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Structural investigation of multivalent carbohydrate–protein interactions using synthetic biomolecules

Valentin Wittmann

Understanding multivalent carbohydrate protein interactions at the molecular level requires access to structural details of these important biological recognition processes. Recent developments toward this goal comprise the use of conformationally defined molecular rulers in combination with binding assays, crystallographic investigation of complexes of multivalent ligands and their target proteins, and distance measurements in the nanometer range by EPR spectroscopy.

Address
University of Konstanz, Department of Chemistry and Konstanz Research School Chemical Biology (KoRS CB), Universitätsstr. 10, 78457 Konstanz, Germany

Corresponding author: Wittmann, Valentin (mail@valentin wittmann.de)

Introduction

Molecular recognition of carbohydrate structures governs a multitude of biological processes such as cell adhesion and signal transduction. Examples include the inflammatory response, viral and bacterial pathogenesis, fertilization, immune system modulation, and cell proliferation [1 3]. The proteins that interact with the carbohydrates are mostly lectins and antibodies. Generation of high-affinity ligands for these proteins is of great medicinal interest for several applications. Blocking carbohydrate protein interactions may be beneficial for the treatment of certain diseases such as intoxications by bacterial toxins or acute or chronic inflammatory diseases. Lectin ligands carrying suitable probes can also serve as diagnostics for certain conditions.

A hallmark of many carbohydrate protein interactions is multivalency [4,5]. Indeed, many lectins contain multiple sugar binding sites mostly because they exist as oligomers or they are clustered on cell surfaces [6]. Binding partners can be multiple carbohydrates within a glycan or a multivalent display of glycans on a (cell) surface. Multivalency can not only result in increased binding affinity (referred to as avidity) but also in increased binding specificity [7,8]. Several types of interactions can occur between multivalent ligands and receptors. The simultaneous binding of a multivalent ligand to several binding sites of a receptor (also referred to as chelating binding) can lead to strongly increased affinity if the spacing of the individual carbohydrate ligands matches the distances of the receptor binding sites [9]. Besides chelating ligands, ligands with a sugar spacing too short to bridge adjacent binding sites can also be effective. The mechanisms discussed to explain increased binding affinity in these cases include statistical rebinding or the bind and slide mechanism [10].

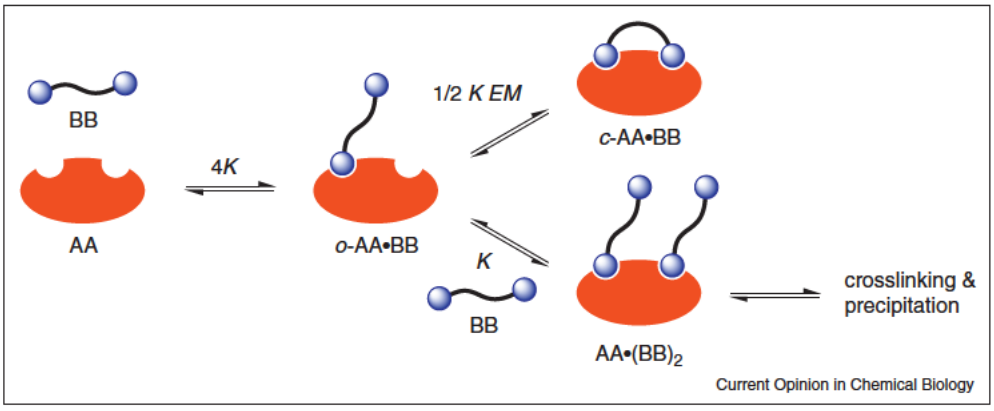
Since chelating binding is especially effective to arrive at high-affinity ligands, many groups focused on the design of suitable ligands [11 14]. Accordingly, a vast variety of scaffolds have been developed for the presentation of carbohydrates including dendrimers, peptides, proteins, nucleic acids, (cyclic) oligosaccharides, small organic scaffolds, quantum dots, nanoparticles, surfaces, and polymers [15•]. Such efforts follow different strategies. Large flexible ligands, such as glycopolymers, can cover wide areas of cell surfaces and bridge multiple membrane-located lectins in a statistical manner (statistical multivalency). On the other hand small oligovalent ligands may bind to several binding sites of an individual (oligomeric) lectin. In principle, with rigid well-designed ligands, selectivity for lectins with matched inter-binding-site distances can be achieved over other lectins with the same carbohydrate selectivity but unsuitable binding site spacing (targeted multivalency). Required for such a design is knowledge about the 3D structure of the protein and the location of the binding sites. This information, however, is often missing. But even if the structure of a lectin is known, different binding modes of a multivalent ligand can occur and it is a challenge to unravel the structural details of such interactions.

