This chapter describes several classes of model systems that have been used for studies of polyvalent binding interactions between carbohydrates and proteins. This review is divided into two parts. The first section provides a mechanistic analysis of polyvalent recognition. The second section utilizes this mechanistic framework to describe and analyze several major classes of model systems. The description of each model system is accompanied by a summary of recent experimental results from the literature. The chapter concludes with a comparison of the model systems and an introduction to several novel model systems that promise to broaden the scope of future studies in polyvalent carbohydrate-protein interactions.

Keywords. Carbohydrates, Polyvalency, Model system, Monolayers
1 Introduction

The interactions of proteins with carbohydrates define and regulate many important biological phenomena, ranging from the adhesion of leukocytes to endothelial cells to the guidance of neuronal growth cones during development [1–3]. Despite the scope and importance of carbohydrates in biology, the difficulty in studying carbohydrate-protein interactions has hindered efforts to develop a mechanistic understanding of carbohydrate structure and function. A major reason for this difficulty stems from the structural complexity of carbohydrates. While the other two classes of biopolymers – nucleic acids and proteins – have a linear arrangement of repeating units, carbohydrate building blocks have multiple points of attachment, leading to highly branched and
stereochemically rich structures. This structural complexity is further increased by post-synthetic modifications, wherein unmodified hydroxyl groups can be sulfated, phosphorylated, acetylated, or oxidized to generate distinct biological activities [4]. Sulfated carbohydrates, for example, function as potent mediators of inflammation [5], while phosphosugars play important roles in cellular signal transduction and metabolism [6].

A second difficulty in studies of carbohydrate-protein interactions is that binding affinities are weak, often with dissociation constants in the millimolar range [7]. In biological contexts, this limitation is often overcome by combining multiple interactions between two or more carbohydrates and a corresponding multimeric protein. These interactions – often described as polyvalent – have several mechanistic and functional advantages over their monovalent counterparts. Among these are the ability to create conformal contact between large biological surfaces, the ability to produce graded responses with a single type of interaction, and the ability to increase the specificity of an interaction [8].

These characteristics, together with the complex nature and widespread importance of polyvalent interactions, have prompted the development of model systems to characterize carbohydrate-protein interactions. This chapter reviews several model systems that have been important in mechanistic studies of glycobiology and in applications that rely on the recognition of carbohydrates. We first describe the principles necessary for understanding polyvalent binding events. We next introduce several model systems that have been important for studies of polyvalent carbohydrate-protein interactions. A description of each model system is accompanied by a brief summary of recent experimental results from the literature. We conclude the chapter with a description of three novel model systems and a comparison of the advantages and limitations of the model systems in the review.

2 Polyvalent Recognition

Polyvalent binding is characterized by the simultaneous interaction between multiple ligands on one entity and multiple receptors on another. Scheme 1 illustrates a polyvalent interaction involving three ligands and three receptors. Note that this scheme can be generalized to \(N\) ligands and \(N\) receptors.

The following section provides a mechanistic description of polyvalent binding interactions, with an emphasis on the distinctions between these interactions and their monovalent counterparts. The remainder of the chapter utilizes these mechanistic principles to describe and analyze several classes of polyvalent model systems that present carbohydrates.

2.1 Mechanistic Features of Polyvalent Binding

The formation of an \(N\)-valent interaction is a stepwise process that involves \(N\) binding events and \((N-1)\) intermediates. This stepwise binding process has two characteristics that are absent in its monovalent counterpart: the ability to reg-
ulate the strength of an interaction through the number of ligand-receptor contacts and the ability to rapidly dissociate the complex with soluble ligand [9]. Whitesides and coworkers illustrated these principles with a trivalent complex between vancomycin and the peptide D-Ala-D-Ala [10, 11]. Whereas the monovalent vancomycin-peptide complex had a dissociation constant of $1 \times 10^{-6}$ mol/l, the trivalent complex had a dissociation constant that was ten orders of magnitude greater ($K_d = 4 \times 10^{-17}$ mol/l). In contrast to the monovalent complex, however, the trivalent complex rapidly dissociated when soluble peptide was added to a solution containing the complex.

Although polyvalent binding is functionally attractive, it complicates the analysis of an interaction because both inter- and intramolecular binding may occur (Fig. 1). After the formation of a monovalent intermediate, intramolecular binding generates a single complex with multiple ligand-receptor interactions, while intermolecular binding affords a crosslinked network of ligands and receptors. Additional intermolecular binding events eventually lead to precipitation of the network from solution. In all cases, each binding event will alter the enthalpy, entropy, and free energy of subsequent interactions since the ligand and receptor are bound together in solution. The precipitation of ligand-receptor networks from solution further increases the complexity of the system.

### 2.2 Factors that Influence Polyvalent Binding

The many possible ways in which $N$ ligands and $N$ receptors can interact makes it difficult to design systems that preferentially follow one binding pathway (i.e., intermolecular vs intramolecular binding). In order for intramolecular binding to predominate, the free energy of an $N$-valent interaction ($\Delta G^{\text{poly}}$) must be more favorable than the free energy of $N$ monovalent interactions ($N\Delta G^{\text{mono}}$). Since the free energy term includes enthalpic ($\Delta H^{\text{poly}}$) and entropic ($\Delta S^{\text{poly}}$) terms, it is necessary to examine the role of these components separately. In a recent review, Whitesides and coworkers presented a system of nomenclature for polyvalent
interactions and described the enthalpic and entropic characteristics of a polyvalent interaction [8]. We provide a summary of this analysis for a divalent system in Fig. 2 and note that this analysis can be extended to $N$-valent interactions using the same principles.

The enthalpy of binding ($\Delta H^{\text{poly}}$) reflects stabilizing interactions between ligand and receptor, less any energetic penalties for non-optimal conformations in the complex and differential solvation of bound and unbound ligand and receptor. After the formation of a monovalent complex, the enthalpy of the second binding event can be greater than, equal to, or diminished relative to the first. If the second ligand and receptor species are properly aligned, the enthalpy of the second binding event will be identical to the first and $\Delta H^{\text{bi}} = 2\Delta H^{\text{mono}}$ (Fig. 2A, Case I). This situation rarely occurs because it is difficult to design scaffolds that optimally position ligands for multipoint binding. In many cases, the second binding event introduces strain in the tether, adding an energetic penalty to the overall enthalpy of interaction (Fig. 2A, Case II). In other systems, favorable secondary interactions between the tether and the receptor during subsequent binding events stabilize the complex, enhancing the enthalpy of the second binding event.

**Fig. 1.** Possible binding pathways in a divalent model system
binding interaction (Fig. 2A, Case III). The binding of cholera toxin (a pentameric protein) to multiple ganglioside lipids is one possible example of this type of interaction [12]. In this system, the binding constant of the second ligand was four times more favorable than that of the first ligand. This effect was due to enthalpy alone since each ganglioside rotated and translated independently.

The entropy of binding ($\Delta S^{\text{poly}}$) reflects changes in the molecular order that occur during a ligand-receptor interaction. Since this term is complex and often poorly understood, it is often divided into entropies of translation ($\Delta S_{\text{trans}}$), rotation ($\Delta S_{\text{rot}}$), conformation ($\Delta S_{\text{conf}}$), and hydration ($\Delta S_{\text{H}_2\text{O}}$). For a bivalent interaction, the sum of these quantities for the second binding event can again be greater than, equal to, or less than the sum observed for the first interaction. Below we consider each case in turn, assuming that the contribution from the entropy of hydration ($\Delta S_{\text{H}_2\text{O}}$) is similar in each situation. If the two ligands and two receptors are connected by a rigid scaffold and are precisely spaced, $\Delta S_{\text{conf}} = 0$ and the interaction will occur with an entropy equivalent to a single monovalent interaction (Fig. 2B, Case I). This situation is unrealistic since most linkers do have conformational degrees of freedom and it is rare that the spacing

