High affinity, specific binding events have dominated most thinking about receptor–ligand interactions [1]. Here, we focus instead on a class of weak interactions that are very important in biology, those that occur when extracellular proteins bind to carbohydrates. Protein–carbohydrate interactions are critical in processes such as fertilization, bacterial and viral pathogenesis, and the inflammatory response. Despite their importance in these specific recognition processes, individual protein–carbohydrate interactions often are of low affinity, and, worse, broad specificity. Why are such low affinity interactions important in biological systems? What determines binding specificity in such events? Researchers have just begun to develop the tools with which to address these significant questions.

Individual interactions of low affinity

Structural studies of several protein–carbohydrate complexes have helped to illuminate the molecular basis for low affinity binding. It is clearly possible to bind to carbohydrate targets with relatively high affinity; for example, the arabinose-binding protein contains a discrete binding pocket for its carbohydrate ligand in which it forms multiple contacts to its ligand [2]. This mode of recognition, however, is not typical for extracellular carbohydrate-binding proteins. Any proteins in this group, such as the serum mannose-binding protein A [3] or E-selectin [4], contain only shallow binding grooves. Their binding sites are largely solvent exposed and the proteins make only a small number of direct contacts with their target ligands (Fig. 1). As expected, these proteins often bind with little discrimination and low affinity to a set of related monovalent carbohydrate structures.

The importance of multivalency

The low affinity and relaxed specificity of individual protein–carbohydrate interactions is difficult to reconcile with the striking diversity of oligosaccharide structures that are involved in specific recognition processes [5]. Given this paradox, many scientists have speculated that multiple protein–carbohydrate interactions could cooperate in each recognition event to give the necessary functional affinity. This means that multiple receptors must be arranged in such a way as to bind efficiently to multiple saccharide ligands. Figure 2 shows several possible ways in which this could be accomplished. The requirement for the spatial arrangement of the receptors and the binding sites on the ligand to be compatible means that specificity in multivalent binding could be achieved, not only via complementarity between individual receptor–ligand pairs, but also by controlling the spatial arrangement between individual recognition elements of a multivalent ligand, or by changing the number of individual interactions (Fig. 2).

Fig. 1. The number of contacts formed between a carbohydrate-binding protein and its ligand affects the affinity of the interaction. (a) Arabinose-binding protein surrounds its monosaccharide ligand (red) [39]. This interaction is relatively high affinity ($K_d = 98 \text{ nM}$ [2]) compared to more typical carbohydrate-binding proteins such as (b) rat mannose-binding protein, shown here in complex with $\alpha$-methyl mannoopyranoside (red) [40]. The low affinity of this interaction ($K_d = 2.9 \text{ mM}$ [41]) is presumably due to the small number of contacts between the ligand and the protein.

Many processes mediated by protein–carbohydrate interactions involve multivalent low-affinity binding, which is inherently difficult to study. New structural templates for the generation of multivalent carbohydrate displays have recently been developed, and tailored multivalent saccharide derivatives can now be used to study and modulate a wide variety of biological recognition events.
cells. What advantages does a multivalent binding system confer in a biological setting? First, since the characteristics of binding can be tuned by alteration of the individual saccharide residues or their relative orientations (Fig. 2), recognition events can be readily and flexibly modulated. Second, the kinetics of multipoint attachment will be different from those involved in the formation of a single receptor–ligand binding event. Finally, multivalent interactions are expected to be more resistant to shear stress, a feature that may be significant for some cell–cell recognition processes.

The power of multivalent binding in carbohydrate recognition was revealed in the antibody selection experiments of Deng et al. [6]. Starting with a monomeric single-chain antibody with a $K_d$ for O-polysaccharide of Salmonella serogroup B of 33 mM, they used phage display to screen for high-affinity mutants. The antibodies they isolated have a $K_d = 10$ nM. The main factor in this increase in affinity seems to be that the mutant antibody molecules exist as a dimer. The increased affinity is presumably due to interactions between the dimeric antibody and adjacent carbohydrate sites within the repetitive O-polysaccharide sequence [6]. The dimeric antibodies may have faster on-rates than the monomeric parent antibodies. This difference in kinetics may also be important in other systems. It has been argued that the selectins, which mediate a loose adhesion between leukocytes and endothelium, allow the leukocyte to 'roll' along the endothelium until it reaches a site of infection, bind their putative carbohydrate ligands with fast on-rates [7]. The valency of these interactions may significantly influence the kinetics and specificity of leukocyte tethering. The results obtained from phage display highlight the effectiveness of the multipoint binding strategies nature has adopted for enhancing the protein–carbohydrate interaction.