Summarized in this article are recent examples aiming at the determination of structural details of multivalent carbohydrate protein interactions using synthetic biomolecules.

Binding modes of multivalent interactions

Carbohydrate-binding proteins can interact with glycans through a variety of mechanisms. Both binding partners (glycan and protein) can be immobilized on a cell surface or one or both binding partners can exist in soluble form leading to different scenarios. Even when looking at the simplest system of a multivalent interaction, the binding

Figure 1



Binding of a divalent ligand BB to a divalent protein AA.

of a divalent ligand to a divalent protein (Figure 1) [16,17], it is challenging to determine or forecast the binding mode of the ligand. The first step of the interaction is the monovalent attachment of the ligand BB to protein AA forming the partially bound 1:1 open complex *o*-AA·BB followed either by intramolecular (chelating) binding to give the fully bound 1:1 cyclic complex *c*-AA·BB or by binding to a second ligand BB forming the 1:2 complex AA·$(BB)_2$. Depending on the conditions, further binding events, such as crosslinking and precipitation, can occur. The tendency of a divalent ligand to form the cyclic complex *c*-AA·BB is expressed by the product ½ *K EM*, where ½ is the statistical factor for the cyclization process, *K* is the microscopic intermolecular association constant, and *EM* is the microscopic effective molarity. Highest values for *EM* are expected for perfectly fitting linkers. Linkers that are too long or too short to support chelating binding have lower *EM* values.

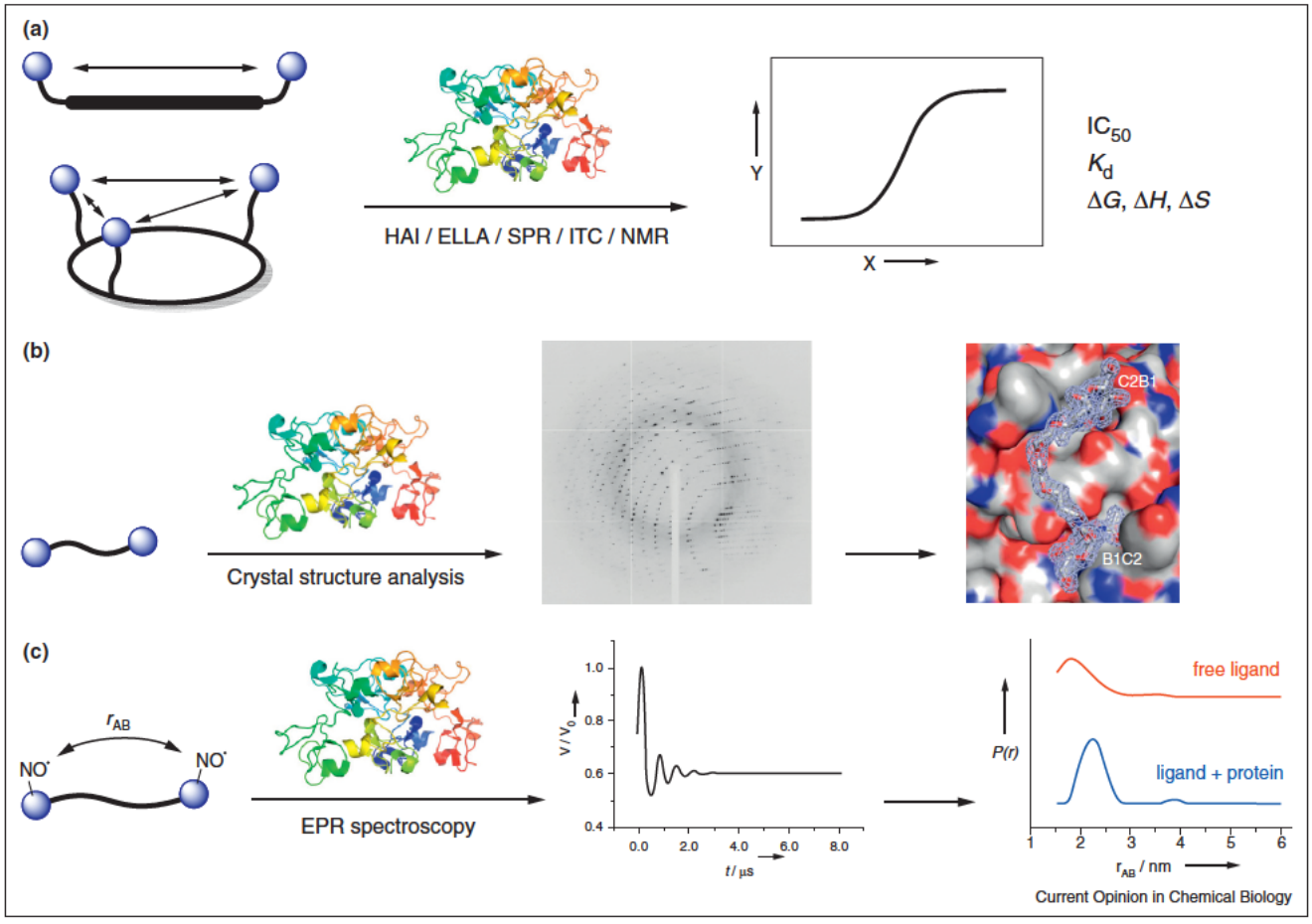
Approaches to structural investigations with synthetic biomolecules