**Fig. 2.** Analysis of enthalpy and entropy of binding in a divalent model system

<table>
<thead>
<tr>
<th></th>
<th>A. Enthalpy ($\Delta H^{\text{poly}}$)</th>
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<tbody>
<tr>
<td>Case I</td>
<td>Optimized tether</td>
</tr>
<tr>
<td>Case II</td>
<td>Strained conformation</td>
</tr>
<tr>
<td>Case III</td>
<td>Favorable secondary interaction</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>B. Entropy ($\Delta S^{\text{poly}}$)</th>
</tr>
</thead>
</table>
| Case I | Optimized tether | $\Delta S_{\text{rot}} + \Delta S_{\text{trans}}$  
$\Delta S_{\text{conf}} = 0$ |
| Case II | Flexible tether | $\Delta S_{\text{conf}} < \Delta S_{\text{rot}} + \Delta S_{\text{trans}}$ |
| Case III | Tether too short, too long, or not flexible | $\Delta S_{\text{conf}} > \Delta S_{\text{rot}} + \Delta S_{\text{trans}}$ |
between a ligand and receptor are matched. In general, $\Delta S_{\text{conf}}$ is unfavorable upon complexation because the binding interaction will constrain many degrees of freedom previously available to the ligand and receptor. If $\Delta S_{\text{conf}}$ is less than the sum of $\Delta S_{\text{trans}}$ and $\Delta S_{\text{rot}}$, the binding is entropically enhanced and intramolecular interaction will be favored (Case II). If $\Delta S_{\text{conf}}$ is greater than the sum of $\Delta S_{\text{trans}}$ and $\Delta S_{\text{rot}}$, intermolecular interactions will be favored (Case III).

This discussion illustrates the ways in which the flexibility of the spacer between ligands (and receptors) is critical to the effective design of a polyvalent model system. The optimal linker will perform two functions: increase the likelihood that interactions will occur without strain and minimize the entropic cost of multiple ligand-receptor interactions. If this balance is not achieved, the formation of cross-linked complexes – rather than a true multipoint attachment – is likely to occur. We also note that there are other strategies by which polyvalent ligands can be directed to assemble into either inter- or intramolecular complexes. For example, intermolecular complexes are favored in cases where the concentration of receptor is large, while intramolecular complexes predominate when the polyvalent ligand is immobilized on a substrate at low density (see Sect. 5.3).

### 2.3 Characterization of Polyvalent Binding

The models outlined above provide a molecular description of polyvalent association. In many cases, however, the mechanism by which binding occurs (see Figs. 1 and 2) is less critical than is the enhancement of binding observed in a polyvalent interaction. To aid in the description of polyvalent interactions, Whitesides and coworkers described a new parameter $\beta$ [8]. This factor is defined as the ratio of the association constant for a polyvalent system (as measured by any number of methods) and the association constant for the monovalent ligand. These relationships are described quantitatively in Eqs. (1) and (2):

$$\Delta G_{\text{poly}} = \Delta G_{\text{mono}} - RT \ln \beta$$

$$\beta = \frac{K_{\text{poly}}}{K_{\text{mono}}}$$

Model systems that have high values of $\beta$ are useful, regardless of the mechanism of action or the nature of individual interactions. In fact, many polyvalent interactions with large values of $\beta$ occur with negative cooperativity – that is, the average free energy of binding for a system of $N$ interactions is less favorable than the free energy of binding for a monovalent interaction ($\Delta G_{\text{poly}}^N / N < \Delta G_{\text{mono}}^N$). A common example in glycobiology is the binding of galactose-containing ligands to the asialoglycoprotein receptor on the surface of hepatocytes [13]. For one trivalent ligand, the value of $K_{\text{mono}} = 7 \times 10^4$ M$^{-1}$ and $K_{\text{tri}} = 2 \times 10^8$ M$^{-1}$. Since $\Delta G_{\text{tri}} / 3$ is less favorable than $\Delta G_{\text{mono}}$ the binding of the trivalent ligand to the receptor occurs with negative cooperativity.

In glycobiology, many model systems demonstrate values of $\beta$ greater than 1. These binding enhancements are generally attributed to the “cluster glycoside
effect” [7], a term that describes interactions in which the binding constant is larger than predictions based on local concentration alone. Despite a vast literature on the subject, the mechanistic basis for the cluster glycoside effect remains unclear. Isothermal titration calorimetry (ITC) has become an important tool for these studies because it measures the valency, free energy ($\Delta G^{\text{poly}}$), and enthalpy ($\Delta H^{\text{poly}}$) of an interaction [14]. Since at equilibrium $\Delta G^{\text{poly}} = \Delta H^{\text{poly}} - T \Delta S^{\text{poly}}$, the entropy of the interaction can be calculated from experimentally determined values. A recent study by Brewer and coworkers used ITC to investigate the thermodynamic basis for the binding enhancements that occur between concanavalin A (Con A) and a series of polyvalent mannosides [15]. They found that the valency of the interaction correlated with the number of carbohydrate ligands on a given scaffold and that the enthalpy of interaction increased linearly with the number of mannose residues on the glycoconjugate. The larger affinities ($K_a$) of polyvalent ligands for the lectin arose from a favorable entropy of interaction (specifically, a more positive $T \Delta S$ term). These data suggest that the polyvalent ligands bind more than one Con A molecule rather than form multiple attachments with a single lectin. These data also show that binding enhancements, while useful, may occur via multipoint binding.

3 Classes of Polyvalent Model Systems

Several classes of model systems have been developed to study polyvalent carbohydrate-protein interactions. To aid in the presentation of this body of literature, we have grouped these models into two classes based on valency (Fig. 3).

Examples in the first class, which we refer to as low valency model systems, present fewer than 20 carbohydrate ligands. Since the types of models in this class are quite diverse, we have further divided them into three groups to facilitate comparison of the different scaffold architectures. Examples in the second class, which we refer to as high valency model systems, present large numbers (often hundreds) of carbohydrate ligands. These model systems include neoglycoproteins, glycopolymers (both soluble and immobilized), hybrid bilayers, and self-assembled monolayers of alkanethiolates on gold. The last two models are functionally distinct from protein- and polymer-based systems because they present carbohydrates in a two-dimensional array.

4 Model Systems with Low Valency

Model systems that present between 2 and 20 carbohydrate ligands have been important for defining the geometric and structural requirements for tight binding to polyvalent receptors [16]. While these models are reasonably well defined and readily modified using synthetic organic chemistry, it is often necessary to prepare large numbers of compounds in order to identify polyvalent ligands that achieve significant binding enhancements. Lee and coworkers, for example, prepared over 100 compounds during the development of high affinity ligands for the hepatic asialoglycoprotein receptor [7, 13, 17]. Schnaar and
coworkers have invested similar efforts toward the development of selective ligands for a lectin from the protozoan *E. histolytica* [18, 19]. These examples reflect the difficulties in designing model systems with low valency.

The following section presents several classes of scaffolds that have been used for the presentation of carbohydrates. We illustrate the utility of these scaffolds with recent examples from the literature that highlight the functional characteristics of each model system. We include two additional pieces of information with each example: an observed binding enhancement of the glycocluster ($\beta$) and a binding enhancement that is corrected for the number of carbohydrate ligands on the glycocluster ($\beta/N$). Due to space considerations, this section only presents glycoconjugates that have undergone biological evaluation and does

---

**Fig. 3.** Graphical summary of model systems presented in this review. Models are divided into systems with low valency and high valency.
not discuss the synthesis of these model compounds. For the preparation of these and other model systems, we direct the reader to several excellent reviews [20–24].

4.1 Clusters

Glycoclusters represent a large group of model systems that present two to five carbohydrate ligands on a small molecular template [20]. These low valency model systems have been extremely important in determining the optimal binding geometries and ligand to ligand distances for a particular multimeric protein. Each figure in this section illustrates a series of glycoclusters that has been used to study a particular polyvalent carbohydrate-protein interaction. This organization facilitates comparisons of the effectiveness of different scaffolds.

Figure 4 illustrates a group of scaffolds that present Sialyl Lewis X (SLex) or derivatives of SLex. These conjugates were designed as tight binding inhibitors for E-selectin, a carbohydrate binding protein that plays a critical role in the migration of leukocytes to sites of injury.