Multivalent saccharide ligands that can span the required distance between binding sites, so that a single ligand can bind to many receptor binding sites, therefore have an advantage over their monovalent counterparts. Alternatively, saccharide-binding proteins may cluster in a particular area of the cell surface either in response to a multivalent ligand or in response to cellular signals. For example, the mammalian asialoglycoprotein receptor, which facilitates the clearance of desialylated serum glycoproteins, is a hexamer that binds 10⁶-times more tightly to tetravalent galactose derivatives than to a related monovalent structure [8]. Influenza virus attacks a target cell through cooperative interactions of hemagglutinin with sialic acid residues on the cell surface; consequently, glycoconjugates bearing multiple sialic acid derivatives are more effective inhibitors of influenza virus binding than is monovalent sialic acid (see below).

Despite the involvement of multivalent protein–carbohydrate interactions in a variety of biological processes, relatively little is known about such binding events. One way to investigate these interactions is to synthesize molecules bearing multiple carbohydrate residues. The possible applications of such tailored saccharide derivatives include the identification of uncharacterized binding events as multivalent, the modulation of carbohydrate-mediated cell or virus binding, the targeting of conjugates to particular cell types, the immobilization of specific cell types, and the inducement of cell responses through selective binding and/or aggregation of cell-surface receptors. Clearly, the generation of non-natural multivalent saccharide derivatives is an important goal. Much progress has recently been made in this area, but the opportunities for creative chemical approaches to make inroads into biological problems are great.

One of the most important insights to come out of the work done so far is the importance of the appropriate design of the scaffold used to display saccharide units for
multivalent recognition. Multivalent saccharide displays have, in the past, been mimicked by the conjugation (usually via reductive amination) of saccharides to bovine serum albumin, to short synthetic peptides or to lipids. The resulting molecules can be used to show that multivalent binding is involved in a biological recognition event [9], but, because it is not easy to control the spacing of the saccharide residues on these scaffolds, it is hard to use them to explore the requirements for binding. Here, we survey some of the non-natural backbones that have been designed to overcome this problem by displaying carbohydrates in a controllable way. We also describe the structural features and applications of a number of the multivalent saccharide derivatives studied so far, which illustrate the insights that can be gained into the basis and consequences of protein–carbohydrate interactions (Table 1).

Small polyvalent displays

Ligands with enhanced functional affinity for target proteins can often be created by the addition of only one or two extra sugar residues. Small templates that allow attachment of a few carbohydrates can offer some of the benefits of multivalent binding while maintaining a defined, low molecular weight structure. Several groups have applied this approach to peptide-based templates. For multivalent carbohydrate display, however, non-natural carbohydrate templates offer advantages over peptide-based templates — they can be of lower molecular weight, more biologically stable, and free of alternative binding sites that can confuse data interpretation. In addition, the spatial relationship between saccharide residues on non-natural scaffolds can be defined, making this class of molecules particularly suitable for structure–function studies of multivalent interactions.

In seminal studies, Lee and coworkers [10] investigated non-natural multidentate carbohydrate ligands directed toward the asialo glycoprotein receptor. The plasma membrane of mammalian hepatocytes expresses the asialo glycoprotein receptor, which recognizes nonreducing, terminal β-D-galactose residues prior to receptor-mediated endocytosis. The goal of this study was to elucidate the features responsible for the high affinity binding of glycoproteins to the asialo glycoprotein receptor. Using the scaffold (6-amino hexanamido)-tris(hydroxymethyl) methane, Lee and coworkers generated a series of multidentate ligands containing one, two or three lactose residues. In a hepatocyte binding assay, increases in functional affinity of 5- to 50-fold were observed correlating with increases in the number of carbohydrate residues within the ligand. By varying the structure of the template and the linker, trivalent lactose derivatives that were 1000-fold more active than lactose were generated [8]. The information thus gained on the structural features of ligands recognized by the asialoglycoprotein receptor may be useful to target molecules to hepatocytes [11].