Variation of the length and flexibility of the linker within multivalent ligands and subsequent evaluation of the effect on binding affinity (Figure 2a) is a common strategy to discover high-affinity lectin ligands [15•,18]. Typical analytical methods for the determination of binding affinities are inhibition of hemagglutination (HAI), enzyme-linked lectin assays (ELLA), surface plasmon resonance (SPR), isothermal titration microcalorimetry (ITC), and NMR spectroscopy [4]. High binding enhancements of divalent ligands over the monovalent reference compounds is generally correlated with the ability of the linker to bridge binding sites (chelating binding mode). These experiments, thus, not only generate good ligands but also provide information on possible distances between binding sites which, in turn, can serve as a blueprint for the design of new ligands. However, strong binding enhancements can also be caused by effects other than chelating binding. An instructive example stems from Pickens et al. who investigated a series of divalent ligands as surface receptor binding inhibitors of the cholera toxin B pentamer [19]. The group observed substantially improved inhibitory potencies for the ligands varying slightly with the linker length although the linkers were too short to allow chelating binding. A combination of solution and crystallographic studies suggested that additional linker protein interactions and steric blocking might be responsible for the enhanced inhibitory potencies. Nevertheless, careful design of ligands with well-defined spacing between sugar epitopes (molecular rulers) can provide valuable information about distances of binding sites as discussed in the next section.

ITC provides the thermodynamic signature of receptor ligand interactions including binding enthalpy, entropy, and stoichiometry. However, in contrast to monovalent interactions the analysis of ITC data of multivalent interactions for relevant thermodynamic parameters is not straightforward because a binding model must first be assumed which can be problematic in cases with mixed binding modes [20,21]. In addition, precipitation often complicates these studies.

Structural information with molecular resolution is obtained from NMR spectroscopy [22] and (more frequently) crystal structure analysis of carbohydrate protein complexes (Figure 2b) [23]. However, although many of such structures have been solved (3D-lectin database; URL: http://lectin3d.cermav.cnrs.fr), there is still only a low number of published examples with multivalently bound carbohydrates. Shenoy et al., for example, studied multivalent binding of high-mannose oligosaccharides of HIV-1 gp120 to cyanovirin-N by NMR spectroscopy [24]. Successful examples of

Figure 2



Structural investigation of multivalent carbohydrate protein interactions.

