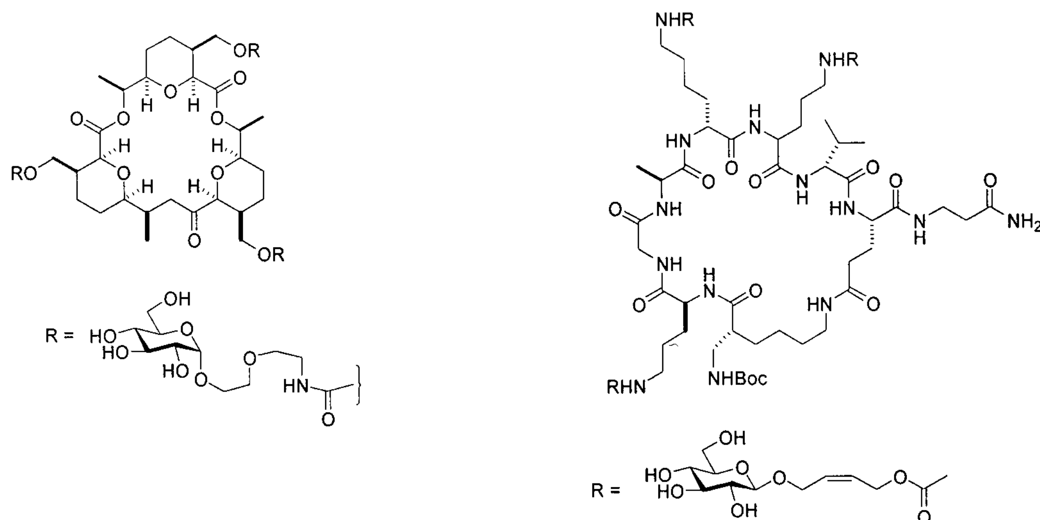

Figure 10. Templated glycoclusters.

phase peptide synthesis of a cyclic peptide containing glycosylated lysines (Figure 10).[61] Takahashi et al. reported the use of metal chelation to organize multivalent dendron fragments into a higher-valent structure.[62] In this approach, multivalent saccharides were grafted onto a diethylenetriaminepentaacetic acid chelate; in the presence of gadolinium(III), two such ligands bind tightly to the metal ion, creating a 12-valent glycoside. Roy and co-workers reported a similar strategy, clustering tetravalent ligands around either iron(II) or copper(II) through bipyridyl ligands.[63] Aoyama and co-workers tethered mono- and disaccharides to a calix[4]resorcarene, yielding octavalent amphiphilic structures: these structures likely adopt higher-order structure in aqueous solution.[64]

A group of so-called two component toxins are responsible for the pathology of the world's most severe enterotoxic diseases; these toxins include the *E. coli* heat-labile toxin, the shiga toxin, and the cholera toxin. Each toxin noncovalently associates a single copy of an enzymatically active 'warhead' subunit with a pentameric lectin; the latter domain facilitates recognition and cell binding. The toxins are structurally and mechanistically related to the tetanus, diphtheria, and pertussis toxins and to the plant toxins abrin and ricin. Because of the potential medical relevance, several multivalent ligands for this group of proteins have been reported. Two fall into the class of low-valent glycoclusters. Fan, Hol, and co-workers reported a pentavalent galactoside

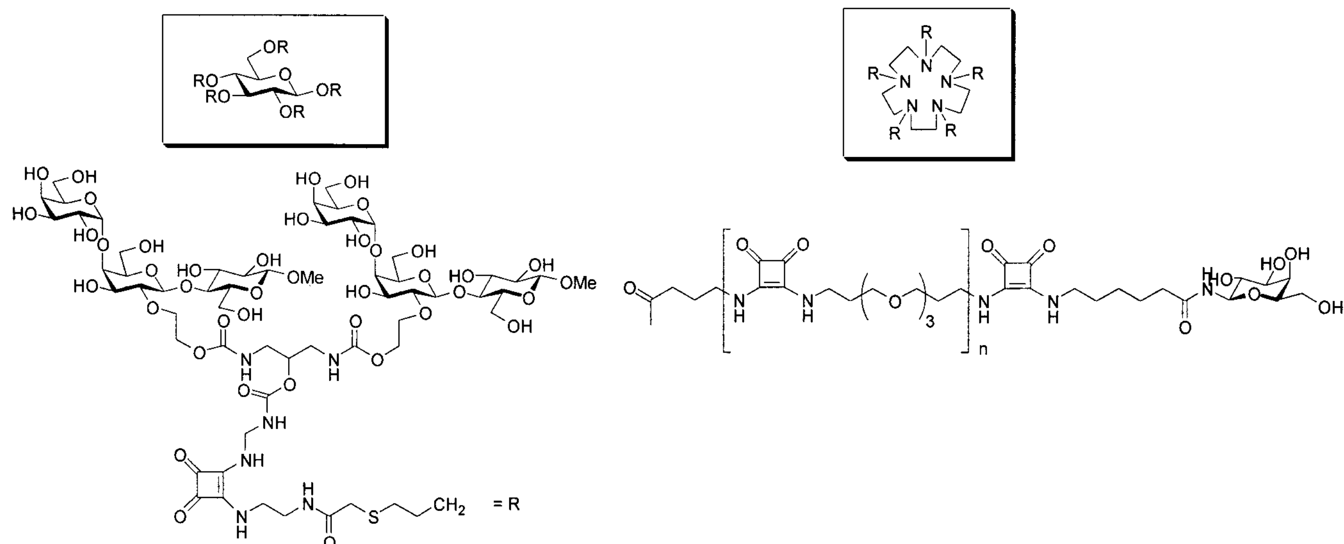

Figure 11. Glycocluster ligands for bacterial two-component toxins. Left: STARFISH, a decavalent ligand for the SLT B-subunit. Right: pentavalent ligand for the *E. coli* heat-labile toxin.

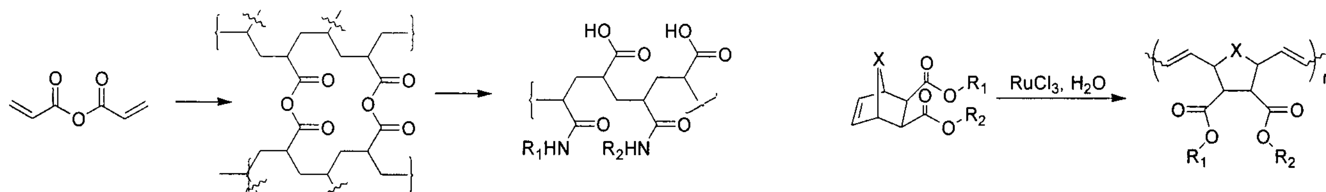

Figure 12. Polymeric glycosidic ligands. Left: synthesis of a cross-linked polyacrylamide suitable for post-polymerization modification. Right: Kiessling ROMP synthesis of glycopolymers.

ligand for the *E. coli* heat-labile toxin based on a macrocyclic core (Figure 11).[65] The spacer arms separating the core from the carbohydrate recognition domain were systematically varied to provide intersaccharide spacings ranging from 50 to 175 Å. Bundle and co-workers utilized a glucose core to anchor 10 copies of the P^k trisaccharide in the search for high-affinity ligands for the shiga toxin.[66] In this design, each arm is terminated in two copies of the trisaccharide, furnishing a decavalent ligand.

2.3. Polymeric Glycoside Ligands

Polymeric carbohydrate-laden ligands have been utilized extensively for the study of multivalency effects in protein–carbohydrate interaction. We group several classes of ligands within this category, including high-valent ligands grafted onto natural or synthetic nominally amorphous linear polymers, high-valent ligands grafted onto existing protein scaffolds, and high-valent ligands affixed to surfaces. Compared to other scaffolds, polymeric ligands offer several advantages, specifically ease of synthesis and very high valency. On the other hand, these ligands are plagued by structural heterogeneity, a property that confounds interpretation of binding data. This heterogeneity takes on several forms. In synthetic polymers, dispersity exists with regard to the degree of polymerization and, in the case of copolymers, microscopic sequence. Many polymerizations create new stereogenic centers during bond formation; this process creates stereochemical heterogeneity in the polymer. Grafting saccharide ligands onto preformed polymers typically proceeds with incomplete functionalization of reactive residues; this incomplete reaction generates microscopic structural heterogeneity. The creation of monolayers, either Languir/Langmuir–Blodgett films or self-assembled monolayers on metal surfaces, produces microheterogeneous surfaces. Here, carbohydrate recognition domains may self-associate, or 'raft', either through favorable carbohydrate–carbohydrate interactions or to minimize solvophobic interactions. Such structure may develop over several length scales, each of which may affect particular classes of interactions. Surface reconstruction may also occur, altering microscopic structures during storage or during exposure to macromolecular binding agents.

The vast majority of amorphous linear polymeric ligands are either copolymers of acrylamide/acrylic acid esters or polymers derived from esters of bicyclo[2.2.1]heptene or heterocyclic variants of this framework by the ROMP methodology (Figure 12). Whitesides, Roy, and Bovin have previously reported the synthesis of sialic acid-bearing polyacrylamides; these polymers showed some of the largest enhancements in affinity on a per mole of saccharide basis ever reported.[67–75] Various groups continue to develop these polymers which, lacking cross-links, retain very high aqueous solubilities. Polymerization of acrylic anhydride yields a reactive backbone that can be reacted with a variety of amine nucleophiles to prepare functionalized polymers.[68] 'Libraries' of polyvalent ligands were prepared by this approach by reacting the reactive core with various side chains

in microtiter plate wells. Carbohydrate recognition domains can be inserted during polymerization in the latter case by displacement of *N*-hydroxysuccinimide esters. Wang and co-workers used this strategy to prepare polymers displaying the αGal epitope, polymerizing acrylamides bearing intact disaccharide.[76]

Kiessling and co-workers have extensively developed ruthenium-catalyzed metathesis polymerization for the preparation of polymeric ligands displaying both mono- and oligosaccharides.[77–82] A significant advantage of the ROMP strategy over other, largely radical, methodologies is the living nature of the process. Exploiting the living polymerization, this group prepared block copolymers to incorporate differential latent functionality at the termini of polymers.[83] Such polymers facilitate construction of polymeric chains of defined length and valency. Although the molecular weight distribution of these polymers is narrower than polymers prepared through radical approaches, considerable polydispersity remains. Additionally, the polymers are created as *E*/*Z* mixtures at each step, creating stereroisomeric heterogeneity. Still, the compounds have been used with great effect.