Direct evidence that the framework used to display the carbohydrates is critical comes from studies such as that of Knowles, Wiley and coworkers [12], who investigated three different classes of templates in generating inhibitors of influenza virus hemagglutinin. Binding of hemagglutinin molecules on the surface of the influenza virus to sialylated lipids and proteins on the target cell is required for infection, and this interaction has therefore been a popular target for multivalent carbohydrate inhibitors. Glick et al. [12] synthesized bivalent sialosides wherein the carbohydrate residues are separated by the progressive addition of either ethylene glycol, glycine, or piperazine spacers. In solution studies, these sialosides exhibited no increase in functional affinity for trimeric hemagglutinin; however, compounds with a spacer of appropriate length and structure have a 100-fold higher affinity for the whole virus than does sialic acid. The most effective inhibitor contains glycine spacer units, which are more rigid than the ethylene glycol derivatives and more flexible than the piperazine components. Undoubtedly the conformational preferences of the linker residues profoundly affect the capacity of the various derivatives to act as multivalent ligands. Thus, no single framework for presentation of multiple ligands can guarantee success.

Multivalent carbohydrate derivatives have also been used to discriminate between related proteins. A recent example involved two homologous proteins, concanavalin A and the lectin from Dioscorea grandiflora, which exhibit similar binding preferences for glucose and mannose residues. Both proteins exist as homotetramers at neutral pH, and both bind monovalent mannose derivatives with low affinity. Surprisingly, a divalent mannose derivative completely discriminates between the two proteins, interacting only with concanavalin A [13]. Although the molecular details of this selectivity are unknown, these results suggest that altering the valency of a saccharide ligand can dramatically affect its specificity.

The examples above highlight the enhancements in binding and specificity that can be achieved by the addition of one or two extra sugar residues. But they also call attention to the importance of each carbohydrate's spatial and conformational orientation in providing this enhancement. Although investigations using defined, low molecular weight structures have provided some of the most detailed knowledge about the structural requirements for multivalent binding, the syntheses of these molecules are challenging, and the work involved in these undertakings can be discouraging when there is no guarantee that a large binding enhancement will be observed [14,15]. Many workers have therefore turned to assembly methods involving polymerization reactions. The structural requirements for multipoint binding can often be met through carbohydrate-substituted polymers, which can display saccharide recognition elements in a variety of topologies. In some cases, only a small section of the polymers may account for most of the binding enhancement, and small changes in their preparation or backbone structure may translate into large differences in their effectiveness. Below, we
<table>
<thead>
<tr>
<th>Template</th>
<th>Sugar</th>
<th>Function</th>
<th>Ref.</th>
</tr>
</thead>
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<tr>
<td><img src="image1" alt="Lactose" /></td>
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<td>Targeting hepatocytes via the asialoglycoprotein receptor</td>
<td>10</td>
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<tr>
<td><img src="image2" alt="Sialic acid" /></td>
<td>Sialic acid</td>
<td>Inhibiting influenza virus hemagglutinin</td>
<td>12</td>
</tr>
<tr>
<td><img src="image3" alt="Sialyl Lewis x pentasaccharide" /></td>
<td>Sialyl Lewis x pentasaccharide</td>
<td>Inhibiting E-selectin</td>
<td>14</td>
</tr>
<tr>
<td><img src="image4" alt="Sialic acid" /></td>
<td>Sialic acid</td>
<td>Inhibiting influenza virus hemagglutinin</td>
<td>17</td>
</tr>
<tr>
<td><img src="image5" alt="Mannose" /></td>
<td>Mannose</td>
<td>Inhibits concanavalin A but not a related lectin</td>
<td>13</td>
</tr>
<tr>
<td><img src="image6" alt="Glucose" /></td>
<td>Glucose</td>
<td>A but not a related lectin</td>
<td></td>
</tr>
<tr>
<td><img src="image7" alt="Sialic acid" /></td>
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<td>Inhibiting influenza virus hemagglutinin</td>
<td>20</td>
</tr>
<tr>
<td><img src="image8" alt="N-acetylglucosamine" /></td>
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<td>Determining critical binding concentrations for hepatocyte binding</td>
<td>28</td>
</tr>
<tr>
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<tr>
<td><img src="image10" alt="Sialic acid" /></td>
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outline the methods used so far to generate large arrays of carbohydrates by polymerization.