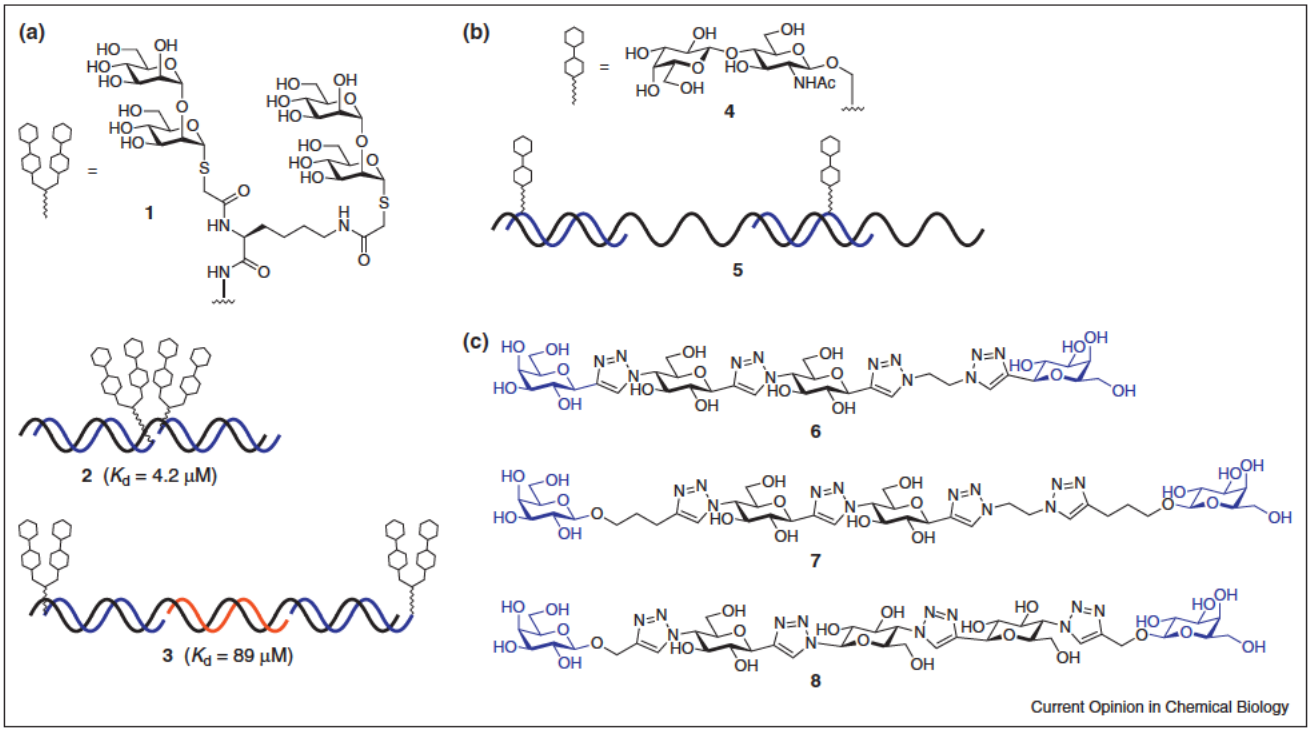
crystal structures of carbohydrates multivalently bound to proteins include complexes of Shiga-like [25] and cholera toxins [26,27]. Some recent examples will be treated below.

The structure determination of carbohydrate protein complexes in solution is demanding, especially if mixed binding modes occur. Recently, distance measurements in the nanometer range by EPR spectroscopy of spin-labeled divalent ligands were employed to analyze multivalent binding to wheat germ agglutinin (Figure 2c) [28••]. Measurements were carried out at 40 K in a glassy state obtained by shock freezing of the protein/ligand solution to which a small amount of glycerol had been added. It is assumed that the distribution of conformers of the biomolecules trapped in this way represents the conformer distribution in solution. With this technique it was not only possible to distinguish monovalent from divalent binding but also to determine differences in binding affinities of individual binding sites of the lectin.

Application of molecular rulers

DNA duplexes, especially those with peptide nucleic acids (PNA) as pairing strand, are interesting scaffolds that have been utilized for positioning carbohydrate epitopes. They are characterized by a high persistence length and the base-pairing rules allow for a defined positioning of the sugars although not every distance can be realized and the sugars may point to different sides of the helical structure. Following initial investigations by the Kobayashi group [29], Winssinger and coworkers used DNA PNA hybrids to generate multivalent carbohydrate ligands with controlled topology for the broadly neutralizing anti-HIV antibody 2G12 [30]. Two Man(α1-2)Man disaccharides were attached via a linker mimicking the substructure of the natural ligand (a high-mannose oligosaccharide) mostly responsible for antibody binding and connected to either the C-terminus or N-terminus of a PNA molecule (Figure 3a). The obtained conjugates were then hybridized to different DNA templates resulting in architectures with varying, yet predictable spacing between the sugar residues, and