Kobayashi and co-workers reported polymeric ligands bearing mono- and disaccharide recognition epitopes based on a polyphenylisocyanide backbone.[84] This backbone was chosen for its rigidity, a trait predicted to affect the lectin binding properties of the polyvalent ligand. Remarkably, the polymers appear to develop regular secondary structure and produce CD spectra indicative of helicies; apparently the saccharide stereochemistry induces stereoregular structure in the complete polymer. Random copolymers containing unglycosylated phenol side chains were also prepared. Roy and co-workers have recently reported a novel series of polyvalent carbohydrate ligands based on a chitosan backbone.[85–87] In this approach, various mono- and oligomeric saccharide recognition domains are tethered through a reactive aldehyde, which is in turn coupled to the free amine of chitosan through a reductive amination. Dendrons containing up to nine monosaccharide residues were successfully grafted to chitosan in this approach.

The coupling of saccharide residues to the surface of proteins has long been used as a straightforward strategy for the creation of high-valence ligands; such approaches are still in use.[88] Typically serum albumin is the protein base of choice, in part due to the 58 lysine residues in its primary sequence. The accessibility of these amines varies, and total loadings typically diminish with increasing steric bulk of the ligand. A variety of coupling chemistries are used for epitope incorporation, including activated esters, most often *N*-hydroxysuccinimides, or isothiocyanates. Periodate cleavage of saccharide residues to produce reactive aldehydes followed by reductive amination in the presence of cyanoborohydride has also been extensively utilized. While the methodology produces medium to high-valent ligands based on a water-soluble core with nanoscopic dimensions, issues of polydispersity present significant impediments to the interpretation of binding data.

Carbohydrate ligands can also be displayed in a polyvalent fashion on two-dimensional surfaces. In many respects such presentation replicates that found on the surface of cells; protein receptors face multiple binding epitopes constrained in a two-dimensional array. Various surfaces have been used, and the coupling can be either covalent or solvophobic. In the former regard, the creation of self-assembled monolayers on gold provides a simple experimental strategy for the controlled preparation of surfaces. The approach follows from the original description by Nuzzo and Allara that alkyl thiols deposit on gold surfaces in a covalent fashion to yield effectively crystalline two-dimensional arrays, a methodology that has become a mainstay of surface science.[89] The preparation of gold monolayers displaying saccharide recognition epitopes has increased markedly with the increasing popularity of surface plasmon resonance assays.

Russell and co-workers prepared a series of monosaccharide monolayers on gold and demonstrated the competence of such surfaces for lectin binding.[90] Kitano and co-workers used a similar approach to anchor multivalent saccharide epitopes, preformed through a radical polymerization, to both silver particles and gold surfaces.[91] Whitesides, Kahne, and co-workers prepared a series of self-assembled monolayers, varying the surface density of carbohydate epitope.[92] Magnusson reported the synthesis of a thiol-terminated glycolipid analogue suitable for coupling to gold surfaces.[93] Bundle and co-workers reported the formation of gold monolayers of the P^k trisaccharide, anchored to a gold surface as mercaptohexadecanyl glycoside, for use as a biosensor,[94] following the earlier report of a similar strategy for detection for the P-fimbriated *E. coli*.[95] Nyquist and co-workers explored the use of GM1-PEG mixed monolayers for biosensor applications.[96] More recently, Penades and co-workers reported a reductive synthesis of gold nanoparticles that produces gold sols with nanometer dimensions uniformly coated with carbohydrate ligands.[97] Disulfide-linked di- and trisaccharides were treated with sodium borohydride in the presence of gold chloride. The preparation produces uniform particles with a mean radius of 1.8 nm and a ratio of 63–97 carbohydrates to 200 gold atoms. The particles are water soluble and stable and provide a novel and interesting platform for the study of multivalency effects.

Various noncovalent adsorptive routes to surface-immobilized multivalent carbohydrates exist. Carbohydrate-containing liposomes and Langmuir–Blodgett films are well-known. More recently, Diederich, Fraser Stoddard, and co-workers reported the formation of stable Langmuir–Blodgett films from fullerene-based carbohydrate dendrons.[98] Saavedra and co-workers have prepared glycosyl ceramide monolayers to study protein–glycolipid binding.[99] The need for novel analytical strategies was the motivating force for efforts by Kiessling and Leckband toward the synthesis of glycolipid monolayers on solid substrata. In the former instance, monolayers of alkanethiols on gold served as substrates for the deposition of glycolipd monolayers; the resulting

surfaces were used in surface plasmon resonance studies of lectin binding.[100] In the latter instance, a similar approach was utilized, depositing glycolipids on a gel phase of 1,2-dipalmitoylphosphatidylethanolamine on mica.[101]

Carbohydrates have also been attached to a wide range of other solid supports, including peptide synthesis resin[102] and silica supports. The attachment of medically relevant saccharide epitopes, including the recognition domains for the shiga and *Clostridium difficile* toxins, to silica supports forms the core technology of the Synsorb corporation.

3. Evaluation of Protein–Carbohydrate Binding

The evaluation of protein−carbohydrate binding constants is not at all straightfoward, and a wide range of assays has been utilized for the measurement of these affinities. An understanding of each assay is vital for the appropriate construction of molecular level models that rationalize the phenomenology of multivalency. Here, we review briefly four of the most widely used techniques; the inhibition of hemagglutination assay, the enzyme-linked lectin assay, isothermal titration microcalorimetry, and surface plasmon resonance, or BIACORE, assays. Our goal is to provide sufficient detail to facilitate critical evaluation of the binding data presented in section 4 and to facilitate the discussion of the molecular basis of the cluster glycoside effect in section 5. In each instance, references to more detailed descriptions of the assay are included.

Typically the goal of protein−carbohydrate binding studies is to relate the structure of the ligand to its activity, that is, how the ligand interacts with the protein receptor. It is important to recall at the outset that the partial molar free energy of a particle is related logarithmically to its *activity*, that is,

$$\mu_I = \mu_I^\circ + RT \ln a_I$$

where μ_I refers to the partial molar free energy of the species and a_I to its activity. The activity of a species is related to its concentration through an activity coefficient (γ):

$$a_I = \gamma_i c_I$$

The activity coefficient describes solute−solute interactions that result in nonideal behavior. The magnitude of the activity coefficient depends on solute concentrations, the choice of concentration scales, the solvent, temperature, and the structure of the solutes. For a 1:1 complex between two species A and B, the thermodynamic equilibrium constant K is related to the activities of each species, that is,

$$K = a_{AB}/a_A a_B$$

In this construction the binding ‘constant’ is a true constant; it is independent of concentration and unitless. On the other hand, since knowledge of activity coefficients is, at best, difficult, it is common to assume activity coefficients of unity and replace activities with concentrations. The assumption of unit activity is usually justified at concentrations below about 0.1 mM. During interpretation of heterogeneous or solid phase assays, it must be appreciated that binding ‘constants’, whatever the measure, are in all likelihood not true constants but rather values highly dependent on the specific construction of the assay.

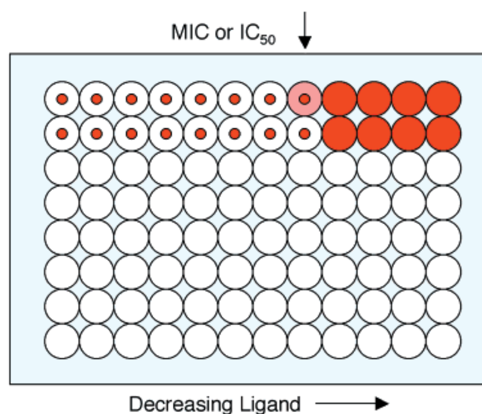

Figure 13. HIA assay. At low soluble ligand concentration (right of plate), multivalent lectin cross-links erythrocytes, producing a gel-phase through the thickness of the well. At high ligand concentration (left side of plate), the hemagglutination reaction is inhibited, and anticoagluated erythrocytes settle at the bottom of the well.

3.1. Inhibition of Hemagglutination (HIA)

Historically, the strength of protein−carbohydrate interaction has been semiquantitatively evaluated by the Landsteiner hapten inhibition assay,[103] often referred to as the inhibition of hemagglutination assay, or HIA (Figure 13). The assay has been known and practiced for decades and is in wide use for the evaluation of virus and viral antigen titer measurements. The test is based on the observation that whole viral particles agglutinate, or aggregate, red blood cells, producing a gelatinous phase throughout the thickness of the test solution. Cells from a variety of mammals and birds are suitable for assay, although chicken or porcine erythrocytes are most commonly used. In the event, erythrocytes are anticoagulated with, for example, citrate and diluted. Addition of soluble lectin, typically at concentrations of 0.1 to 0.01 mg mL^{-1}, produces a cross-linked matrix. Addition of soluble lectin in the presence of a soluble ligand results in diminished level of precipitate. Ultimately the precipitin reaction is prevented entirely, and the assay reports the minimum concentration of a carbohydrate that inhibits the hemagglutination reaction. The test is often generalized to use the aggregation and precipitation of other multivalent ligands, including glycogen, yeast mannan, and starch granules. Since the test typically utilizes serial dilutions, the error is at least ± one well, or a factor of 2. While the order of inhibitory potency is generally robust to variables such as the source and age of erythrocytes, absolute IC_{50} values vary widely.

