Chapter 29. Principles for Multivalent Ligand Design

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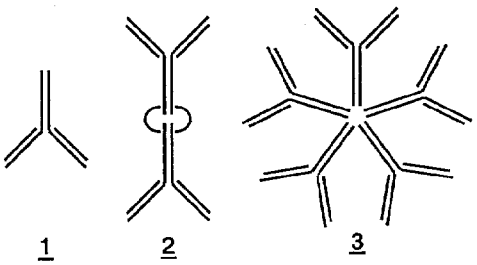
Multivalent interactions control a wide variety of cellular processes including cell surface recognition events (1). Examples of specific cell – cell binding events can be found in diverse processes, such as inflammation, tumor metastasis, and fertilization. An understanding of the mechanistic principles that underlie multivalent binding events would facilitate the generation of new classes of therapeutic agents and biomaterials. Synthetic multivalent ligands can be used to illuminate and exploit biological processes that benefit from multipoint contacts. This review focuses on the principles for designing synthetic multivalent ligands and the interplay between ligand structure and biological activity

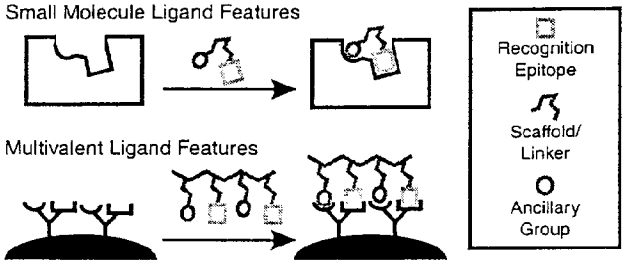
Antibodies are the perhaps most widely used tools for studying multivalency in biological systems. Because of their quaternary structures, antibodies have multiple recognition sites. There are many antibody isotypes, that vary in size, shape, orientation of binding sites, and valency. Although IgG, IgD, and IgE are dimeric 1, IgA and IgM form higher order oligomers. IgM is a decamer 3, and IgA ranges from a tetramer 2 to an octamer. These differences in antibody size and quaternary structure directly influence their resulting biological activities (2).



The use of antibodies can provide information about the involvement of multivalent interactions in a particular process; however, this information is limited. Understanding the structural requirements for multivalent ligand activity requires a wider variety of multivalent ligands whose structure, including size and valency, can be controlled and tailored. It is chemical synthesis that provides access to molecules that possess a variety of sizes, shapes, and valencies.

The flexibility offered by chemical synthesis is illustrated by design strategies that have been used to create small molecule drug candidates. Given a natural product lead, chemical synthesis can be used to install critical molecular features, thereby creating small molecule analogs of a more complex structure. The display of functional groups and other features (i.e. hydrophobicity and flexibility) can be optimized to create ligands with higher affinity or specificity for the chosen target. The principles of small molecule drug design have been reviewed extensively, including a recent discussion of peptidomimetic design (3).

Small molecule ligand design can be analyzed to emphasize common principles important for multivalent ligand design. A primary recognition epitope, which may contain important binding features identified in a natural product, or be derived from a common pharmacophore, or act as a transition state analog, serves as the lead structure (square). In addition, ancillary functional groups that exploit interactions not initially identified in the lead structure or that



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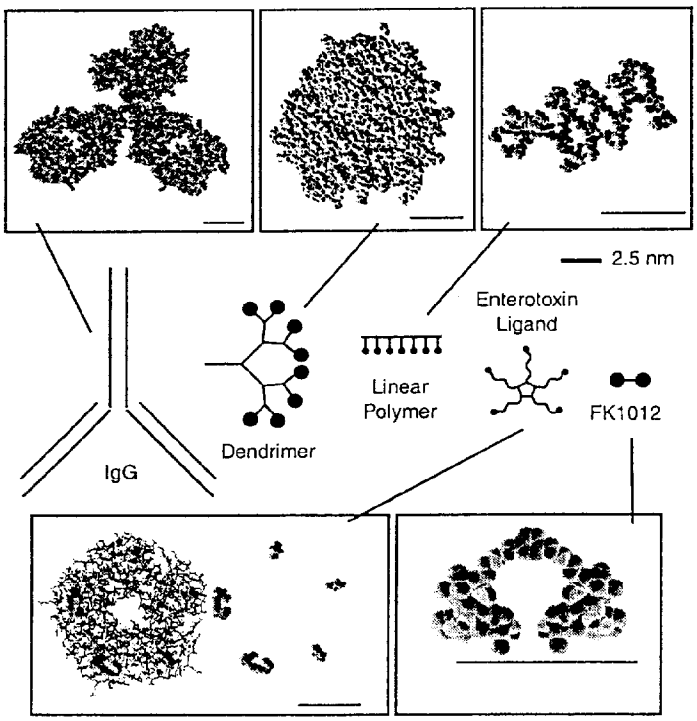
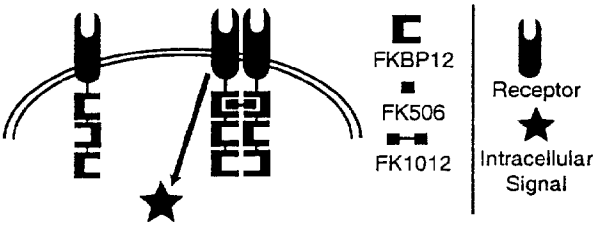
restrict the overall ligand conformation often lead to ligands with significantly increased potencies (circle). The features of the scaffold (or the linker) that presents the groups that occupy the binding site(s) on the protein target also are important determinants of productive binding. These are basic design principles that can be used to guide the preparation of larger, multivalent ligands.