Figure 3



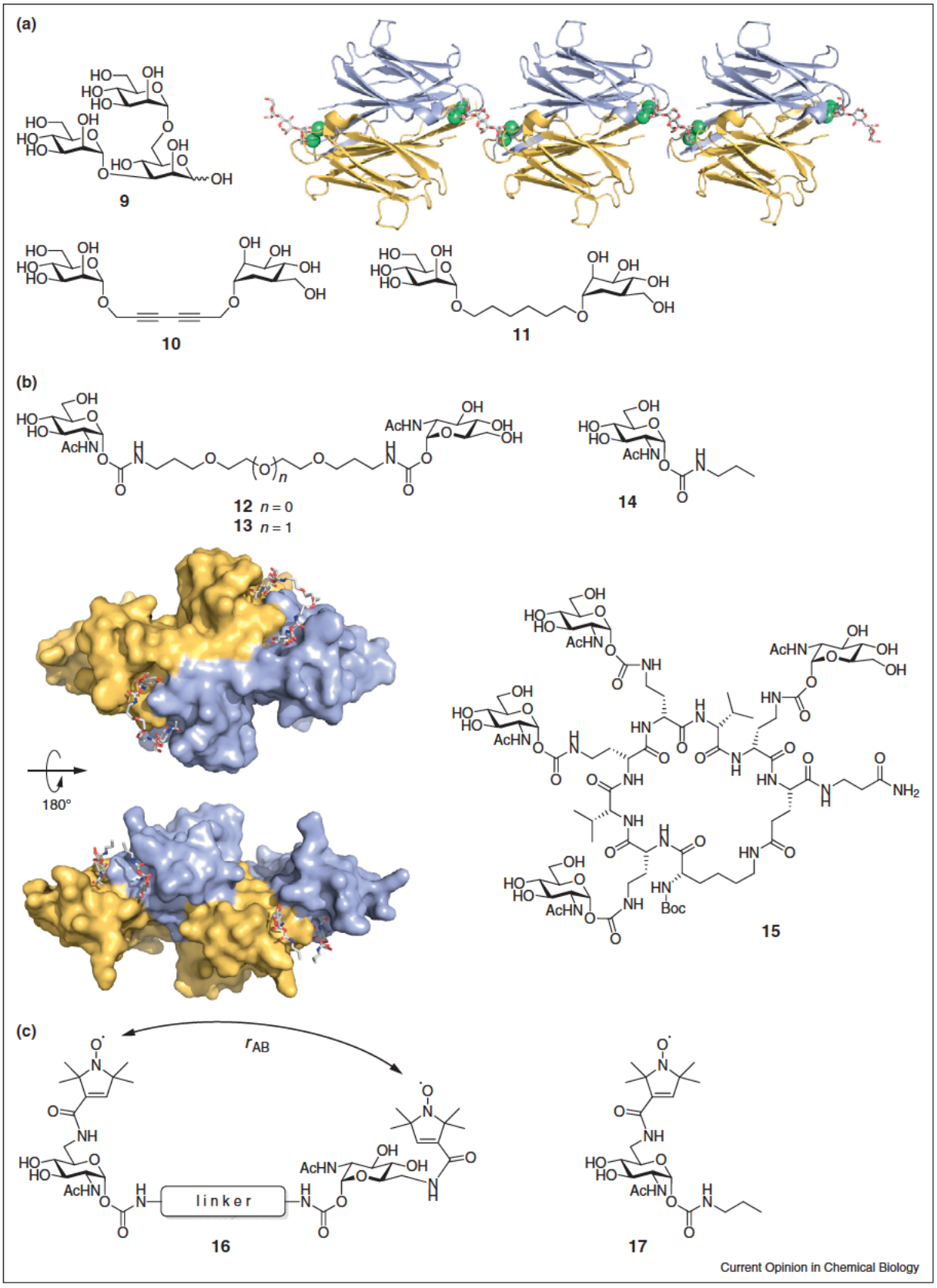
Spatial screening of lectin ligands with molecular rulers.

binding affinity for 2G12 was determined by SPR. It was found that only oligosaccharide mimics of type **1** resulted in 2G12 ligands. As expected, the topology of the supramolecular architecture had a significant impact on binding affinity. Complex **2** with a spacing of 38.5 Å between the branching points joining the disaccharides was the best binder. Ligands with longer distances as in **3** bound weaker in line with the required distance of approx. 31 Å that had been determined from a previous crystal structure of 2G12 in complex with two oligomannosides.