It has become common to consider IC_{50} values from agglutination studies as a surrogate for dissociation constants; such parallels are unjustified. In addition to concerns regarding activities, such equalities

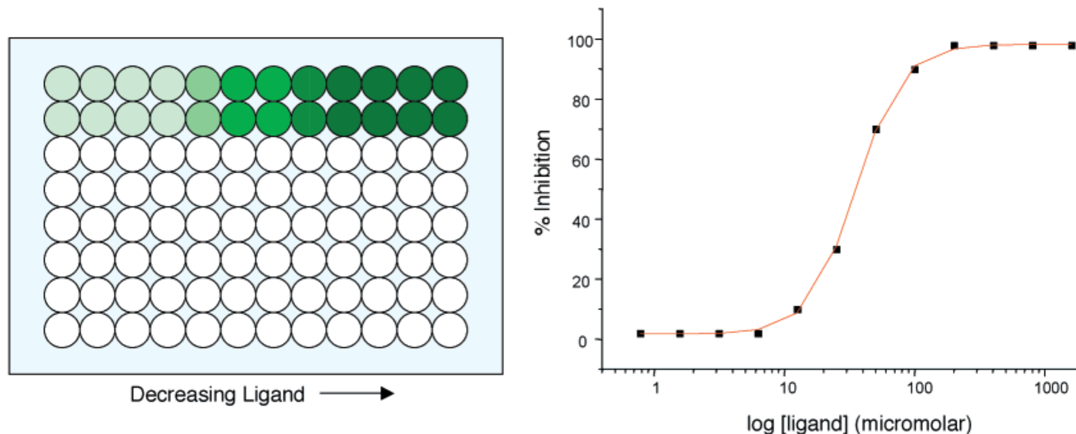

Figure 14. The ELLA assay. Soluble ligand competes with ligand immobilized for sites on a lectin−enzyme conjugate. Following incubation, the wells are evacuated and a prodye substrate is added; the amount of color formed is proportional to the concentration of lectin−enzyme conjugate in the well and inversely proportional to the affinity of the soluble ligand. Curve fitting (right) provides an IC_{50} value.

require the assumptions of reversibility, a condition that may or may not be met, and equivalent concentrations of competing ligands. Deviations from this latter condition vary widely from assay to assay. In short, the inhibition of hemagglutination assays is a quick, simple assay that orders soluble ligands in a rough order of binding affinity. Attempts to extract information beyond this from the assay are problematic.

3.2. Enzyme-Linked Lectin Assay (ELLA)

The enzyme-linked lectin assay is a variation of the well-known enzyme-linked immunosorbent assay (ELISA).[104] In ELLA, soluble and immobilized ligands compete for binding sites on a lectin−enzyme conjugate, usually horseradish peroxidase (Figure 14). In the typical assay, microtiter plate wells are coated with a high molecular weight polymeric saccharide, for example, yeast mannan or a polyacrylamide/acrylic acid ester. To the wells are added lectin−enzyme conjugate and soluble ligand. Following an incubation period, the wells are evacuated and refilled with a prodye substrate for the enzyme conjugate. The development of color, proportional to the concentration of retained lectin and inversely proportional to the affinity of the soluble ligand, is read spectrophotometrically and an IC_{50} determined by curve fitting. For the most part, relative inhibitory powers are similar to those deduced from agglutination assays, although marked differences in IC_{50} values are common. These differences again serve as powerful warning against assuming either value is simply related to a true binding constant.

ELLA avoids many of the problems associated with HIA. First, IC_{50} values are determined by curve fitting, improving the precision associated with serial dilution. Second, although one of the binding partners of the competitive couple is bound to a solid phase, the assay does not involve aggregation and the formation of high molecular weight cross-linked complexes. This feature of the assay ameliorates concerns regarding kinetic components of the reaction associated with irreversibility. Nonetheless, other aspects of the assay are troublesome. First, complete inhibition of lectin binding to the plate walls is seldom observed, and maximal inhibition occurs at considerably less than 100%. The reasons for this behavior are obscure but might be rooted in multiple classes of microscopic binding sites on the microtiter plate surface. Second, concerns regarding the equation of IC_{50} values with binding constants are essentially identical to those noted for the HIA. Finally, from a practical perspective, the assay requires lectin−enzyme conjugate; most lectins are not commercially available in conjugate form. Still, ELLA removes many of the problems associated with agglutination assays and stands as an attractive alternative methodology.

3.3. Isothermal Titration Microcalorimetry (ITC)

Titration calorimetry has long served as a powerful methodology for the evaluation of binding constants.[105,106] During the early 1990s, several instruments with milliliter cell volumes became commercially available, making the technique accessible to nonspecialists. Titration calorimetry operates by evaluating the heat evolved during ligand binding as a function of titrant concentration. Typically the macromolecule (lectin) is placed in the cell, and ligand is added in a series of 20 to 50 injections. Most instruments utilize a continuous power compensation design, as opposed to passive thermal conductivity. In this configuration, a sample and reference cell are brought to thermal equilibrium and then heated at a constant rate (typically 0.1 °C per hour). Addition of ligand to the sample cell perturbs this equilibrium in a fashion dependent on the enthalpy characterizing binding. A compensating voltage returns the cells to equilibrium; raw data from the calorimetric experiment is thus in units of power versus time (Figure 15). Integration with respect to time provides the more familiar titration curve, relating enthalpy evolved to ligand concentration.

The shape of the integrated curve is a function of the concentration of binding sites in the cell and the binding constant. A model of binding is assumed; this model is then used to deconvolute the binding curve to provide a binding constant, enthalpy of binding,

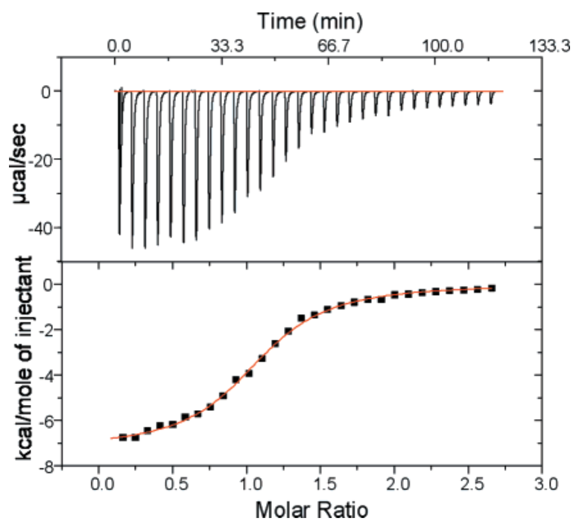

Figure 15. Raw (top) and integrated data from a typical calorimetric titration. The shape of the curve is determined by the unitless parameter *c*, numerically equivalent to the product of the binding constant and the concentration of binding sites in the cell. In the example shown here, $c = 14$.

and stoichiometry of binding for each class of binding sites assumed in the model. The curve fitting process functions optimally when the product of the binding constant and the concentration of binding sites ranges from 10 to 50, but at least from 1 to 1000. These values place practical limits on the systems amenable to study by ITC. At the low-affinity limit, the technique fails for material availability and/or solubility concerns: a millimolar dissociation constant requires at least millimolar binding sites. At the high-affinity limit, the technique fails over sensitivity issues: a nanomolar dissociation constant requires no more than micromolar binding site concentrations. Practically, ITC is effective for systems with binding constants ranging from roughly 10^3 to 10^7 M^{-1}, although methodologies exist to expand this range.[107−109]

ITC provides many advantages for the evaluation of protein−carbohydrate compared to other methodologies. Most importantly, ITC yields a concentration binding constant, rather than an IC_{50}. With the sole assumption of unit activities, a reasonable assumption for the dilute solutions typically employed, values from ITC are thus directly relatable to the free energy change for the binding event. ITC is the only method capable of providing binding enthalpies directly; while in principle measurement of the temperature dependence of binding constants provides enthalpies of binding, in practice changes in heat capacity accompanying binding in aqueous solution make such protocols all but impossible. Evaluation of the enthalpy of binding as a function of buffer ionization enthalpy provides information regarding the change in protonation state of the ligand and receptor during binding. Evaluation of the enthalpy of binding as a function of temperature provides a direct measure of the change in molar heat capacity accompanying binding; this value is vital during considerations of the effect of solvent reorganization to the thermodynamics of ligand binding.[110−112]

Despite these advantages, ITC is no panacea. First, compared to other methods, the amount of material required is high; typically milligram quantities of both protein and ligand are required. Second, great care must be taken during data reduction. Calorimetry provides no structural information regarding the bound complex. Knowledge of this structure is critical to data reduction, since the model adopted affects the functional relationship between enthalpy and ligand concentration. Thermodynamic parameters are state functions, and the enthalpy recorded is the sum of all processes that occur in the reaction cell. Thus, changes in protonation state or coupled equilibria are all reported as a single binding enthalpy. While such information is useful in developing an understanding of the microscopic events that comprise 'binding', they can also be misleading if not fully exposed.