The examples in this figure illustrate that the success or failure of a particular model system depends on the nature of the scaffold and the length of the tether between the scaffold and the carbohydrate. Scaffolds based on butane or pentane, for example, demonstrated no binding enhancements over monovalent SLex [25], while more conformationally rigid scaffolds such as quaternary carbon 1 [26], carbohydrate 2 [25], peptide 3 [27], and aromatic ligand 4 [28] provided better results. In all cases, however, the enhancement of binding was quite sensitive to the length of the tether between the scaffold and SLex. Scaffold 5, based on a flexible ethylene glycol tether, demonstrated a fivefold enhancement over SLex when the number of glycol units was either two or five but demonstrated negligible enhancements when other ethylene glycol spacers were used [29]. Collectively, these examples illustrate the importance of conformational flexibility and ligand to ligand spacing in multivalent interactions. They also demonstrate the difficulty in designing the appropriate polyvalent ligand for a particular system.

It is instructive to compare these results with those in Fig. 5, which depicts a series of compounds (6a–e) that were tested for their ability to inhibit the binding of the bacterium Staphylococcus suis to Galα(1,4)Galβ motifs on epithelial cells of the urinary tract [30]. These data represent the minimum concentration of multivalent compound required to inhibit the crosslinking of red blood cells by the bacterium.

In this series, the nature and valency of the scaffold is less important than the length of the spacer between the scaffold and the carbohydrate ligands. Compound 6b, for example, was 20-fold more potent than monovalent carbohydrate 6a in inhibiting binding. The introduction of a three carbon segment (compound 6c) resulted in an additional 50-fold enhancement. Trivalent system 6d, based on a quaternary carbon scaffold, was a less potent inhibitor than 6c, while tetravalent ligand 6e inhibited bacterial adhesion at a twofold lower concentration than 6c.
An important, and often overlooked, factor in the evaluation of multivalent models is the assay used to determine binding enhancements. A number of assays are available for these measurements in glycobiology [8], and the choice of a particular assay depends on the interaction being studied, the quantity of available material, and access to specialized equipment. To illustrate the importance of the assay used to measure a polyvalent interaction, Toone and coworkers evaluated a series of carbohydrates with a hemagglutination inhibition assay.

<table>
<thead>
<tr>
<th>R</th>
<th>IC50</th>
<th>β</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLe(^x)-OH</td>
<td>600 μM</td>
<td>1</td>
</tr>
<tr>
<td>SLe(^x), n = 2</td>
<td>140 μM</td>
<td>4.3 (2.15)</td>
</tr>
<tr>
<td>SLe(^x), n = 3</td>
<td>370 μM</td>
<td>1.2 (0.6)</td>
</tr>
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<table>
<thead>
<tr>
<th>R</th>
<th>IC50</th>
<th>β</th>
</tr>
</thead>
<tbody>
<tr>
<td>R(_1 =) SLe(^x), R(_2 =) H</td>
<td>200 μM</td>
<td>1</td>
</tr>
<tr>
<td>R(_1 =) R(_2 =) SLe(^x)</td>
<td>200 μM</td>
<td>3 (1.5)</td>
</tr>
<tr>
<td>R(_1 =) R(_2 =) SLe(^x) (Naphth), n = 1</td>
<td>30 μM</td>
<td>6.7 (3.35)</td>
</tr>
<tr>
<td>R(_1 =) R(_2 =) SLe(^x) (Naphth), n = 2</td>
<td>60 μM</td>
<td>3.3 (1.65)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>R</th>
<th>IC50</th>
<th>β</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLe(^x)-OH</td>
<td>393 μM</td>
<td>1</td>
</tr>
<tr>
<td>SLe(^x), n = 1</td>
<td>228 μM</td>
<td>1.7 (0.85)</td>
</tr>
<tr>
<td>SLe(^x), n = 2</td>
<td>70 μM</td>
<td>5.6 (2.8)</td>
</tr>
<tr>
<td>SLe(^x), n = 3</td>
<td>179 μM</td>
<td>2.2 (1.1)</td>
</tr>
<tr>
<td>SLe(^x), n = 4</td>
<td>176 μM</td>
<td>2.2 (1.1)</td>
</tr>
<tr>
<td>SLe(^x), n = 5</td>
<td>81 μM</td>
<td>4.9 (2.45)</td>
</tr>
</tbody>
</table>

**Fig. 4.** Glycoclusters for studies of the interaction between Sialyl Lewis X and E-selectin. Absolute and valency-corrected binding enhancements over monovalent analogs are provided.
The former assay measures the ability of a multivalent inhibitor to prevent the cross-linking of erythrocytes (red blood cells) by a lectin (in this case Con A). The latter method measures the ability of a multivalent inhibitor to prevent binding of a lectin to an immobilized multivalent ligand.

The results in Fig. 6 show that 7d and 7e are potent inhibitors of hemagglutination but only modest inhibitors of lectin binding by ELLA. The authors attribute this dramatic difference to the lectin used in the assays. In the ELLA, the Con A lectin was conjugated to horseradish peroxidase (HRP; a 40-kD protein) while in the hemagglutination assay, an unconjugated lectin was used. The large HRP moiety is believed to prevent effective crosslinking of the protein by the

\[ R = \text{Gal} \alpha(1,4)\text{Gal} \beta \]

<table>
<thead>
<tr>
<th>Inhibitory Conc. (nM)</th>
<th>( \beta )</th>
</tr>
</thead>
<tbody>
<tr>
<td>6a ( R \cdot \text{TMS} )</td>
<td>1800</td>
</tr>
<tr>
<td>6b</td>
<td>90</td>
</tr>
<tr>
<td>6c ( \text{S-NHR} \cdot \text{SR} )</td>
<td>6</td>
</tr>
<tr>
<td>6d ( \text{RHN} \cdot \text{NO}_2 \cdot \text{NHR} )</td>
<td>25</td>
</tr>
<tr>
<td>6e ( \text{RHN} \cdot \text{S-NHR} \cdot \text{S-NHR} \cdot \text{S-NHR} )</td>
<td>3</td>
</tr>
</tbody>
</table>

**Fig. 5.** Glycoclusters for studies of the interaction between galabiose and *S. suis*. Absolute and valency-corrected enhancements of binding over monovalent analogs are provided.
multivalent ligand, reducing the potency of the glycoconjugate. This hypothesis was confirmed with ITC, which showed that the free energy of association between unconjugated Con A and 7d and 7e did increase (relative to α-methyl mannose) and that this increase arose primarily from a more positive entropy of interaction (see Sect. 2.3). This example illustrates the importance of the assay in the evaluation and interpretation of binding enhancements in polyvalent glycoconjugates. For the remainder of the chapter we will include the assay used for binding in our description of each model system.

### Table 1: Comparison of Binding Assays for Polyvalent Glycoclusters that Present Mannosides

<table>
<thead>
<tr>
<th>R =</th>
<th>Potency relative to α-Me Mannose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HAI</td>
</tr>
<tr>
<td>7a</td>
<td>0.81</td>
</tr>
<tr>
<td>7b</td>
<td>3.6 (1.8)</td>
</tr>
<tr>
<td>7c</td>
<td>8.6 (2.9)</td>
</tr>
<tr>
<td>7d</td>
<td>186 (47)</td>
</tr>
<tr>
<td>7e</td>
<td>672 (112)</td>
</tr>
</tbody>
</table>

### Fig. 6. Comparison of binding assays for polyvalent glycoclusters that present mannosides. Absolute and valency-corrected enhancements of binding over monovalent analogs are provided.
4.2 Cyclodextrins and Calixarenes

Cyclodextrins are cyclic oligosaccharides containing α-(1,4)-linked glucopyranosyl units. These structures present a hydrophobic interior that is capable of binding small organic molecules and a hydrophilic exterior [32, 33]. Hydroxyl groups on the exterior of the cyclodextrin can be modified so that ligands are presented on a single face of the structure with control over ligand-to-ligand spacing (Fig. 7A).