**Spherical carbohydrate displays**

Dendrimers, which are highly branched oligomers, bridge the gap between the small molecules described in the previous section and high molecular weight polymers [16]. As templates for the display of polyvalent molecules, dendrimers offer some unique features. For example, large dendrimers can adopt globular shapes, a characteristic that may be used to sterically interfere with cell-cell recognition. Synthetic methods can also be used to engineer dendrimers with specific properties, such as an affinity for membranes, that could be valuable in biological applications.

Efforts toward the application of dendrimer technology to the creation of multivalent ligands are in their infancy. While searching for inhibitors of influenza virus hemagglutinin, Roy et al. [17] pioneered the synthesis and use of carbohydrate-substituted dendrimers, using solid-phase methodology to synthesize sialic acid decorated dendrimers. These dendrimers, containing 2, 4, 8, or 16 sialic acid residues, all inhibited the agglutination of erythrocytes by influenza virus (which is caused by hemagglutinin-mediated crosslinking of the erythrocytes) in the micromolar concentration range. These results demonstrate the potential of dendrimers to act as polyvalent ligands, and more applications are likely to be forthcoming [18].

Liposomes also provide a spherical display, and have found a wider variety of uses than dendrimers. Sialic acid modified liposomes have been used as inhibitors [19,20] and as sensors of influenza virus binding [21]. The ability of mannose-coated liposomes to target specific cell surface receptors has been investigated, but the effectiveness of this strategy has not been established [22]. A more promising application of such liposomes may be as adjuvants to activate cell-mediated immunity after vaccination [23].

In a study of the inhibitory properties of liposomes, Sopvak et al. [20] synthesized lipids terminated with sialic acid residues attached through C-glycoside linkages. Liposomes with varying amounts of the saccharide-substituted lipid were then generated by covalent crosslinking of diacetylene functional groups in the lipid tails. The resulting liposomes, which present a range of sugar densities, were then tested in hemagglutination assays with the virus, and in infectivity assays in cell culture. Although the liposomes containing 5–10 % sialic acid are the most effective inhibitors of hemagglutination, the liposomes containing 1–5 % sialic acid are more active in cell culture. A major conclusion of this work is that the optimization of hemagglutination does not guarantee the desired biological response has been maximized. Charych and coworkers [21,24] have modified this technology to give cross-linked liposomes that can be used to detect colorimetrically the binding of influenza virus.

**Linear carbohydrate displays**

Although small and spherical saccharide displays have found important uses, the most popular method for generating multivalent ligands is through the polymerization of modified acrylamides [25–28]. Saccharide-substituted acrylamide derivatives have diverse uses as multivalent inhibitors of cell or virus binding, surfaces for cell-specific binding, artificial antigens, and targeted drug delivery agents. There are two basic approaches to the synthesis of carbohydrate-modified polyacrylamides, differing in whether the carbohydrate residues are conjugated before or after polymerization. Through acrylamide copolymerizations, differently substituted monomers can be incorporated to afford polymers with customized properties. For example, Lee, Roseman and coworkers [29] derivatized activated acrylamide gels with galactose and N-acetyl glucosamine; the former materials bind rat hepatocytes, while the latter bind chicken hepatocytes.

Reporter groups, such as fluorescent naphthalene derivatives and biotin, have been incorporated into saccharide-substituted copolymers. Materials modified with these groups have been used in enzyme-linked immunosorbent assays to quantitate binding or to monitor the cellular distribution of a particular polyvalent ligand [25]. The flexibility and simplicity of the acrylamide polymerizations has spawned a variety of applications of these materials to investigate and manipulate biological systems.