3.4. Surface Plasmon Resonance (SPR)

At the contact surface of a metal and a dielectric, an evanescent longitudinal propagating wave is generated; this wave is known as a surface plasmon. These surface waves absorb some amount of incident plane-polarized light and ultimately affect the total internal reflectance of the system in a quantifiable fashion. Thus a scan of total internal reflection light as a function of either incident or reflection angle produces a sharp minimum. The position of this minimum is ultimately dependent on the refractive index and thickness of the surface layer: the technique is thus a sensitive measure of changes that occur at that surface (Figure 16). Although surface plasmon resonance was first observed in 1902,[113] its use for biosensors began in earnest only in the mid 1980s. In this application, a flow cell is created that passes one-half of a binding couple over an immobilized layer of its cognate partner, affixed to a gold substratum. Changes in the refractive index of the adsorbed layer as the ligand binds are recorded, and with knowledge of the bulk concentrations and adsorbed amounts, on-rates are readily deduced. Passage of buffer over the bound surface produces an off-rate; the ratio of these rate constants yields a binding constant. Today the technique is widely used for the study of interacting biological systems, facilitated by the commercial availability of several instruments.[114−117]

Like all other techniques, SPR offers several unique advantages. SPR uses very small amounts of material, expanding the range of systems amenable to study. Second, SPR provides rate constants for both forward and reverse reactions and is the only technique described here which does so. More recently a number of novel instrument configurations have been reported, most notably coupling of the SPR instrument to a MALDI TOF mass analyzer. On the other hand, great care is required when designing the SPR experiment and interpreting the results of these experiments.[118−120] Surface loading of the functionalized chip is crucial; at higher surface capacities mass transport issues provide anomolously low off-rates. On the other hand, the low-capacity surfaces that avoid mass transport problems minimize sensitivity and instrument response as well as increase

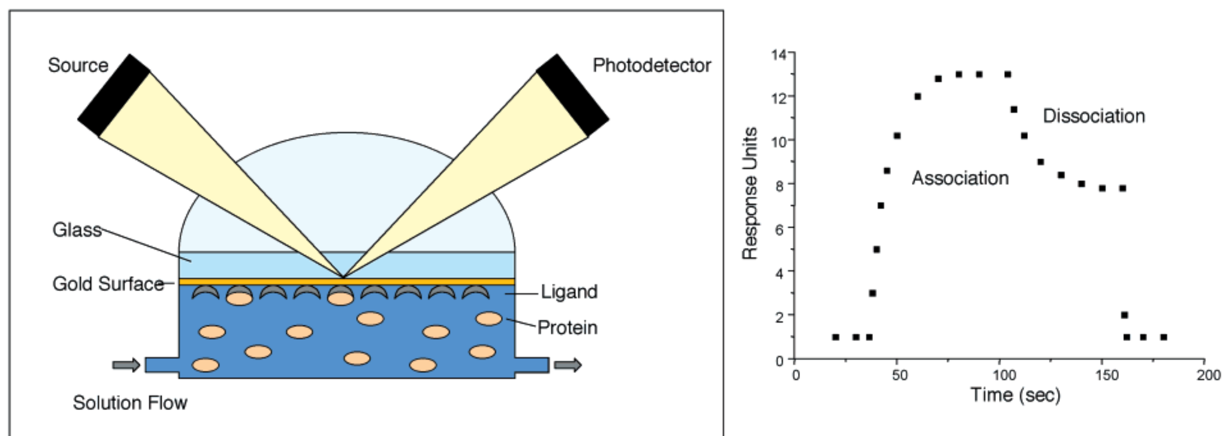

Figure 16. The SPR experiment. A lectin solution flows over a chip displaying immobilized ligand. Changes in refractive index at the surface, indicative of binding, are monitored as a function of time, yielding a binding on rate. Flow of buffer removes bound protein, providing an off rate. The ratio of on and off rates provides a binding constant.

nonspecific binding. In general, low molecular weight ligands provide lower sensitivities, and the low molecular weight partner of the couple is typically immobilized. The orientation of ligands with respect to the cell surface and flow vector also affects apparent on and off rates. Great care must be taken to conduct appropriate blanks, since small changes in refractive index between buffer and ligand solutions can provide spurious results.

4. Binding of Multivalent Saccharide Ligands: The Cluster Glycoside Effect

Myriad reports of the binding of multivalent saccharides to lectins have appeared during the past 10 years. Here, we focus on those reports that provide sufficient detail to facilitate a clear measure of the enhancement on a valency-corrected basis; Table 1 below summarizes several such reports. We have *not* attempted to generate an exhaustive tabulation of binding data. For clarity we note only the greatest reported enhancement. We also include the number of compounds analyzed and the magnitude of the enhancements reported.

The table reveals several trends worthy of note:

• For the most part *all* multivalent ligands, regardless of ligand valence, lectin valence, ligand construction, or binding assay, show some enhancement in activity over the monovalent reference on a valence-corrected basis.

• *There exists tremendous variation in the magnitude of the cluster glycoside effect*, and enhancements on a per mole of saccharide basis range from zero to 10^6. Although enough variability exists that unambiguous conclusions are impossible, there appears to be a trend of increased enhancement with increasing valency. Thus enhancements observed for linear polymers are near 10^5, for dendrimers near 10^3 fold, and for glycoclusters near 10^2. Beyond this broad categorization, relationships between valence and affinity are obscure. Among low-valent ligands, at least through a valency of 20, there is no discernible relationship between valency and affinity; enhancements for bivalent ligands as large as 1000 and for nonavalent ligands as low as 0.4 have been reported.

• *There may exist a relationship between the magnitude of the cluster glycoside effect and the assay utilized*. The average enhancement measured by ITC is 86, by SPR 150, by ELLA 45 000, and by HIA 150 000. There are large deviations from these averages, however, and the data set is insufficient to permit unambiguous conclusions. Still, given that the assays operate in vastly different concentration regimes and measure different physical properties, it is worth noting that the magnitude of reported cluster glycoside effects may be linked to the measurement.

• *There is no apparent relationship between lectin valence and the magnitude of the cluster glycoside effect*. To be sure, the largest enhancements are observed for polymeric ligands against viral particles. The effect of both ligand valence and the HIA assay presumably contribute to this trend, and the extent to which lectin valence further contributes to these enhancements is unclear.

• In instances where binding was evaluated by more than one methodology, considerable discrepancy between the derived values is apparent. Toone and co-workers evaluated the binding of a series of mannoside-laden dendrimers by ITC, ELLA, and HIA (Table 2).[39] These discrepancies hardly seem surprising since each technique measures a phenomenon that includes a binding event as we typically imagine it but does not report solely on a binding phenomenon. These observations provide another cautionary note for the interpretation of binding data.

5. Molecular Interpretation of Cluster Glycoside Effects

That multivalency in carbohydrate ligands improves the performance of those ligands in a range of assays is unassailable; the cluster glycoside effect is by now well-demonstrated. On the other hand, the mechanisms by which multivalent ligands achieve this performance is unclear. In this section, having considered the synthesis of mulitvalent ligands, the methodology used to evaluate protein−carbohydrate binding and the enhancements in apparent affinity observed for various ligand classes, we turn our

Table 1

ligand class	carbohydrate epitope	number of compared ligands	protein	assay	magnitude of enhancement	maximum enhancement[a] (valency)	reference ligand
glycocluster	β-Gal[53]	6	*K. bulgaricus* cell wall lectin	turbidity	10^0	1.5 (7)	monovalent β-Gal cyclodextrin
glycocluster	β-Gal[65]	5	heat-labile enterotoxin	ELISA	10^0-10^4	20800 (5)	β-Gal
glycocluster	α-Man[50]	5	Con A	ELLA	10^0-10^1	16.6 (6)	monovalent α-Man CD
glycocluster	β-Glc[185]	4	pea lectin	ELLA	10^0-10^1	15 (7)	βMeGlc
glycocluster	α-Man[186]	10	*E. coli* type 1 fimbriae	HIA	10^0-10^1	260 (2)	αMeMan
glycocluster	α-Man[60]	3	Con A	FRET	10^0	3.3 (3)	monovalent α-Man ligand
glycocluster	vancomycin[187]	1	NAc-L-Lys--Ala-D-Ala tripeptide	SPR	10^3	1000 (2)	vancomycin
glycocluster	C-linked Galα1−4Galβ1−4Glc (Pk trisaccharide)[167]	5	SLT-1 B-subunit	ITC	10^0-10^1	5.5 (2)	Pk trisaccharide
glycocluster	β-GlcNAc[55]	4	wheat germ agglutinin	HAI	10^0-10^1	40 (7)	GlcNAc
glycocluster	α-Man[188]	10	Con A	ITC	10^0-10^1	9 (2)	trimmanoside
glycocluster	α-Man[188]	10	Dioclea lectin	ITC	10^0-10^1	13 (2)	trimmanoside
glycocluster	sialyl Lews x[189]	10	P-selectin	ELISA	10^0-10^1	5 (2)	sialyl Lewis x
glycocluster	Man-α1−6-[Man-α1−3] Man[190]	12	Con A	ELLA	10^1-10^2	289 (4)	αMeMan
glycocluster	β-GalNAc[191]	4	*Vicia villosa* B_4 lectin	ELLA	10^0-10^1	5.1 (3)	GalNAc
glycocluster	Pk trisaccharide[66]	2	SLT-1 B-subunit	ELISA	10^1-10^6	880000 (10)	Pk trisaccharide
glycocluster	α-Man[192]	5	*E. coli* type 1 fimbriae	ELISA	10^1-10^2	354 (3)	αMeMan
dendritic	β-Gal(1−3) α-GalNAc[75]	5	murine antibody JAA-F11	ELISA	10^0-10^1	32 (4)	monovalent β-Gal ligand
dendritic	Gal-β1−3GalNAc-β1−4[sialic acid α2−3]-Gal-β1−4Glc[23]	6	cholera toxin B-subunit	solid phase	10^0	2 (7)	GM1
dendritic	Galβ1−3GalNAc[17]	4	murine T-antigen antibody	ELISA	10^2	115 (32)	Galβ1−3GalNAc-α-allyl
dendritic	β-Glc[19]	3	wheat germ agglutinin	HAI	N/A	N/A (1024)	GlcNAc
dendritic	β-lactose[18]	7	*Viscum album* lectin	ELLA	10^3-10^4	10345 (64)	lactose
dendritic	α-Man[32]	4	Con A	ELLA	10^0	0.4 (9)	αMeMan
dendritic	α-Man[40]	5	Con A	ITC	10^0	1.3 (6)	αMeMan
dendritic	α-Man[40]	5	Con A	HAI	10^0-10^1	12.7 (6)	αMeMan
dendritic	α-Man[39]	10	Con A	ITC	10^0-10^2	197 (6)	αMeMan
dendritic	α-Man[39]	10	Con A	HAI	10^0-10^2	>112 (6)	αMeMan
dendritic	α-thiosialic acid[193]	5	*Limux flavus* lectin	ELLA	10^0-10^1	6.7 (32)	α-thiophenylsialic acid
dendritic	β-LacNAc[194]	6	WGA	ELLA	10^0-10^1	21 (8)	β-allyl-GlcNAc
dendritic	α-thiosialic acid[195]	8	*Limux flavus* lectin	ELLA	10^0-10^1	15 (12)	α-azido-sialic acid
dendritic	α-Man[196]	4	pea lectin	ELLA	10^0-10^2	86 (16)	*p*-nitrophenyl-αMan
linear polymer	β-lactose[80]	1	galectin-3	ELLA	10^1	5 (20)	lactose
linear polymer	3′ sulfated β-Gal[79]	4	P-selectin	cell binding	10^0-10^1	20 (24)	sialyl Lewis x
linear polymer	α-Man[77]	8	Con A	HIA	10^2-10^3	∼2500 (143)	monovalent α-Man ligand
linear polymer	3′,6′-disulfo Le^x(Glc)[82]	4	L-selectin	ELISA	10^0-10^1	5.8 (15)	sialyl Lewis x
linear polymer	α-Man[100]	3	Con A	SPR	10^1	38 (143)	αMeMan
linear polymer	α-C-Man[197]	4	Con A	HIA	10^3-10^5	100000 (N/A)	αMeGlc