Fig. 7A–C. Cyclodextrins that present carbohydrates. (A) General structure of β-cyclodextrin; (B) structure of the monosubstituted hexavalent structure prepared by Defaye and coworkers [37]; (C) structure of persubstituted hexavalent cyclodextrin presenting β-D-glucosides and data for the inhibition of binding of pea lectin to poly(acrylamide-co-allyl-α-D-mannoside) [38]
Although several synthetic methods for the preparation of glycosylated cyclodextrins have been reported [34, 35], few have evaluated the activity of these conjugates. In a recent report, Nishimura and coworkers prepared a series of perglycosylated cyclodextrins and showed that a hexavalent N-acetylglucosamine conjugate inhibited the agglutination of human erythrocytes at a 240-fold lower concentration than its monomeric counterpart [36]. Defaye and coworkers prepared a cyclodextrin derivatized with dendritic mannoses and showed that the hexavalent conjugate inhibited the binding of Con A to yeast mannann with an IC_{50} of 8 µmol/l, a 100-fold increase in potency over α-methylmannoside [37] (Fig. 7B). In another study, Vargas-Berenguel and coworkers synthesized a series of β-cyclodextrins modified with either β-d-glucopyranose, β-d-galactopyranose, β-d-N-acetylglucopyranosylamine, or α-d-1-deoxy-1-thiomannopyranoside [38]. The inhibitory potency of these compounds was evaluated using an ELLA with the appropriate plant lectin and immobilized polymers presenting multiple carbohydrate ligands. Figure 7C illustrates two glucosylated cyclodextrins that inhibited the binding of pea lectin to poly(acrylamide-co-allyl-α-d-mannoside). When the carbohydrate was attached directly to the cyclodextrin scaffold, a modest binding enhancement over β-methylglucose was observed. The introduction of an acetamido spacer group between the carbohydrate and the cyclodextrin significantly enhanced the potency of the glycoconjugate.

Calixarenes are cyclic oligomers of substituted aromatic rings. Like cyclodextrins, these scaffolds are able to bind hydrophobic organic molecules and can be modified with carbohydrate ligands on one face of the molecule with control over ligand to ligand spacing (Fig. 8A). These conjugates have been useful for both site-directed drug delivery [39] and for studies of water-monolayer surface interactions [40]. Roy and Kim, for example, prepared a series of dendritic p-tert-butylcalix[4]arenes presenting from 4 to 16 N-acetylgalactosamine ligands [41] (Fig. 8B). In an ELLA, the hexadecamer was 12 times more potent than allyl-α-GalNAc in inhibiting the binding of the agglutinin from Vicia villosa to asialoglycoporphin. More recently, Aoyama and coworkers prepared a calix[4]arene presenting eight galactosides and showed that this glycoconjugate could deliver a fluorescent dye to the surface of rat hepatoma cells (Fig. 8C) [42].

4.3 Dendrimers and Dendrons

Dendrimers represent a class of polymer composed of a core structure that is modified with several regularly hyperbranched units (Fig. 3A). Dendrons are a subclass of dendrimers in which the core structure is modified with only one hyperbranched unit [43]. These structures are useful templates for the design of polyvalent ligands for two reasons. First, dendrimers and dendrons permit access to valencies between glycoclusters and model systems that present very large numbers of carbohydrates. Second, synthetic chemistry permits wide flexibility in adjusting the valency, size, and even shape of a dendron [20–24].
Despite this flexibility in design, dendrimers and dendrons sometimes fail to display large enhancements in binding affinity toward carbohydrate-binding proteins. Roy and coworkers, for example, found that dendrons presenting N-acetylglucosamine displayed a valence-dependent enhancement of binding to the agglutinin from wheat germ (WGA) (Fig. 9). The same scaffold presenting N-acetyllactosamine (LacNAc), by contrast, showed little binding enhancement toward the lectin from *E. cristagalli*.

Many authors reason that the poor binding enhancements reflect an improper spacing or geometry of the carbohydrates presented on these scaffolds [13, 45]. It is possible, however, that larger dendrons and dendrimers (with valency greater than four) sterically prevent the cross-linking of carbohydrate-binding proteins. Further mechanistic studies using ITC will provide answers to this and
related mechanistic questions. For more discussion of these model systems, we
direct the reader to other chapters in this volume and to several recent reviews [24, 46, 47].

4.4 Designs Based on Structural Information

Some of the most potent polyvalent systems with low valency have been
designed using detailed structural information of the carbohydrate binding
protein. While this approach is limited to systems for which crystallographic or
NMR structural data are available, it significantly reduces the time required to
develop a polyvalent system that presents ligands with the appropriate geome-

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<thead>
<tr>
<th></th>
<th>IC$_{50}$</th>
<th>Enhancement</th>
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<tbody>
<tr>
<td>allyl-α-D-GlcNAc</td>
<td>15000 µM</td>
<td>1</td>
</tr>
<tr>
<td>n = 2</td>
<td>3100 µM</td>
<td>4.8 (2.4)</td>
</tr>
<tr>
<td>n = 4</td>
<td>510 µM</td>
<td>25.4 (6.4)</td>
</tr>
<tr>
<td>n = 8</td>
<td>88 µM</td>
<td>170 (21.2)</td>
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<tr>
<th></th>
<th>IC$_{50}$</th>
<th>β</th>
</tr>
</thead>
<tbody>
<tr>
<td>azido-β-D-LacNAc</td>
<td>430 µM</td>
<td>1</td>
</tr>
<tr>
<td>n = 2</td>
<td>340 µM</td>
<td>1.3 (0.65)</td>
</tr>
<tr>
<td>n = 4</td>
<td>140 µM</td>
<td>3 (0.75)</td>
</tr>
<tr>
<td>n = 8</td>
<td>86 µM</td>
<td>5 (0.63)</td>
</tr>
</tbody>
</table>

Fig. 9A, B. Comparison of dendrons presenting. (A) N-acetylglucosamine and (B) N-acetyllactosamine. Each series of compounds was evaluated for its ability to inhibit binding of wheat germ agglutinin (A) or Erythrina cristagalli (B) to microtiter plates coated with porcine stomach mucin. Values in parentheses represent enhancements that have been corrected for the valency of the dendron. While the compounds in (A) shows binding enhancements (per ligand) over the corresponding monomeric carbohydrate, the compounds in (B) do not [44]
try and spacing. Two recent reports have utilized this approach in the design of synthetic inhibitors for members of the AB$_5$ family of bacterial toxins. These toxins, which include the heat-labile enterotoxin from *E. coli*, shiga toxin, shiga-like toxin, and pertussis toxin, consist of five identical carbohydrate-binding subunits arranged around a core subunit in a star-like fashion [48]. To mimic the structure of heat-labile enterotoxin, Hol and coworkers prepared an azamacrocycle presenting β-galactose and varied the length of the spacer between the macrocycle and carbohydrate groups (Fig. 10) [49]. The most potent analog demonstrated an IC$_{50}$ of 560 nmol/l, which represents an enhancement of $10^5$ over monomeric galactose. This degree of enhancement is remarkable since the linker between the carbohydrate and the scaffold is quite flexible.

Bundle and coworkers recently prepared a decavalent sugar cluster for inhibition of the binding of Shiga toxin to gangliosides on the cell surface (Fig. 11) [50]. In this work, the authors used α-glucose as a scaffold for the attachment of ligands and varied the number of diethyl squarate moieties in the spacer arm to achieve optimal binding. The most successful inhibitor, named STARFISH, had an IC$_{50}$ of $4 \times 10^{-10}$ mol/l. This value is comparable to the estimated affinity of the native ganglioside-pentamer interaction ($K_d = 10^{-9}$ mol/l) and is $10^6$ more potent than the monomeric carbohydrate ($\beta = 10^6$). Interestingly, the authors obtained a crystal structure of the toxin-inhibitor complex and found that each STARFISH ligand bound two toxin pentamers. They attributed this finding to the lack of flexibility in the STARFISH oligomer. This example illustrates that even the most carefully designed interaction may behave unpre-

<table>
<thead>
<tr>
<th>n</th>
<th>IC$_{50}$ (µM)</th>
<th>β (relative to galactose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>242 ± 91</td>
<td>240 (48)</td>
</tr>
<tr>
<td>2</td>
<td>16 ± 8</td>
<td>3600 (720)</td>
</tr>
<tr>
<td>3</td>
<td>6 ± 4</td>
<td>10 000 (2000)</td>
</tr>
<tr>
<td>4</td>
<td>0.56 ± 0.06</td>
<td>104 000 (20 800)</td>
</tr>
</tbody>
</table>

*Fig. 10. Structure of a pentameric inhibitor of the heat-labile enterotoxin from *E. coli* [49]. Increasing the length of the tether between the carbohydrate and the macrocycle increases the inhibitory potency of the compound. Values in parentheses represent enhancements that have been corrected for the valency of the dendron.*
### Fig. 11. Structure of a potent decameric inhibitor of shiga-like toxin [50]. Values in parentheses represent enhancements that have been corrected for the valency of the dendron.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ (µM)</th>
<th>$\beta$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P^k$ trisaccharide</td>
<td>2100</td>
<td>1</td>
</tr>
<tr>
<td>$P^k$ dimer</td>
<td>55</td>
<td>38 (19)</td>
</tr>
<tr>
<td>STARFISH</td>
<td>0.00024</td>
<td>$8.75 \times 10^6$ (875000)</td>
</tr>
</tbody>
</table>
dictably. It also illustrates that the interpretation of experimental results is often ambiguous, even in the most controlled setting.