Seitz and coworkers followed a similar strategy and attached *N*-acetyllactosamine (LacNAc) units **4** to DNA PNA duplexes to generate multivalent ligands for *Erythrina cristagally* lectin (ECL) (Figure 3b) [31,32]. Screening of a series of divalent ligands revealed an optimal distance between the disaccharides of ca. 104 Å which corresponds to the known distance between the binding sites when taking the curvature of the protein surface into account. Increasing the flexibility of the nucleic acid scaffold by introduction of nick sites or single-strand regions as in **5** was beneficial for binding most likely because it facilitates bending of the DNA PNA complex around the protein. However, it is obvious that distance determinations with flexible ligands are less precise. More recently, the same group applied LacNAc-modified DNA PNA hybrids for the spatial screening of *Ricinus communis* agglutinin (RCA_{120}) [33•]. Again, highest binding affinity was observed for a divalent ligand with a separation of the LacNAc residues (140 Å) matching the required distance (≥130 Å) to bridge the binding sites seen in the crystal structure. Interestingly, the screening process unraveled a second, much shorter distance (62 Å) that also led to high affinity indicating a potential secondary binding site that has so far not been described. The procedure was also applied to structurally ill-defined assemblies of L-selectin on nanoparticles and leukocytes using sialyl Lewis^x (sLe^x) and a DNA aptamer, respectively, as ligands [33•]. Although the observed binding enhancements were less dramatic, differences in the sLe^x surface density on both surfaces could be detected. Duplex DNA was also used by Schlegel et al. for the defined presentation of carbohydrates although no spatial screening was carried out [34].

The lectins LecA and LecB (or PA-IL and PA-IIL) of the pathogen *Pseudomonas aeruginosa* are virulence factors involved in biofilm formation. Pertici and Pieters designed divalent ligands to bridge the distance of 26 Å between the two closest binding sites of LecA [35,36•]. Rigidified spacers of different length equipped with flexible ends were terminated with two galactose residues (Figure 3c). An ELISA-type assay revealed that even small variations of the linker structure had a

Figure 4


(a)
9
10
11
(b)
12 n = 0
13 n = 1
14
180°
15
(c)
rAB
linker
16
17
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tremendous effect on inhibitory potency. Whereas divalent **6** had an even worse potency compared to a monovalent reference compound, the somewhat elongated **7** was a 545-fold (272-fold per sugar) and second-generation ligand **8** (IC_{50} = 2.7 nM) was a 7555-fold (3780-fold per sugar) better inhibitor. Molecular modeling confirmed that both **7** and **8** can bind in a chelating binding mode to LecA whereas **6** is slightly too short to bridge both binding sites.

In the examples discussed above systematic spatial screening of carbohydrate-modified molecular rulers confirmed the distances between binding sites of structurally characterized proteins. The findings indicate that the use of such molecular rulers is a valid approach to scan also undefined proteins for inter-binding-site distances. Highest accuracy is obtained with conformationally restricted, rigid linkers. In this case, however, even small deviations from the matching case may lead to a significant or even complete loss of binding enhancements. Therefore, several groups developed combinatorial approaches to identify multivalent ligands from large ensembles of spatially diverse glycoclusters [37 41].

Crystallographic investigation of multivalent interactions

X-ray crystallography of complexes of multivalent ligands and their target proteins provides the most comprehensive picture of the mode of interaction between the binding partners. A common problem in generating co-crystals of multivalent ligands and receptors is the formation of random aggregates. Consequently, structural information on such complexes is rare.

Lameignere et al. investigated BC2L-A, a *Burkholderia cenocepacia* soluble lectin related to the lectin LecB from *P. aeruginosa* [42••]. BC2L-A is a monovalent lectin that forms dimers. It has a specificity for mannosides with a K_d in the micromolar range and is hypothesized to play a role in biofilm formation. Examined ligands included three differently linked mannose disaccharides, the trisaccharide Man(α1-3)[Man(α1-6)]Man **9** and two synthetic divalent ligands with either a rigid (**10**) or a flexible linker (**11**) (Figure 4a). ITC measurements indicated that only the rigid divalent ligand **10** had a significantly increased binding affinity over α-methyl mannoside and induced clustering of the lectin (protein monomer/ligand ratio 2:1) whereas the trimannoside **9** was a ten times weaker binder and bound in a 1:1 stoichiometry without inducing clustering. Co-crystallization of the protein with trisaccharide **9** required several weeks. Surprisingly, the high-resolution structure revealed that the trisaccharide exists in an stretched conformation in which it can bridge two adjacent protein dimers leading to infinite chains (protein monomer/ligand ratio 2:1) (Figure 4a). Obviously, the different time scales of the ITC experiment and the crystallization process are the reason for the different binding mode.