Table 1 (Continued)

ligand class	carbohydrate epitope	number of compared ligands	protein	assay	magnitude of enhancement	maximum enhancement[a] (valency)	reference ligand
linear polymer	α-Man[76]	6	*E. coli*	agglut.	10^1-10^2	150 (N/A)	α MeMan
linear polymer	β-Gal[198]	3	RCA_{120}	HIA	10^1-10^2	200 (N/A)	galactose
linear polymer	α-C-Man[81]	4	Con A	HAI	10^1-10^3	1935 (N/A)	αMeMan
linear polymer	vancomycin[199]	2	D-Ala-D-Ala dipeptide	SPR	10^0-10^1	16 (2−15)	monomeric vancomycin ligand
linear polymer	β-LacNAc[200]	2	RCA_{120}	HIA	10^1-10^3	1000 (N/A)	LacNAc
linear polymer	α-Man[201]	6	Con A	HIA	10^2-10^3	550 (N/A)	αMeMan
linear polymer	Lysoganglioside GM_3[202]	2	hemagglutinin	ELISA	10^0-10^3	526 (N/A)	GM_3
linear polymer	α-sialic acid[68]	41	hemagglutinin	HIA	10^2-10^6	2×10^6 (N/A)	sialic acid
linear polymer	α-sialic acid[69]	2	hemagglutinin	HIA	10^4-10^6	750000 (N/A)	sialic acid
linear polymer	α-sialic acid[69]	2	hemagglutinin	ELISA	10^3-10^6	1000000 (N/A)	sialic acid
branched polymer	α-sialic acid[203]	17	hemagglutinin from Sendai virus	HIA	10^2-10^4	40000 (N/A)	sialic acid
spherical polymer	sialy Lewis x[204]	5	E-selectin	cell binding	10^4	5000 (N/A)	sialyl Lewis x
spherical polymer	β-Glc[91]	2	Con A	turbidity	10^1	15 (N/A)	monovalent β-Glc ligand
spherical polymer (protein based)	sialyl Lewis x[205]	2	E-selectin	ELISA	10^2	90 (11)	sialyl Lewis x
spherical polymer (protein based)	β-GalNAc[206]	3	*E. histolytica* lectin	membrane binding	10^2	95.5 (20)	GalNAc
spherical polymer (protein based)	GT1b[207]	1	murine sialoadhesin	cell binding	10^1	25 (140)	GT1b

[a] Enhancement corrected for the valency of the ligand.

Table 2

ligand valency	valence-corrected enhancement		
	ELLA	HIA	ITC
2[a]	2.1	1.3	1.1
3[a]	1.1	1.0	1.0
4[a]	0.7	0.8	1.0
6[a]	1.4	0.6	ND
2[b]	2.3	1.8	1.1
3[b]	1.8	2.9	0.6
4[b]	3.0	47	8.1
6[b]	2.5	>112	2000

[a] Peptide-linked dendritic ligand. [b] PEG-linked dendritic ligand.

attention to a consideration of the mechanisms through which cluster glycoside effects arise. We consider three mechanisms for the enhancement: an intramolecular, or chelate, binding, an intermolecular aggregative process, and steric stabilization. We note at the outset that, in the absence of detailed thermodynamic data on the binding event, structural information regarding the bound complex or, better yet, both, unambiguous conclusions regarding the mechanism of enhancement are difficult to reach. Nonetheless, data suitable for such interpretation is now accumulating. Below we outline the salient features of each binding mechanism and speculate on the likelihood of its contribution to the observed phenomonology.

5.1. Intramolecular Binding: The Chelate Effect

The notion of a bivalent ligand binding to a bivalent receptor with an affinity greater than that of the monovalent counterpart is well-accepted in chemistry, particularly in inorganic chemistry where myriad natural and synthetic metal chelates are known. Any thermodynamic parameter characterizing a bivalent association is related to the corresponding term for monovalent association by an interaction energy, that is,

$$\Delta J_{bi} = 2\Delta J_{mono} + \Delta J_{int}$$

where ΔJ represents the change in any thermodynamic property during binding, “bi” refers to that parameter for the bivalent ligand, “mono” refers to that parameter for the monovalent ligand, and “int” refers to the interaction parameter, or the energetic consequence of physical linkage.[121] We are ultimately

concerned with determining the interaction free energies. Because it is sometimes more intellectually accessible to consider the enthalpic and entropic contributions to the interaction energy, we make this division here.

5.1.1. Entropic Contributions to Interaction Free Energies

Interaction energies for multivalent complexation are traditionally considered in entropic terms. The overall entropy of a particle can be considered as the algebraic sum of four components; namely the translational, rotational, conformational, and solvation-associated entropies. Similarly, an interaction entropy can be considered as the sum of a similar set of values.

Translational and Rotational Entropy. Prior to ligand binding, both ligand and receptor are free to translocate in three dimensions and to rotate on three principal axes. Following binding, a single aggregate particle retains these motional freedoms. Typically translational and rotational entropies are considered as a single term.

The translational entropy of a particle in the gas phase is described by the Sakur–Tetrode equation

$$S = k_B N_i \left[\left(\ln \left(\left(\frac{2\pi i m_1 k_B T}{h^2} \right)^{3/2} V \right) + \frac{3}{2} \right) - \ln(N_i - 1) \right]$$

where k_B is the Boltzman constant, T is the temperature, h is Planck's constant, m_1 is the mass of the monomeric molecule, and N_i is the number of monomers in the aggregate particle. Importantly, translational entropies vary as the natural logarithm of the molecular weight. Similarly, the rotational entropy of a particle is given by the expression

$$\frac{S}{R} = \log \frac{e^{3/2}\sqrt{\pi}}{\sigma} \left(\frac{T^3}{\theta_a \theta_b \theta_c} \right)^{1/2}$$

Here θ_a, θ_b, and θ_c represent the rotational degrees of freedom about the three principal axes and σ is the symmetry number of the molecule. A symmetrical molecule ($\sigma > 1$) will have a rotational entropy diminished by an amount equal to $R \log(\sigma)$. Again, the rotational entropy has a logarithmic relationship to the rotational degrees of freedom, in turn related to the molecular dimensions and, indirectly, to the molecular weight of the ligand.

The logarithmic relationship between molecular weight and translational and rotational entropy provides that tethering two (or more) ligands produces a multivalent ligand with a translational and rotational entropy roughly equivalent to that of the monovalent ligand. *The binding of a bivalent ligand thus proceeds with a favorable contribution to the interaction free energy equivalent to the translational and rotational entropy of the monovalent ligand*. The value of this term is crucial, since it presumably accounts for a large fraction of the interaction free energy. Although evaluation of the translational and rotational entropy in the gas phase is a relatively straightforward process, the situation is considerably more complex in condensed phase. The ability of a molecule to move in three dimensions is greatly diminished in solution relative to the gas phase; a highly interacting liquid such as water might reasonably be expected to exert an especially strong localizing effect. The gas-phase translational entropy for typically sized organic molecules is near 15 kcal mol^{-1} near room temperature. Estimates of the corresponding value in aqueous solution vary widely, ranging from 2.1 to >15 kcal mol^{-1}.[122] The often quoted value of Jencks of 10.5 kcal mol^{-1} [123] is at best a rough estimate and was probably never intended as a hard and fast value. Most estimates are centered at the lower end of the range, and the favorable contribution of ΔS_{T+R} to the interaction free energy is likely no greater than 6 kcal mol^{-1}.