4.5
Other Models

A variety of other scaffolds have been utilized for the preparation of glycoconjugates. These include glycopeptoids [51, 52], azamacrocycles [53], linear peptides [54, 55], cyclic peptides [56, 57] and others [58, 59]. Due to space considerations, we refer the reader to the original literature for further information.

5
Model Systems with High Valency

Many applications in glycobiology require model systems that present large numbers of carbohydrates, including vaccination [60], chromatography [61], and the inhibition of interactions that occur over large areas (such as cell-cell and cell-pathogen interactions). Examples of the latter include the binding of leukocyte selectins to endothelial cells (cell-cell) [5], the adhesion of influenza virus to cells of the upper airway (cell-virus) [62], and the adhesion of E. coli to cells of the urinary tract (cell-bacteria) [63]. In the following section we outline several model systems that present large numbers of carbohydrates.

5.1
Neoglycoproteins

Neoglycoproteins remain one of the most widely used classes of polyvalent glycoconjugates. These models are naturally occurring proteins that have been synthetically modified with carbohydrate ligands. Many synthetic methods for the preparation of neoglycoproteins are available, and a large number of these methods provide reasonable control over valency [18, 21–23]. These approaches have the limitation, however, that the sites of glycosylation are not strictly defined and therefore yield heterogeneous glycoforms. To overcome this limitation, several methods have been developed to produce defined glycoforms (Scheme 2).

Davis and coworkers, for example, have used site-directed mutagenesis to introduce cysteine residues in defined locations, which are then selectively coupled with methanethiosulfonate-derivatized carbohydrates [64, 65]. While useful for determining the role of glycosylation in modifying the activity of a protein, this and other chemoselective ligation methods [66–69] are not yet routine and have not been used to generate neoglycoproteins with large numbers of carbohydrates.
5.2 Neoglycopolymers

Polymers derivatized with carbohydrates continue to be important tools in mechanistic studies of polyvalent carbohydrate-protein interactions. Polymers have been attractive scaffolds because synthetic approaches for their preparation permit control over the molecular weight of the polymer and the density of one or more ligands on the polymer backbone [22, 23, 70, 71]. This model system has the limitation that ligands are not presented in defined environments and that the number of interactions is often unknown. For studies that do not require strict mechanistic interpretations, these considerations are less important. We have separated the discussion of these glycoconjugates into two classes of model systems: soluble and immobilized polymers. The former have emerged as potent inhibitors of polyvalent carbohydrate-protein interactions, while the latter have found extensive analytical applications in glyco-biology.

5.2.1 Soluble Polymers

The properties of a soluble polymer depend on a number of factors, including the structure of the backbone and the molecular weight. Several backbones have been used in the literature, including polyacrylamide, polylysine [72], polystyrene [73, 74], dextran [75], and several others [76–79]. Of these, poly-
Acrylamide has been the most widely used because of its biocompatibility and ease of preparation [80]. Whitesides and coworkers, for example, have prepared polyacrylamides presenting a C-glycoside of sialic acid and shown that these conjugates strongly inhibited hemagglutination of erythrocytes by the influenza virus ($\beta = 10^{10}$ M$^{-1}$) [81–83]. The potency of these polymeric inhibitors was attributed to two mechanisms of action: polyvalent competitive inhibition and steric stabilization (Fig. 12). The latter effect is due to the ability of the water-swollen polymer to interfere sterically with the erythrocyte-virus interaction.

In subsequent work, these polymers were found to be more potent inhibitors of hemagglutination in the presence of a monomeric inhibitor of the enzyme neuraminidase [84]. This enzyme is also present on the surface of the virus particle and competes with hemagglutinin for sialic acid groups on the polymer (Fig. 13). Interestingly, the enhancement was proportional to the inhibition constant of the monomeric inhibitor (Fig. 14A). The authors suggested that the monomeric inhibitor displaces the polymer from the erythrocyte, enhancing its ability to inhibit sterically the virus-erythrocyte interaction (Fig. 14B). More potent monomeric inhibitors were more effective at displacing the polymer and increasing the observed value of $K_{HAI}^{\text{HAI}}$ for the two-component system.

Wang and coworkers utilized polyacrylamides derivatized with Galα(1,3)Galβ(1,4)Glcβ epitopes to inhibit the immune recognition of porcine xenografts [85]. Using an inhibition ELISA with purified human anti-Gal antibody, they found that the polymers showed greater enhancements for classes of antibody that display greater numbers of binding sites (IgM > IgA > IgG). In addition, the polymers were shown to inhibit binding of anti-Gal IgM to pig kidney cells in a
concentration-dependent manner whereas the trisaccharide alone had no effect, even at a concentration of 1 mmol/l.

Polyacrylamides decorated with sialic acid and sialic acid derivatives have also been used for studies of selectin-mediated interactions [86–88]. Hayashi and coworkers, for example, prepared a homopolymer from a modified SLex acrylamide conjugate and examined its activity against HL-60 cells in vitro and in a murine model in vivo [88]. The polymer inhibited adhesion more readily than the monomeric sugar (IC50 = 1.5 mg/ml for Sialyl Lewis X; IC50 = 0.01 mg/ml for the polymer), but the ability of the polymer to inhibit inflammation in vivo was only slightly greater than the monomer.

The use of ring-opening metathesis polymerization (ROMP) has also found application in the construction of glycopolymers. ROMP is a method for generating “living” polymers and permits the preparation of block copolymers with excellent control over the composition and length of each component. The living nature of the polymerization also permits the preparation of polymer chains that are end-labeled with different functional groups (Scheme 3) [89,90].

Kiessling and coworkers have used ROMP to prepare several classes of polyvalent glycoconjugates. Initial work developed short polymers presenting sul-

![Fig. 13](image-url)
Fig. 14. A More potent monomeric inhibitors enhance inhibition of hemagglutination more readily than weaker inhibitors [84]. \([\text{Neu2en}]_{1/2A}\) represents the concentration of monomeric inhibitor required to enhance the inhibition of hemagglutination by 50%. B Proposed mechanism for enhancement of inhibition of hemagglutination by monomeric inhibitors.

Scheme 3. Preparation of glycopolymers using ROMP
fated galactose moieties as inhibitors of the binding of leukocyte selectins to glycoconjugates on the surface of high endothelial venules [91–93]. They later found that these polyvalent oligomers, but not their monovalent counterparts, were able to induce the release of L-selectin from the surface of lymphocytes [94]. Subsequent work expanded this approach to the study of binding to concanavalin A [95, 96]. In these studies, the authors found that longer polymers were more effective than shorter polymers at inhibiting hemagglutination of erythrocytes by Con A (Fig. 15).

Recent work has taken advantage of the living nature of the polymerization to generate polymers presenting carbohydrate ligands and fluorescent reporter groups. Polymer 9 has been used to visualize cell-surface selectins [97], while polymer 10 has been used to examine the chemotactic response of E. coli [98].