A number of researchers have contributed to the synthesis and testing of sialoside-containing polyacrylamide inhibitors of the influenza virus [30–32]. Whitesides and coworkers [32,33] have performed the most comprehensive series of structure-activity studies so far on this class of structures. They used a broad range of sialic acid substituted acrylamide copolymers to probe the mechanism of inhibition of hemagglutination by multivalent carbohydrates. As anticipated, the materials form multiple sialic acid-hemagglutinin interactions at the virus surface. The inhibition is not solely due to cooperative binding, however; the binding of polymers to the virus also sterically blocks access to viral receptors at the cell surface. The Whitesides group expand on their model of steric inhibition in this issue of Chemistry & Biology [34]. They demonstrate that the addition of a monovalent neuraminidase inhibitor enhances the efficacy of their polyvalent sialosides and hypothesize that the higher activities are due to increases in steric inhibition resulting from an expansion of the polymeric gel bound to the viral surface. Such an expansion may occur in conjunction with changes in the ligation state of neuraminidase.

These observations on the mode of action of the polyvalent sialosides provide a foundation for the design of inhibitors of influenza, and insights into the mechanisms through which natural polyvalent ligands might act.

Most studies of carbohydrate-substituted polymers have focused on the polyacrylamide backbone. For some recognition events, alternative templates for saccharide presentation may afford more effective multivalent ligands. The paucity of methods for the synthesis of
Saccharide-modified materials may be attributed to the density of functional groups possessed by saccharides. The reactive intermediates involved in many polymerization chemistries cannot survive exposure to the polar, reactive groups that carbohydrates possess. Recently, a polymerization method that tolerates a wide range of function groups, the ring-opening metathesis polymerization (ROMP), has been applied to the synthesis of carbohydrate-substituted materials [35, 36]. Like acrylamide polymerizations, ROMP can be effected in polar solvents (such as water), and the carbohydrate residues need not be protected. ROMP is more versatile than acrylamide polymerizations for example, after all the initially added monomer is consumed the polymerization can be continued by adding a new type of monomer. In addition, the polymer can be end-labeled with various reporter groups for immobilization or detection. Moreover, the polymer backbone is more rigid than that of the polyacrylamides, and its structural features are very different. The unique features of ROMP offer opportunities to generate new types of biologically active materials.

Explorations of the potential of ROMP for the synthesis of saccharide-substituted materials have just begun. Mortell et al. [35, 37] demonstrated that ruthenium-catalyzed ROMP could be used to polymerize monomers with glucose and mannose substituents. The resulting materials were effective inhibitors of concanavalin A-mediated hemagglutination. For example, a polymer bearing mannose residues was 10^-fold more effective on a per sugar residue basis than the corresponding monovalent derivative.

Structure-function studies with polyvalent glucose and mannose O- and C-glycosides generated by ROMP using a ruthenium catalyst, have also provided information regarding the specificity of multivalent binding [37]. In a concanavalin A-mediated hemagglutination assay, no difference in the inhibitory potency of monomeric glucose and mannose C-glycosides can be detected; however, the difference between the glucose and mannose-substituted polymers is more than 100-fold. Thus, small changes in the structure of the saccharide residue can have large effects in a polyvalent context. This type of recognition specificity may regulate many cell-cell interactions in nature.

Where do we go from here?
Recent additions to the scaffolds for multivalant carbohydrate display add to the chemical tools available to explore multivalent protein–saccharide interactions. Most work so far has focused on increasing the functional affinity of protein–carbohydrate interactions through multivalency. These efforts have been driven by interest in converting low affinity inhibitors into effective modulators of saccharide-mediated recognition. But it is now clear that if this goal is to be accomplished, a more detailed understanding of the specificity of these interactions must be obtained. The new templates for carbohydrate presentation may provide the means to uncover the determinants of specificity. As well as being useful to explore biological recognition processes, multivalent carbohydrate derivatives can be used as probes of biological function. For example, the interaction of L-selectin with sulfated glycolipids, such as sulfatides, activates signal transduction pathways [38]. This observation suggests a potential new use for non-natural multivalent carbohydrate ligands — the activation and investigation of signaling pathways. Undoubtedly, other applications of multivalent saccharide ligands will be discovered. Just as protein–carbohydrate interactions gain strength through increasing numbers of participants, our knowledge of these interactions will strengthen as the number of tools and their uses increase.

References
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