Conformational Entropy. The restriction of conformational degrees of freedom during ligand binding reduces the overall entropy of the molecule. While the term is intellectually accessible, at least from the statistical perspective, its evaluation is again fraught with uncertainty. The entropy associated with a rotational mode is related to the internal partition function

$$Q = \frac{1}{n} \left(\frac{8\pi I_r k T}{h^2} \right)^{1/2}$$

where I_r is the reduced moment of inertia about the rotational axis and n is the symmetry number for the internal rotation. The entropy associated with this partition function is then given by the expression

$$S(Q) = R(0.5 + \ln Q)$$

Jencks suggested a value near 4.3 eu for the rotational entropy of a completely unrestrained rotor, providing a maximum loss of entropy for localization of 1.4 kcal mol^{-1} near room temperature;[124] other estimates are higher yet.[125,126] More recently, Mammen, Shakhnovich, and Whitesides proposed a novel model for the evaluation of conformational entropy; this model suggests a value roughly half that suggested by Jencks.[127] This model has been criticized as incomplete.[128] Other computational and experimental studies fall between these limits.[129,130]

An experimental approach to the measurement conformational entropy in proteins has been proposed. The approach begins from order parameters, in turn derived from magnetic resonance experiments.[131−134] Briefly, both dipolar and chemical shift anisotropy mechanisms of relaxation are coupled to molecular motion; evaluation of relaxation at various fields can then be related to a spectral density or the density of these motions. The spectral density can be converted to an order parameter S, in turn related to the conformational entropy by the expression

$$S_{conf} = k_B \ln[\pi(3 - \sqrt{(1 + 8S)})]$$

While for the most part this work has focused on backbone entropies, observing the amide NH vector, some work has been centered on the evaluation of changes in side chain entropy, using ^{13}C and ^{2}H relaxation parameters. The work has not been extended to small molecule studies.

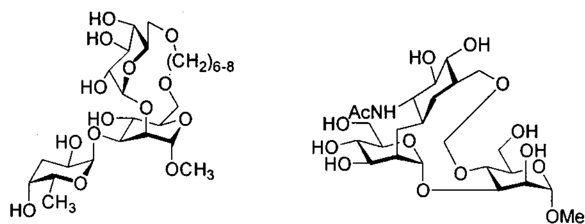

Figure 17. Tethered oligosaccharide ligands. Left: abequose trisaccharide for antibody Se 155.4. Right: trisaccharide ligand for concanavalin A.

Evidence continues to accumulate that conformational entropy losses during ligand binding are smaller than previously appreciated; even the Whitesides approach may significantly overestimate the term. A range of unnatural β- and γ-amino acids have been reported that form stable helical structures, despite a significant increase in the conformational entropy penalty for doing so.[135−144] The binding of propylenediamine tetraacetate to divalent calcium is some 5 kcal mol^{-1} less favorable than the corresponding binding of ethylenediamine tetraacetate: the loss in free energy is virtually all enthalpic (Christensen and Toone, unpublished results). Similarly, Bundle and Boons have independently synthesized a variety of conformationally 'locked' oligosaccharides designed to avoid losses in conformational entropy during ligand binding (Figure 17).[145,146] In the event, all such ligands bound with no greater affinity than the native flexible ligand. Although it is possible that such efforts lock the ligand in an unfavorable orientation, leading to enthalpic reduction in binding free energies, a calorimetric study by Bundle and co-workers found no such effect; to the extent that binding free energies were diminished relative to untethered ligands, the losses were entropic in nature.[145] Finally, NMR studies of overall protein conformational flexibility during ligand binding show a range of changes. While many proteins show the expected decrease in flexibility, others show *increased* flexibility following ligation.[147−150] In several other instances, increases in conformational flexibility at sites remote from the binding site apparently compensate for losses at the actual binding site.[151−153]

That losses in conformational entropy are, at least in some cases, considerably less than expected is somewhat surprising. Two plausible explanations rationalize the observation. First, bound ligands might retain significant flexibility. Second, the free ligand might access considerably fewer conformational states than would a completely unrestrained ligand. Such preorganization might arise from favorable interactions between portions of the ligand remote in sequence or from solvophobic effects that collapse the ligand to minimize accessible surface area. We note parenthetically that the ligand need not be preorganized in a favorable conformation for binding to minimize losses in conformational degrees of freedom.

Solvation-Associated Contributions to ΔS_i. Perhaps the largest contribution to uncertainty in multivalent binding arises from the solvation-associated term. The molecular basis of the change in entropy associated with the solvation of various solutes remains poorly understood. The best experimental measure of the thermodynamic consequence of solvent reorganization during processes in aqueous solution remains the change in molar heat capacity that accompanies the process.[112,154,155] On the other hand, the relationship between ΔC_p and solvation-associated enthalpy (and, by extension, entropy) is weak, and significant differences could well be invisible to this measure. Still, the observation of a significant difference in ΔC_p during the binding of related ligands likely signifies a significant contribution of solvation thermodynamics to the overall binding process.

5.1.2. Enthalpic Contributions to Interaction Free Energies

Although interactions energies are typically considered from an entropic perspective, enthalpic effects also play important roles in determining the overall affinity of a multivalent ligand. Two effects could, in principle, contribute to this term. First, the linker domain might alter the position of the ligand within the binding site; this effect would contribute unfavorably to the interaction enthalpy. Second, the linker itself could provide a contribution, either by interacting with the surface of the protein at the periphery of the binding site or over the space between sites or through unfavorable steric interactions that develop during restriction of the linker domain. The unfavorable contribution from the former term is relatively simple to overcome; linkers simply need to incorporate sufficient length and flexibility to facilitate optimal positioning of the carbohydrate recognition domain within the binding site. In the vast majority of examples listed in Table 1 the linker domain is of insufficient length to span two sites on a single protein. In such cases an effectively infinite unfavorable contribution to ΔH_I precludes intramolecular binding.

In instances where linker domains do not preclude an intramolecular bivalent binding, the interaction enthalpy consists of contributions arising from contacts between the linker domain and the surface of the protein and unfavorable steric interactions that arise in the linker during binding. There is every expectation that the former effect will be significant. Several groups have successfully replaced portions of a carbohydrate binding domain with peptide or protein analogues; clearly molecular motifs other than carbohydrates can interact in a favorable fashion with a lectin binding site.[156−163] Although favorable enthalpies arising from contacts between the linker and the protein surface could in principle amount to several kcal mol^{-1}, the effect is indeterminate in the general case, and difficult to predict. There are also intriguing suggestions that restriction of linker domains during intramolecular chelate-type binding can lead to significant and unfavorable enthalpic terms, as linker dihedrals are locked into gauche or eclipsed orientations. In retrospect, such effects seem inevitable and likely to make important contributions to overall interaction energies. A methyl−methyl gauche interaction provides roughly 1 kcal

mol^{-1} of unfavorable enthalpy; double gauche pentane is disfavored by 4 kcal mol^{-1} relative to the all anti form. Eclipsed conformers are even more problematic; the barrier to rotation about the carbon–carbon bond of ethane is 3 kcal mol^{-1} while butane eclipsed about the C_2-C_3 bond is 5–6 kcal mol^{-1} above the anti staggered form. Given the magnitude of such effects, it is hardly surprising that even small deviations from minimum energy conformations in the bound form could provide substantial unfavorable enthalpic contributions to interaction free energies. The 5 kcal mol^{-1} diminution in binding free energy of propylenediamine tetraacetate to Ca(II) relative to EDTA is virtually all accounted for by a loss in enthalpy; in conjunction with crystallographic studies of the metal chelate it seems likely that the formation of an eclipsing interaction in the propylene backbone is responsible for much of this loss (Christensen and Toone, unpublished results). Penel and Doig recently suggested that such strain energies induced as amino acid side chains restricted away from low-energy conformers contribute significantly to the overall free energy, opposing helix formation in short peptides.[164] This study suggested that this effect was at least as large as the loss in configurational entropy and may, in fact, be significantly larger.

5.1.3. Importance of Intramolecular Binding in Protein–Carbohydrate Interaction

To bind in an intermolecular fashion, the binding free energy for bivalent complexation need only be larger than that of the monovalent binding event; put another way, the interaction energy cannot be more unfavorable than the absolute value of the free energy of binding of the weaker of the two recognition epitopes. In practice, this condition is severe for protein–carbohydrate interaction, where monovalent interaction energies are small and distances between binding sites are large. For the vast majority of the ligands described in Table 1, the linker domain is too short to span two sites on a single protein, and a chelate-type binding motif is excluded a priori. In some instances, however, intramolecular binding seems likely. Most examples of this model involve either bacterial two-component toxins or polymeric ligands.

The bacterial two component toxins—specifically the *E. coli* heat-labile toxin, the Shiga and Shiga-like toxins (SLT), and the cholera toxin—present special opportunities for the construction of high-affinity ligands that operate through a chelate effect. Unlike most lectins, the bacterial toxins direct all five binding sites along a single axis; as a result they do not aggregate erythrocytes and are, presumably, less susceptible than plant lectins to aggregative behaviors with multivalent ligands. Additionally, the SLT is unusually small, with a subunit molecular weight of only 7700 Da. The binding subdomain apparently contains three sites per monomer capable of interacting with the trisaccharide receptor, and intersite distances are as small as 10 Å.[165] Fan, Hol, and co-workers reported a pentavalent ligand for the *E. coli* heat-labile toxin (Figure 11).[65] The activities of these ligands were evaluated in an ELISA protocol; the best ligands showed an enhancement of 10^4 over monovalent ligand on a valence-corrected basis. The authors ruled out an aggregative mechanism of enhancement through the use of dynamic light scattering studies, although solutions were passed through a filter small enough to remove large aggregates prior to evaluation. Distances between binding sites on this protein are roughly 45 Å, making construction of chelate-type ligands challenging. The authors made use of the concept of effective length, recently described by Kramer and Karpen.[166] In this rubric, entropic penalties resulting from restriction of conformational degrees of freedom are limited through the use of linkers considerably longer than required by the intersite distances. The work was motivated by entropic concerns; here we have suggested that such concerns may not be as severe as those imposed by enthalpic issues arising from unfavorable steric interactions. Longer linkers would, of course, also minimize enthalpic penalties by providing access to a much larger ensemble of conformations.