**Fig. 15.** Relationship between the length of a mannoside polymer and its ability to inhibit hemagglutination by Con A [95, 96]. The 50-mer exhibits a significant enhancement of inhibition relative to the 25-mer and the 10-mer, perhaps due to its ability to span two binding sites of the lectin
5.2.2

**Immobilized Polymers**

Immobilized polymers have found use as solid supports for studies in cell biology and as scaffolds that present ligands for studies of polyvalent binding interactions. The most common example of the first application uses glycopolymers for the culture of hepatocytes [99–102]. Weisz and Schnaar, for example, used polyacrylamide derivatized with galactose to study the adhesion of hepatocytes and the localization of the asialoglycoprotein receptor during adhesion [100]. Schnaar and coworkers also used gels presenting gradients of the carbohydrate N-acetylglucosamine to study the migration of melanoma cells [101]. More recently, Griffith and Lopina prepared a class of “star” poly(ethylene oxide) gels presenting galactose [102]. These gels presented the sugar in clusters and enabled the investigators to estimate the minimum ligand to ligand distance required for efficient function of the hepatocytes.

Immobilized polymers have been particularly important model systems for studying the kinetics of carbohydrate-protein interactions. Surface plasmon resonance (SPR) is an analytical technique that is well suited for these studies because it measures binding interactions in situ and without the need for molecular or enzymatic labels. SPR has been reviewed elsewhere [103, 104], and its use for studies of carbohydrate-protein interactions is the subject of another chapter in this volume. It is, however, important to describe this technique in brief here. SPR measures changes in the refractive index of the medium near a thin film of gold or silver. Since the association of proteins or other small molecular weight ligands with groups presented near the interface influences the local refractive index of the medium, this technique can monitor binding interactions in real time (Fig. 16).

The majority of studies using SPR are conducted by coupling protein or small molecules to an immobilized carboxymethylated dextran polymer. In the case of carbohydrate studies, carbohydrate-specific antibodies, lectins, or neoglycoconjugates have been coupled to the polymer. An appropriate binding partner is flowed over the polymer and binding interactions are measured. Vliegenthart and coworkers, for example, immobilized sialic acid binding lectins from *S. nigra* and *M. amurensis* to the dextran matrix and examined the interaction of these proteins with a series of oligosaccharides [105]. Other studies have grafted preformed glycopolymers onto self-assembled monolayers on gold or silver [106, 107]. We will discuss the use of SPR to study interactions between lectins and well-defined organic surfaces later in the review.
Self-assembled monolayers are model systems that present one or two layers of amphiphilic molecules in a two-dimensional array [108]. Several classes of monolayers have found application in the study of biological recognition, including hybrid bilayers on glass, hybrid bilayers on gold and self-assembled monolayers of alkanethiolates on gold. Supported lipid bilayers are also an important model system in this class, but this model has not been used extensively for studies of multivalent carbohydrate-protein recognition. Each of these model systems presents carbohydrate ligands with control over the density of the ligand (but not necessarily over ligand-ligand spacing). An important feature of these models is that the immobilization of the ligand at a surface prevents intermolecular associations of multiple polyvalent ligands and receptors (as shown in Fig. 1). This characteristic simplifies the mechanistic analysis of polyvalent interactions. The following section presents recent developments in the use of these monolayers for studies of carbohydrate-protein interactions.

Fig. 16A, B. Surface plasmon resonance. (A) schematic of the optical biosensor and (B) example of a sensorgram that shows the association of a protein with an immobilized ligand.

5.3
Self-Assembled Monolayers

Self-assembled monolayers are model systems that present one or two layers of amphiphilic molecules in a two-dimensional array [108]. Several classes of monolayers have found application in the study of biological recognition, including hybrid bilayers on glass, hybrid bilayers on gold and self-assembled monolayers of alkanethiolates on gold. Supported lipid bilayers are also an important model system in this class, but this model has not been used extensively for studies of multivalent carbohydrate-protein recognition. Each of these model systems presents carbohydrate ligands with control over the density of the ligand (but not necessarily over ligand-ligand spacing). An important feature of these models is that the immobilization of the ligand at a surface prevents intermolecular associations of multiple polyvalent ligands and receptors (as shown in Fig. 1). This characteristic simplifies the mechanistic analysis of polyvalent interactions. The following section presents recent developments in the use of these monolayers for studies of carbohydrate-protein interactions.
5.3.1

Hybrid Bilayers on Glass

Hybrid bilayers on glass are a class of self-assembled monolayers that are formed by allowing phospholipid vesicles to associate with a monolayer of hydrophobic alkylsiloxanes [109]. This system is referred to as a hybrid because it contains both a bilayer leaflet and a synthetic leaflet (Scheme 4).

![Scheme 4. Architecture of a hybrid bilayer. For a bilayer on gold, X = sulfur. For a bilayer on glass, X = Si(O) Group for molecular recognition Phospholipid or sphingolipid Hydrophobic undecyl group Substrate](image)

Hybrid bilayers are good model systems for studying cell-cell and cell-pathogen interactions because ligands presented in the bilayer are free to diffuse laterally, much like ligands found in cellular membranes [110]. While this property allows the formation of ligand clusters, it may also facilitate the exchange and nonspecific adsorption of cell surface proteins. The extent to which changes in the composition of these substrates changes over time has not been determined. The utility of supported bilayers in mechanistic studies of carbohydrate-protein interactions was demonstrated by Stevens and coworkers in the development of a sensor for bacterial toxins [111]. This work utilized a hybrid bilayer presenting 10,12-pentacosadiynoic acid (PDA), a sialic acid-PDA conjugate, and a ganglioside lipid (Fig. 17).
Fig. 17. Colorimetric detection of toxins using a supported bilayer [111]. The extensively conjugated poly(acetylene) network on the left exhibits a blue color. Binding of a toxin to the ganglioside in the bilayer decreases the length of the conjugated network and changes the color of the film from blue to red. This figure shows binding of the toxin but not changes in the structure of the bilayer.
Ultraviolet illumination of the bilayer afforded an extended network of conjugated polyacetylenes having a blue color. Binding of a bacterial toxin to the ganglioside ligand perturbed the structure of the monolayer, decreasing the length of the conjugated backbone, and resulting in a color change from blue to red. This colorimetric readout enabled the rapid and sensitive detection of cholera toxin and the heat-labile enterotoxin from *E. coli* and has been used in a solution phase format in which liposomes are used in place of the hybrid bilayers [112, 113].

Schmidt and coworkers used hybrid bilayers on glass to study the rolling of leukocytes on immobilized SLe\(^\alpha\) [114, 115]. In one study, a series of glycolipids was prepared in which SLe\(^\alpha\) was connected to a glycerol backbone either directly or via tri-, hexa-, or nona(ethylene glycol) groups [114]. At low densities of ligand, groups with longer spacer arms were still able to support the cell rolling, while groups with shorter spacer arms did not. This result demonstrates that both the accessibility and the flexibility of the ligand are important in cell adhesion.

### 5.3.2 Hybrid Bilayers on Gold

Hybrid bilayers on gold are prepared by allowing a phospho- or sphingolipid vesicle to associate onto a hydrophobic self-assembled monolayer on gold (Scheme 4 where X = sulfur) [116]. These models are often preferred over the hybrid bilayers on glass because the underlying gold film permits the use of surface plasmon resonance for studies of real time ligand-receptor interactions.

Kiessling and coworkers used SPR to investigate the binding of Con A to mannosides presented in a hybrid bilayer [117]. By incubating the lectin with different concentrations of \(\alpha\)-methyl mannose, the authors were able to determine the apparent binding constant of the lectin for the immobilized mannoside. This study also investigated the ability of polymeric inhibitors (prepared by ROMP) to inhibit the binding of Con A to the monolayer (Fig. 18).

The authors found that polymers were more effective inhibitors than monomers and that longer polymers were more potent inhibitors than shorter polymers. Preincubation of the polymers with the lectin significantly improved their potency. It is still unclear, however, whether the enhanced inhibition of lectin binding by longer polymers was due to polyvalent attachment or to crosslinking of the protein in solution.