Schwefel et al. studied the interaction of several di-, tri-, and tetravalent ligands with wheat germ agglutinin (WGA) by an ELLA and by X-ray crystallography [43••]. WGA exists as a dimer with eight binding sites (four primary with high affinity and four secondary) for *N*-acetylglucosamine (GlcNAc). Divalent **12** (Figure 4b) showed a remarkable multivalency effect (1170-fold binding enhancement per sugar). X-ray crystallography of the complex of WGA and the second best, closely related ligand **13** explained this affinity. Four molecules of **13** simultaneously bind to the protein with each ligand bridging pairs of adjacent binding sites. This structure showed for the first time that all eight binding sites of the WGA dimer are simultaneously functional. In contrast, the crystal structure of WGA in complex with monovalent GlcNAc derivative **14** revealed that only the primary (high-affinity) binding sites were occupied. Thus, the increased binding affinity of a divalent, chelating ligand provides a means for the identification of secondary (low-affinity) binding sites. Reported was also tetravalent neoglycopeptide **15** with a binding potency 25,500 times higher than that of GlcNAc (6400 times per sugar). X-ray crystallography revealed that **15** binds to WGA with two sugars in a chelating binding mode. Comparison of the X-ray structure with the NMR-derived solution structure of **15** suggests that the peptide is preorganized in solution in a way supporting chelating binding.

Application of EPR spectroscopy

Since binding mechanisms of multivalent interactions in a densely packed crystal may differ from those in solution, Braun et al. employed distance measurements in the nanometer range by using EPR spectroscopy of spin-labeled WGA ligands to study multivalent binding in (frozen glassy) solution [28••]. Double electron electron resonance techniques (DEER or PELDOR) delivered distance distributions between two nitroxide labels at opposite ends of divalent ligands **16** (Figure 4c). Analyses of the distance distributions showed a detailed picture of the binding mechanisms of the divalent ligands. For example, a ligand with a flexible linker gave rise to a broad distance distribution when free in solution. Upon chelating binding to a protein, its flexibility was reduced leading to a narrower distance distribution with a maximum indicative for the distance between the binding sites (cf. Figure 2c). In this way, chelating binding is

(Figure 4 Legend) Multivalent lectin ligands used in crystallographic and EPR spectroscopic investigations. **(a)** Synthetic ligands for BC2L A and crystal structure of **9** in complex with the protein (PDB ID: 2WRA). **(b)** Mono , di , and tetravalent WGA ligands and crystal structure of **13** in complex with the protein (PDB ID: 2X52). **(c)** Spin labeled WGA ligands for EPR spectroscopic distance measurements.

directly detected and can be differentiated from monovalent binding of multiple molecules. In addition, it was possible to use increasing concentrations of spin-labeled monovalent ligand **17** to effect successive binding to the multivalent protein. This approach allowed to measure distances between binding sites and to detect differences in the binding affinities of individual binding sites.

Conclusions

The generation of high-affinity multivalent lectin ligands is increasingly recognized as promising approach to obtain drugs for therapeutic and diagnostic applications. Although a wealth of scaffolds has been elegantly designed and many of the obtained ligands show tremendous binding enhancements over their monovalent constituents, the underlying mechanisms of the affinity gain are often not well understood. It is generally accepted that chelating binding leads to strong multivalency effects and potentially also to enhanced selectivity, especially with rigid, perfectly fitting linkers between the sugar residues. The rational design of such linkers, however, requires knowledge of the protein structure. Systematic scanning of distances between binding sites is possible to some extent with molecular rulers that allow for precise control of spatial presentation of carbohydrates. Much deeper insight in the structural details of multivalent interactions is gained by crystal structure analysis. However, co-crystallization may be hampered by precipitate formation. Therefore, innovative methods for structural investigation of multivalent interactions such as distance measurements by EPR spectroscopy are urgently needed. They will be a further step toward a deeper understanding of multivalent interactions.

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