Toone and co-workers evaluated the binding of several multivalent ligands to the B-subunit of the SLT.[41,167] Although many ligands provide significant enhancements in activity compared to their monovalent counterparts, the mechanism of enhancement depends on the nature of the linker domain. Calorimetric evaluation of the binding of two bivalent C-linked peptide-linked ligands, one identified through a solid-phase screen, showed that while one ligand apparently functions through an chelate-type mechanism the other exerts its activity through an aggregative process. Here the binding motif is controlled by the nature of the peptide linker; while a hydrophobic linker results in chelate-type binding, a hydrophilic linker suppresses this binding mode and results in the formation of aggregates. Aromatic dendritic ligands produced strong enhancements in apparent affinity during ITC studies. These enhancements in free energy were accompanied by a diminution in binding enthalpy, a signature event we have previously linked to aggregation.[39,40] ITC studies at varying protein concentrations and mass spectrometric evaluation of ligand binding demonstrated that the enhancements in apparent activity were completely attributable to aggregation. These studies provide an important salutary caution regarding the development of binding models based on a single assay of activity.

The most impressive gains in activity on a per mole of saccharide basis are observed with polymeric ligands; many of these ligands are of sufficient length to span two sites on a single lectin, and the chelate binding model must at least be considered. On the other hand, given the well-known propensity of polymeric glycosides to aggregate and precipitate a wide range of multivalent lectins, the invocation of chelate binding requires additional extrinsic evidence beyond performance in a single binding assay. Kiessling and co-workers prepared series of polymeric mannosides and observed a striking increase in activity in HIA assays against concanavalin A as a function of average degree of polymerization or spacing between terminal residues.[77] The attainment

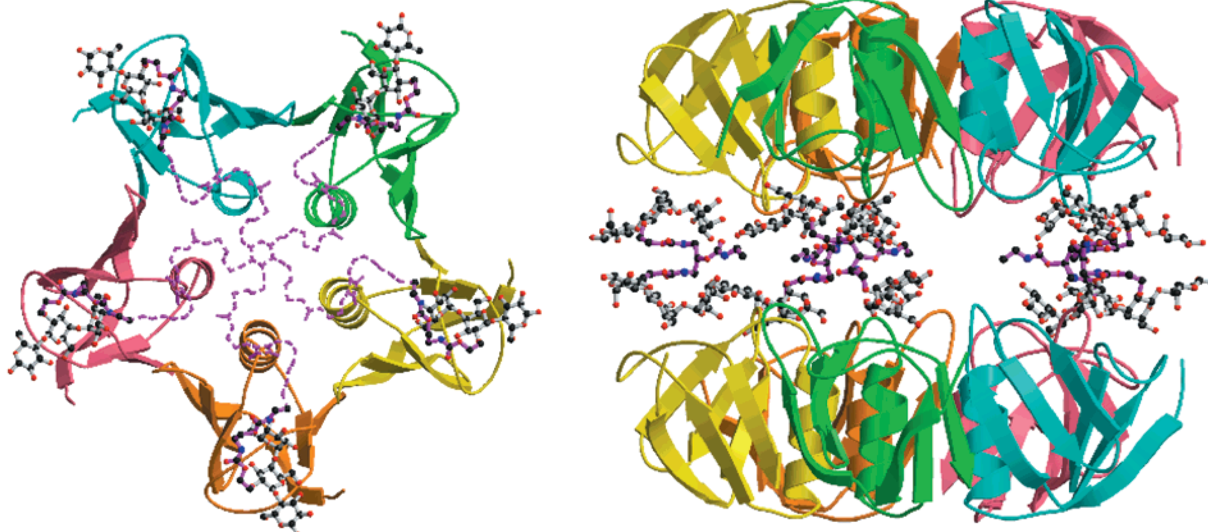

Figure 18. Crystal structure of the STARFISH ligand bound to the SLT.

of maximal activity seems to coincide with an average length sufficient to span two lectin binding sites. Consistent with other observations that losses in conformational degrees of freedom may not be as important as previously thought, ligands with different flexibilities showed roughly the same activity. Interpretation of the binding studies is complicated by the complex mixture of stereoisomers present in the assay mixture. Similarly, the actual linker length required to span two sites on concanavalin A is unclear. The commonly quoted intersite distance of 65 Å is a shortest-path distance that describes a chord to the Arc that a linker must take over the surface of the protein to access two sites simultaneously. Still, the results are provocative and are at least consistent with a chelate-type binding, if not uniquely consistent with this model. There is no convincing evidence that other polymeric ligands function through a chelate type binding, although they could in principle do so.

Perhaps the most striking example of an affinity enhancement involving chelate binding was reported by Bundle and co-workers.[66] Here a decavalent ligand, designated STARFISH (Figure 11), inhibits SLT in ELISA assays with a 10^6-fold enhancement over monomeric ligand, again on a valence-corrected basis. Crystallographic evaluation of the bound complex revealed a cross-linking of two B-subunit pentamers by a single decavalent ligand (Figure 18); the ligand thus shows activity by both aggregative and chelate mechanisms. The energetic consequence of each effect—intermolecular chelate binding and aggregation of two proteins—is unclear. Also unclear is whether this species is uniquely populated in solution; it is possible that more complex aggregates are populated or that, at low protein concentration, a 1:1 complex still forms at high affinity. As was the case in the Fan ligands, the STARFISH ligand utilizes linker domains considerably longer than required to span two binding sites in an extended conformation. Although sufficient examples to reach unambiguous conclusions about the relationship of linker length and affinity, the results in toto may point to important design features for the construction of high-affinity ligands.

5.2. Intermolecular Binding: Aggregation and Precipitation

Multivalent ligands can also bind multivalent receptors in an intermolecular fashion, potentially leading to the formation of large aggregates that precipitate from solution. Such an effect is the basis for the familiar precipitin reaction. Assuming linkers of sufficient length to facilitate intramolecular binding, an intermolecular binding will still predominate when the overall interaction energy for aggregate formation is more favorable than for an intramolecular assoication. Aggregates may be stabilized by a range of forces, including protein–protein interactions. A diminished solubility of the complex also contributes to the overall equilibrium, and kinetic effects arising from irreversible precipitation are coupled to apparent binding energies.

That multivalent saccharide ligands cross-link and aggregate multivalent lectins has long been recognized; indeed the quantitative precipitin reaction of multivalent lectins has been used extensively to characterize lectins. During the 1980s, Brewer and co-workers presented a series of papers that detailed the remarkable selectivity that accompanies the formation of insoluble cross-linked aggregates.[168−170] Apparently the microscopic structure of the aggregates facilitates the formation of homogeneous cross-links in the presence of multiple carbohydrate recognition epitopes, essentially magnifying the specificity of the binding.[171−173] The extent of precipitation is highly dependent on a range of factors, including the concentration of both ligand and macromolecule, the affinity constant characterizing the interaction, the valency of both ligand and macromolecule, the structure of both the ligand and macromolecule, and details of the solution, including ionic strength, pH, and temperature. The ratio of ligand to receptor, in addition to the concentrations of each, is also vital; large insoluble precipitates form only when the two are present in equimolar amounts. At proportions

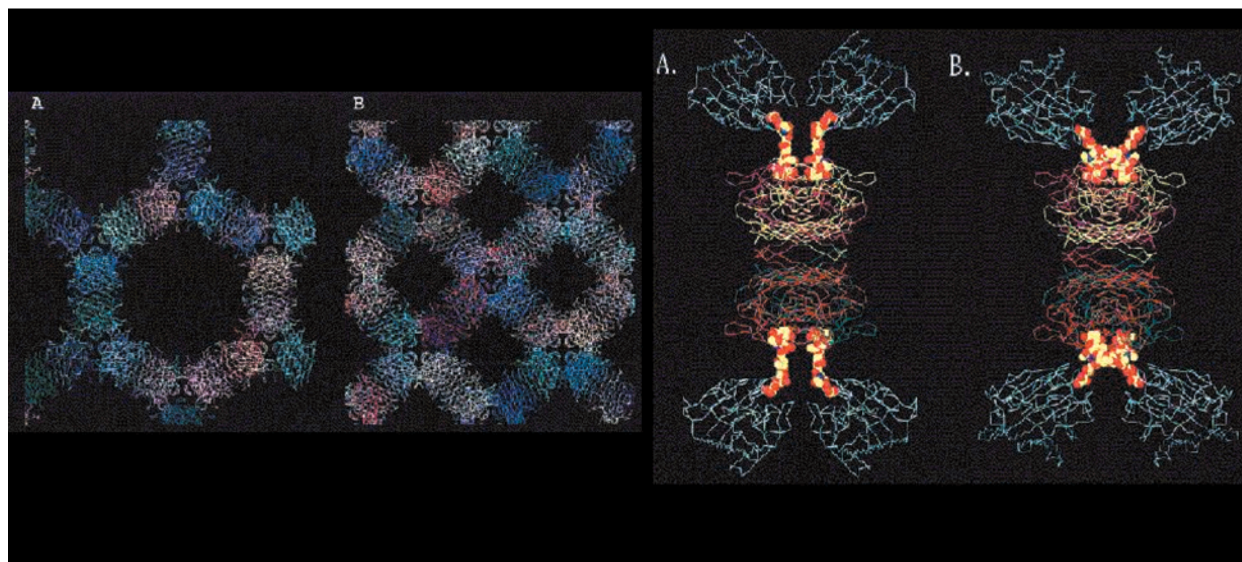

Figure 19. Cross-linking of the soybean lectin by isomeric pentasaccharides. Left: macroscopic cross-links produced by βGal(1→3)βGlcNAc(1→3)(βGal(1→4)βGlcNAc(1→6))βGalOR and βGal(1→3)βGlcNAc(1→2)(βGal(1→4)βGlcNAc(1→4))βGalOR. Right: Detail of cross-links produced by βGal(1→3)βGlcNAc(1→2)(βGal(1→4)βGlcNAc(1→3))βGalOR and βGal(1→3)βGlcNAc-(1→3)(βGal(1→4)βGlcNAc(1→6))βGalOR.

outside of this narrow stoichiometry, small soluble species are formed; over some concentration ranges presumably the 1:1 complex is populated. Various attempts have been made to model the precipitin reaction, but the lack of defined structure and the variable and unknown nature of the interactions between macromolecules during aggregate growth preclude a general analytical solution.[174−177] At a qualitative level, however, the system is intuitive. The nature of the system also suggests that results of precipitin assays will depend on concentrations, affinities, valencies, and ratios of interacting species through a complex series of interdependent relationships.