### 5.3.3 Self-Assembled Monolayers of Alkanethiolates on Gold

Self-assembled monolayers of alkanethiolates on gold (SAMs) are a class of model substrates that are especially well suited for studies of polyvalent binding at interfaces [118, 119]. These monolayers form spontaneously by the adsorption of alkanethiols from solution onto a clean surface of gold. Since the properties of the monolayer depend upon the terminal functional group of the precursor alkanethiol, virtually any surface can be prepared using organic synthesis (Scheme 5). SAMs that present oligo(ethylene glycol) groups have the addition-
al advantage that they are effective at resisting the nonspecific adsorption of protein, which allows an unambiguous characterization of specific carbohydrate-protein interactions [120].

For mechanistic studies of polyvalent carbohydrate interactions, monolayers offer three advantages over other model systems. First, the properties of monolayers enable molecular level control over the structure, density, and environ-

![Graph](image_url)  

**Fig. 18.** Inhibition of binding of Con A to a hybrid bilayer presenting mannose groups [117]. Longer polymers were more effective than shorter polymers at inhibiting binding to the bilayer. All polymers were more potent inhibitors when they were preincubated with the lectin.

![Scheme 5](image_url)  

**Scheme 5.** Architecture of a self-assembled monolayer of alkanethiolates on gold.
ment of ligands. This feature is particularly important for carbohydrate-protein interactions, which are often sensitive to subtle changes in the presentation of ligand. Second, the ligands are immobilized and presented in an environment that is inert to nonspecific adsorption of protein. This characteristic eliminates changes in the structure of the substrate over time and is particularly important for studies that utilize mammalian cells. Finally, these substrates are compatible with SPR, making the characterization of ligand structure and ligand-receptor interactions possible.

Kahne and coworkers used monolayers presenting different densities of carbohydrate to characterize a lectin “specificity switch” [121]. Two carbohydrate ligands were tested in this study: a native ligand for the *B. purpura* lectin and a lead compound that bound this ligand in a library screen (Fig. 19).

A.

![Diagram](image)

**Natural Ligand**  **Hit Ligand**

B.

<table>
<thead>
<tr>
<th>$\chi_{\text{sugar}}$</th>
<th>IC$_{25}$ (Natural Ligand)</th>
<th>IC$_{25}$ (Hit Ligand)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>150 $\mu$M</td>
<td>130 $\mu$M</td>
</tr>
<tr>
<td>0.6</td>
<td>80 $\mu$M</td>
<td>190 $\mu$M</td>
</tr>
</tbody>
</table>

Fig. 19A, B. Density-dependent binding of the lectin from *B. purpura* to two carbohydrate ligands [121]. (A) the structure of the two monolayers presenting the two ligands; (B) at low densities of sugar ($\chi_{\text{sugar}} = 0.1$), the immobilized native ligand interacts more strongly with the lectin, while at higher densities ($\chi_{\text{sugar}} = 0.6$), the hit ligand from a library screen binds more tightly.
Using SPR, they found that the lectin preferred the native ligand at low densities but bound the lead compound more tightly at higher density. This finding suggests that cells may regulate their interaction with extracellular proteins through changes in the density of a particular carbohydrate in the glycocalyx.

We have used monolayers to study the interaction between cellular glycosyltransferases and glycosylated proteins of the extracellular matrix. One example of this interaction occurs during the migration of malignant cell lines that express $\beta$-(1,4)-galactosyltransferase (GalTase) in their plasma membrane [122, 123]. This enzyme, traditionally found in the Golgi apparatus, plays a crucial role in the post-translational modification of proteins. When expressed at the cell surface, GalTase transfers galactose to N-acetylglucosamine (GlcNAc) residues on the extracellular matrix protein laminin. The enzyme modifies the matrix only during migration, suggesting that it may play a mechanistic role in the migration process.

We utilized monolayers that present GlcNAc as a model system to investigate the enzymatic modification of surfaces by GalTase [124]. In initial work, we prepared monolayers presenting different mole fractions of GlcNAc and investigated the enzymatic incorporation of $^{14}$C-labeled galactose onto the monolayer (Fig. 20). The modification of the substrate by GalTase showed a striking dependence on the density of ligand on the monolayer (Fig. 20B). At mole fractions less than 0.7 ($\chi_{\text{GlcNAc}} < 0.7$), galactose incorporation increased linearly with the density of carbohydrate. At higher densities, however, incorporation of $^{14}$C galactose decreased rapidly. Monolayers presenting carbohydrate alone, for example, incorporated the same density of $^{14}$C as monolayers with $\chi_{\text{GlcNAc}} = 0.2$. This result suggests that ligand crowding interferes with further enzymatic modification at high values of $\chi_{\text{GlcNAc}}$.

In order to characterize the enzymatic modification, we investigated the binding of two lectins to monolayers presenting either GlcNAc or the product of the enzymatic reaction $N$-acetyllactosamine (LacNAc) (Fig. 21). Before the modification only the lectin from $B.\ simplicifolia\ II$, which recognizes GlcNAc, associated with the monolayer. After the modification, however, only the lectin from $E.\ cristagalli$, which recognizes LacNAc, bound to the surface. To determine if the lectins could be used to monitor the reaction, we examined the binding of $B.\ simplicifolia$ over time and found that there was indeed a time-dependent decrease in binding as the enzymatic modification proceeded (Fig. 22).

These results show that the combination of monolayers and SPR is a powerful technique for mechanistic studies of carbohydrate-protein interactions at interfaces. These methods can also be extended to the study of other glycosyltransferases and glycosidases. We are currently studying these processes as well as the modification of the carbohydrate by migrating cells.
Fig. 20. (A) Enzymatic modification of monolayers that present GlcNAc [124]. (B) Relationship between the density of carbohydrate on the monolayer ($\chi_{\text{GlcNAc}}$) and the transfer of $^3$H galactose onto the monolayer.
Fig. 21. Data from SPR for the binding of *Bandeiraea simplicifolia* BS-II lectin (*dashed curve*) and *Erythrina cristagalli* lectin (*solid curve*) to monolayers presenting (A) GlcNAc and (B) LacNAc among tri(ethylene glycol) groups. The BS II lectin bound monolayers presenting GlcNAc groups, while the *E. cristagalli* lectin bound only monolayers presenting LacNAc moieties. Binding of both lectins could be competitively inhibited by soluble carbohydrate ligand [124]
Fig. 22. (A) The *E. cristagalli* lectin (solid curve), but not the BS II lectin (dashed curve), binds LacNAc groups resulting from treatment of GlcNAc groups with GalTase. (B) Plot of BS II lectin binding vs time. All data are reported as a percentage of the binding response observed between the lectin and an untreated monolayer presenting GlcNAc [124].
6 Novel Model Systems

Several groups have begun to develop new model systems that extend the scope of current research on polyvalent carbohydrate-protein interactions. These include dynamic substrates, combinatorial methods, and the use of mammalian cells that present defined glycoforms on their surface. Each has unique capabilities and limitations that are described below.

6.1 Dynamic Self-Assembled Monolayers

Dynamic substrates are a class of model substrates that can change, in real time, the identity or density of ligands that participate in a polyvalent association. These substrates are important for modeling interactions that are altered by an external stimulus, such as the initiation of cellular or bacterial migration in response to a change in the density or structure of an immobilized carbohydrate [101]. Self-assembled monolayers on gold are ideal platforms for the development of dynamic substrates because the underlying metal film serves as an electrode that can affect oxidation-reduction chemistry at the interface [125]. Our group has employed the hydroquinone-quinone redox couple because the quinone group reacts efficiently and selectively as a dienophile in the Diels-Alder reaction, while the hydroquinone group is unreactive under the same reaction conditions [126, 127] (Fig. 23A).

By tethering a carbohydrate to cyclopentadiene, it is possible to turn on the immobilization of the sugar (Fig. 23B). We have used this methodology to immobilize GlcNAc to monolayers and have shown that the lectin from *B. simplicifolia* interacts with the monolayer only after the immobilization. This methodology makes possible the development of substrates for investigating a wide range of carbohydrate-mediated phenomena, including the up- or down-regulation of carbohydrate binding proteins on cell surfaces or in solution.