5.2.1. Importance of Aggregative Binding in Protein−Carbohydrate Interaction

At the outset, an aggregative model of enhanced activity seems by far the most reasonable with which to rationalize the cluster glycoside effect. The well-studied precipitin reaction, the ubiquitous and well-studied cross-linking behavior of myriad lectins with a structurally diverse series of multivalent ligands, the low monovalent affinities characterizing protein−carbohydrate interaction, and the large intersite distances found in high molecular weight multivalent lectins together conspire to bias such systems strongly toward aggregative behavior. Indeed, the importance of aggregative processes promoted by protein−carbohydrate interaction in vivo suggests that carbohydrate-mediated recognition is important in biology precisely because such multivalent interactions lead to clustering.[178−181] The vast majority of ligands described in Table 1 cannot span two sites on a single multivalent lectin, and it seems highly unlikely that any of these species provide enhancements in activity over those of the monovalent ligand by any mechanism other than aggregation.

Aggregation has been demonstrated in crystallographic studies of lectins. In 1997, Brewer and co-workers reported a crystal structure of soybean agglutinin cross-linked by four biantennary blood-group analogues that effectively function as bivalent ligands (Figure 19).[182] In each instance, the structure of the bivalent ligand induces a unique regular aggregate structure, despite the observation that the orientation of the carbohydrate epitope within the binding site is essentially equivalent. These results demonstrate at a molecular level the profound effect exerted on the regular lattice structure by modest changes in the structure of the scaffolding outside the carbohydrate recognition domain. Generalizing these results to multivalent ligands based on non-carbohydrate scaffolds, the results provide a ready rationalization for the significant effect of relatively small changes in aglycone structure on the activity of a multivalent ligand. In the general case, the formation of insoluble aggregates is too fast to allow crystal growth; presumably, however, some level of microscopic structure is preserved and accounts for observed differences in activity.

Naismith, Toone, and co-workers recently reported structural and energetic studies of concanavalin A binding to dendritic ligands.[40] Ligands with valencies greater than three showed enhancements in activity in agglutination, but not calorimetric, assays. On the other hand, calorimetric titration of tetra- and hexavalent ligands showed substantially diminished enthalpies of binding and visible cloudiness, observations consistent with the formation of aggregates. Crystals of both bi- and trivalent ligands grew in the presence of succinylated concanavalin A, a form of the protein known to exist in dimeric form. Surprisingly, the cocrystal structure shows the protein exclusively in tetrameric form with protein−protein contacts essentially identical to those present in native tetrameric protein (Figure 20). Apparently succinylation shifts the dimer−tetramer equilibrium sufficiently to facilitate crystal growth, rather than the formation of macroscopically amorphous aggregates. Again, the

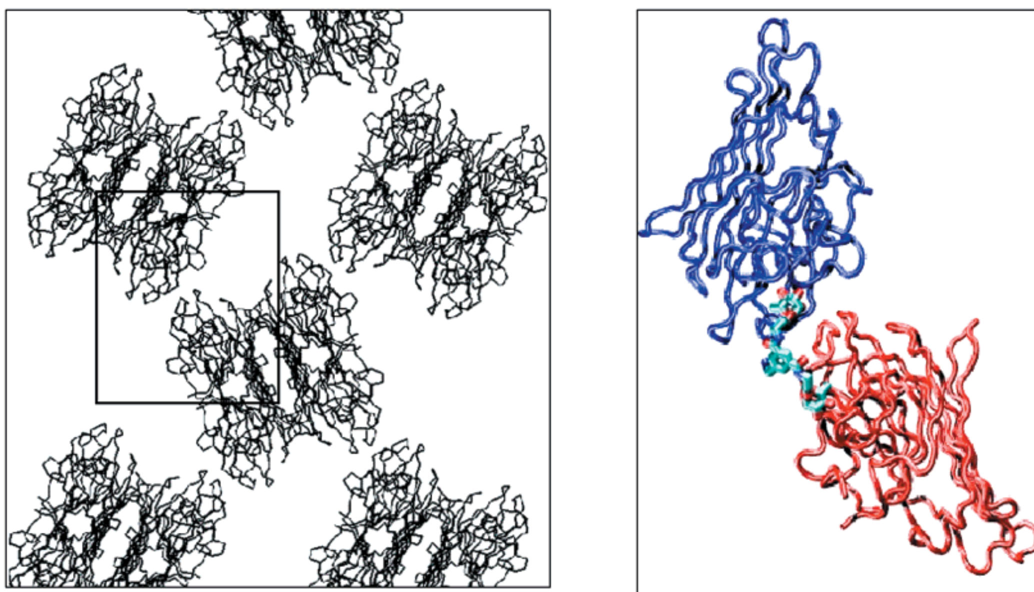

Figure 20. Cross-linked crystals of concanavalin A. The infinite sheets of tetrameric lectin (left) are held in place by intermolecular bivalent binding (right).

orientation of the carbohydrate within the binding site is identical to that of monomeric ligand, an observation that bolsters suggestions that diminished enthalpies of binding of multivalent ligands are the result of aggregation. The structures of cocrystals with bi- and trivalent ligands are identical, and density for the third carbohydrate residue of the trivalent ligand is not visible. Apparently the constraints imposed by the macroscopic structure of the cross-linked lattice preclude binding of all three saccharide residues. The diminished binding constant and subunit stoichiometry observed during ITC studies with this ligand are consistent with this observation and strongly suggest that cross-linked aggregates in solution have structures similar to those in the crystalline phase.

Intermediate between the formation of 1:1 complexes and large aggregates lie soluble complexes of a stoichiometry greater than 1:1. The STARFISH ligand of Bundle and co-workers described above is an example of such a binding motif, although the energetic consequence of dimerization compared to chelate binding of carbohydrate recognition domains is unclear. Burke, Kiessling, and co-workers recently reported the preparation of a templated trivalent ligand for concanavalin A (Figure 10).[60] This work suggests that ligand binding promotes the formation of small aggregates which, in turn, bind immobilized ligand in an SPR assay with greater affinity than does isolated tetravalent protein. The energetic consequences of aggregate formation are unclear, although the observations certainly suggest that additional studies on the behavior of small clustered ligands is warranted.

5.3. Steric Stabilization

Colloidal particles are kinetically stabilized when surfaces are coated with both polyelectrolytes and polymers that extend out into solution. Proteins have also been stabilized by a similar strategy, especially through surface incorporation of such hydrophilic polymers as methoxy polypropylene glycol.[183,184] Such proteins show enhanced thermal stability, resistance to denaturation during freezing, and resistance to proteolytic degredation, in each case presumably as a result of diminished protein–protein contacts available.

Whitesides and co-workers have suggested steric stabilization as a third motif by which multivalent ligands might show enhanced activity in some assays. In this motif, binding of a large species near the surface of a carbohydrate binding protein would prevent the approach of other macromolecules. Such effects should be most significant for polymeric ligands, species that provide significant steric bulk, in aggregation assays, where the approach of multiple macromolecules or particles form the basis of the assay. Indeed, examples supporting this motif fit these criteria. While the mechanism is well-documented in colloid science and seems reasonable, the evidence offered in support of its relevance to the cluster glycoside effect remains indirect. A variety of calorimetric and mass spectrometric assays might shed further light on this mode of interaction and its applicability to the general case of protein–carbohydrate interaction.

6. Conclusions and Future Outlook

Many pathological interactions are mediated by protein–carbohydrate recognition events. The low affinity of carbohydrate binding proteins for their mono- or oligosaccharide ligands frustrates efforts toward the development of carbohydrate therapeutics, species that would act through the inhibition of pathological recognition and adhesion. By now the cluster glycoside effect—an enhancement in valence-corrected binding activities of multivalent saccharide ligands—is well-demonstrated. The mechanisms by which such ligands act is slowly coming into focus. The most important single mechanism of action for

those ligands reported to date is aggregation; multivalent ligands cross-link and precipitate multivalent lectins. The stringent demands imposed by lectin structure and the nature of protein–carbohydrate interaction, namely weak monovalent binding activities and large distances between binding sites, make construction of multivalent ligands that bind with high affinity in an intramolecular sense a daunting task. Additionally, the assays used to evaluate protein–carbohydrate interaction each evaluate a range of different process, either because the assay itself measures a phenomenon that incorporates additional interactions beyond simple binding or because the assays operate under different concentration regimes. The evaluation of binding by a variety of techniques facilitates a better understanding of the molecular basis by which multivalent ligands exert their effects.

At this point it seems reasonable to assume, in the absence of evidence to the contrary, that enhancements in activity over monovalent ligands result from aggregation and/or precipitation. On the other hand, recent reports of high-affinity multivalent ligands for bacterial two-component toxins are promising and suggest that the creation of biologically relevant high-affinity ligands is far from impossible. Additional studies of small molecule model compounds in aqueous solution are required to better understand the energetic consequences of multivalent binding. The role of small cluster formation in the creation of stable complexes and, as a result, effective high-affinity ligands remains unclear and requires further study. Finally, the role of additional mechanisms of action, including steric stabilization, requires further clarification. Together, these studies will provide a basis on which to consider the general phenomenon of association in aqueous solution, in addition to a framework for the synthesis of useful biological compounds.

7. Acknowledgment

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