6.2 Combinatorial Approaches

Advances in combinatorial chemistry have permitted the rapid discovery and optimization of ligands for carbohydrate-binding proteins [128–130], minimizing the significant time investments often demanded by traditional synthetic approaches (Sect. 4). Kahne and coworkers, for example, prepared a library of disaccharides on solid support and screened this library for binding to the lectin from *Bauhinia purpurea* [131, 132]. The screen identified a novel ligand that bound the lectin more tightly than the native ligand when presented on beads.

Whitesides and coworkers have used a combinatorial approach to libraries of polyacrylamides presenting derivatives of sialic acid [133]. Evaluation of these compounds identified a ter-polymer that inhibited the hemagglutination of chicken erythrocytes at subnanomolar concentration. In another study, Roy and coworkers generated a series of polyacrylamide gels presenting mono- and di-
saccharides and evaluated the modification of these polymers with several glycosyltransferases in a 96-well format [134]. They demonstrated that this assay was both accurate and more efficient than its solution-phase counterpart. Recently, Ramstrom and Lehn utilized disulfide exchange to generate a small library of divalent carbohydrates and screened this library for its ability to bind Con A [135]. A bis-mannoside was selected from the mixture as the most potent ligand for the lectin.

The preparation of carbohydrate libraries on two-dimensional solid support also holds great promise for studies of polyvalent carbohydrate-protein interactions [128–130, 136, 137]. Jobron and Hummel, for example, generated a series of glycopeptides on cellulose using traditional methods in peptide synthesis [138]. This study did not, however, utilize the array to investigate multivalent

Fig. 23A, B. Diels-Alder reaction for the immobilization of carbohydrates to monolayers. (A) Hydroquinone groups can be reversibly oxidized to the quinone group, which undergoes a Diels-Alder reaction with a substituted cyclopentadiene. (B) Immobilization of GlcNAc by the Diels-Alder reaction of quinone and a conjugate of the carbohydrate and cyclopentadiene
recognition at the solid support. Our group is currently using self-assembled monolayers on gold for the preparation of carbohydrate arrays. By immobilizing a series of carbohydrates onto distinct regions of the monolayer, we are generating a “sugar chip” that can be modified either chemically or enzymatically. Target structures on the chip can be evaluated by immunofluorescence microscopy. Since monolayers permit control over the presentation and density of ligands, this approach makes it possible to define unambiguously ligands for bacterial and cellular adhesion.

6.3 Cells, Bacteria, and Viruses

An intrinsic feature of the model systems described above is that they do not present carbohydrates in their native cellular environment. Several approaches have been used to overcome this limitation. First, mammalian cells have been enzymatically tailored to present non-native carbohydrate structures [139–141]. Palcic and coworkers, for example, used Lewis α (1 → 3,4)-fucosyltransferase to transfer a preassembled trisaccharide onto the surface of erythrocytes [141]. This approach can, in principle, be extended to both bacterial and viral systems. A more recent methodology developed by Bertozzi and coworkers uses the cell’s own metabolic machinery to present modified carbohydrates at the cell surface [142–144]. In this approach, the enzymes responsible for the synthesis of sialic acid process a derivative of N-acetylmannosamine substituted with a ketone group. The modified sialic acid is transported to the cell surface where it can be selectively modified using nucleophilic hydrazide, aminooxy, or thiosemicarbazide groups. While this method is selective and biologically relevant, it is limited to a small number of cellular pathways that tolerate non-native functional groups. We believe, however, that these cellular models will continue to become more useful and powerful in the near future.

7 Comparison of Model Systems

An overview and comparison of model systems is presented in Fig. 24.

8 Future Directions and Conclusions

This chapter establishes a mechanistic framework for understanding polyvalent carbohydrate-protein interactions and describes several classes of model systems for the evaluation of these interactions. While the examples in this review demonstrate the diversity and complex nature of carbohydrate-protein interactions, they also emphasize the need for the development of new analytical tools, novel synthetic methods, and more sophisticated model systems. We believe that the approaches described in this review, together with combinatorial methods and advances in materials science, promise to expand further the rate and scope of new discoveries in glycobiology.
A. Overview of Model Systems

Low Valency Model Systems

**Principle Use:** Determination of structural and geometric requirements for tight binding to a multivalent protein

**Examples:** Glycocusters, cyclodextrins, calixarenes, dendrons and dendrimers

High Valency Model Systems

**Principal Use:** Chromatography, vaccination, inhibition of interactions that occur over large surface areas

**Examples:** Neoglycoproteins, soluble polymers

**Principal Use:** Mechanistic studies of interactions that occur at interfaces

**Examples:** Immobilized polymers, hybrid bilayers on glass or gold, self-assembled monolayers

B. Comparison of Model Systems

<table>
<thead>
<tr>
<th>Model System</th>
<th>Valency</th>
<th>ELLA</th>
<th>HAI/ITC/ACE</th>
<th>SPR</th>
<th>Stability$^d$</th>
<th>Homogeneity$^e$</th>
<th>Synthetic Effort</th>
</tr>
</thead>
<tbody>
<tr>
<td>glycocusters</td>
<td>&lt;7</td>
<td>Yes</td>
<td>Yes</td>
<td>No$^c$</td>
<td>++++</td>
<td>++++</td>
<td>Significant</td>
</tr>
<tr>
<td>cyclodextrins</td>
<td>2-20</td>
<td>Yes</td>
<td>Yes</td>
<td>No$^c$</td>
<td>++++</td>
<td>++++</td>
<td>Significant</td>
</tr>
<tr>
<td>calixarenes</td>
<td>2-20</td>
<td>Yes</td>
<td>Yes</td>
<td>No$^c$</td>
<td>++++</td>
<td>++++</td>
<td>Significant</td>
</tr>
<tr>
<td>dendrimers</td>
<td>2-20</td>
<td>Yes</td>
<td>Yes</td>
<td>No$^c$</td>
<td>++++</td>
<td>++++</td>
<td>Significant</td>
</tr>
<tr>
<td>neoglycoproteins</td>
<td>2-40</td>
<td>Yes</td>
<td>Yes</td>
<td>No$^c$</td>
<td>++++</td>
<td>++++</td>
<td>Significant</td>
</tr>
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<td>soluble polymers</td>
<td>&gt;10</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>+++</td>
<td>++</td>
<td>Minimal</td>
</tr>
<tr>
<td>immobilized polymers</td>
<td>&gt;100</td>
<td>Yes$^b$</td>
<td>No</td>
<td>Yes$^d$</td>
<td>+++</td>
<td>++</td>
<td>Minimal</td>
</tr>
<tr>
<td>hybrid bilayers on glass</td>
<td>&gt;100</td>
<td>Yes$^b$</td>
<td>No</td>
<td>No</td>
<td>++</td>
<td>+++</td>
<td>Moderate</td>
</tr>
<tr>
<td>hybrid bilayers on gold</td>
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<td>Yes$^b$</td>
<td>No</td>
<td>Yes</td>
<td>++</td>
<td>+++</td>
<td>Moderate</td>
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<tr>
<td>self-assembled monolayers</td>
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<td>No</td>
<td>Yes</td>
<td>+++</td>
<td>+++</td>
<td>Moderate</td>
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</tbody>
</table>

$^a$HAI = hemagglutination inhibition assay; ELLA = enzyme-linked lectin assay; SPR = surface plasmon resonance; ITC = isothermal titration calorimetry; ACE = affinity capillary electrophoresis.

$^b$An ELLA can be used if the model surface, rather than a ligand in solution, is evaluated.

$^c$SPR can be used if the conjugate is immobilized directly or indirectly on a thin metal film.

$^d$If the polymer is immobilized directly or indirectly on a thin metal film

$^e$Stability depends on the nature of chemical bonds in the structure. For example, esters and thioureas are less stable than amides.

$^f$++++ = excellent; +++ = good; ++ = fair; + = poor

$^g$A homogeneous system is one in which all ligands are equally accessible to a multimeric receptor

$^h$These structures are more homogeneous, but less easily prepared, if chemoselective ligation methods are used.

**Fig. 24.** A Summary of high valency and low valency model systems. B Comparison of model systems presented in the review
9 References

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