In multivalent displays, the identity of the monovalent recognition epitopes is dictated by the biological interaction to be studied. These epitopes can be derived from a natural ligand or from identification of small molecule agonists or antagonists. After selecting the recognition epitope, the choice of scaffold or backbone from which these epitopes are displayed is a critical design parameter. This choice determines many of the overall ligand features including size, three-dimensional shape, and valency. The relative sizes of the scaffolds most commonly used to study multivalent interactions can be estimated using structures derived from X-ray crystallography, molecular modeling, or electron microscopy. Models and scaled schematic depictions of scaffolds discussed in this review are depicted (4). In addition to their differences in size, scaffolds also vary in three-dimensional shape, as exemplified in the comparison of linear polymers and more spherical dendrimers. The number of attachment points available on a scaffold determines the maximum valency, or number of recognition epitopes, that can be displayed. This feature, combined with the size and shape of the scaffold, dictates the spacing of recognition epitopes. Valency, spacing, and the flexibility and hydrophilicity of the framework can have significant effects on the biological activity of the ligand. The linker, which also can influence activity, must be attached to the recognition epitope in such a way that receptor-ligand binding is not disrupted. Finally, the incorporation of ancillary groups, which contribute binding energy but are not found in the original recognition epitope, can be exploited for multivalent ligand design as with small molecule targets. The interplay of all of these ligand features can result in molecules with unique biological activities. A change in ligand structure can alter the mechanisms by which a ligand functions. For example, changing the maximum distance between terminal binding epitopes on a synthetic scaffold can influence the number of receptors that can bind simultaneously to a single ligand, thereby altering biological responses to the ligand. The mechanisms that underlie multivalent ligand activity have been discussed elsewhere (1,5).

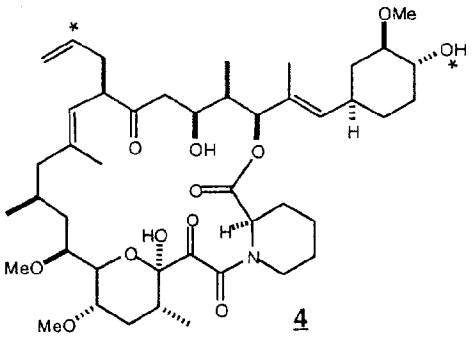


To illustrate the influence of multivalent ligand design features, we present examples in which one or more multivalent ligand design features have been systematically varied and the biological activity of the resulting ligands explored. Two

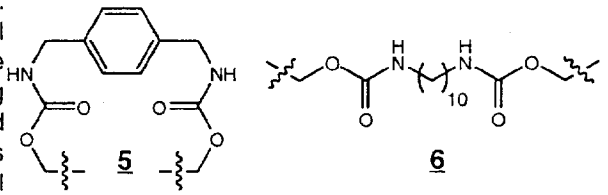
examples of defined divalent molecules will be discussed as will additional examples of higher valency ligands. Dimeric ligands are discussed separately because the strategy used to display the recognition epitopes is different. Specifically, dimeric ligands often consist of recognition elements connected by a linker, as opposed to higher valency compounds that generally display recognition epitopes from a scaffold. Descriptions of strategies for multivalent ligand synthesis have appeared elsewhere, including a discussion of bioactive polymer assembly (6).

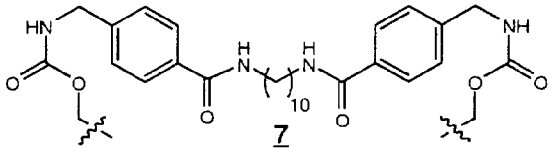


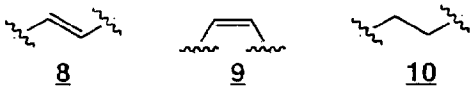
Defined divalent molecules have been generated to explore protein dimerization and the signal transduction events that result. The chemical induced dimerization (CID) strategy developed by Schreiber, Crabtree and coworkers uses cell permeable synthetic dimers of natural products with known protein receptors (7). A target receptor is fused to the natural product-binding domain, and the divalent natural product ligand is able to oligomerize the target receptor. A cell line transfected with the fusion protein can be treated with synthetic dimers and the biological effects of receptor oligomerization measured. This controlled association can mediate intracellular events, such as cytoplasmic calcium concentration, gene transcription, or kinase activity (8, 9).



The first demonstration of the CID strategy utilized FK506, a cell permeable immunosuppressant. FK506, 4, binds to the peptidyl-prolyl cis-trans isomerase FK506-binding protein (FKBP12); this complex is, in turn, a ligand for the serine/threonine phosphatase calcineurin (10). The binding of the FK506-FKBP12 complex to calcineurin is responsible for the immunosuppressive activity of FK506. The CID strategy exploited the interaction between FK506 and FKBP12 while minimizing the interaction of the FK506-FKBP complex with calcineurin. The functional groups of FK506 that contribute to FKBP12 and calcineurin binding are known, and sites involved in calcineurin binding were chosen as points of modification for dimerization of FK506 (11). Two of the FK506 functional groups that do not appear to be required for FKBP12 binding are the hydroxyl group (*) and the allyl group (*). Linkers appended through hydroxyl group modification obstructed binding to FKBP12 (12). Modification of the alkene did not interfere with the interaction of FK506 and FKBP12; therefore, the terminal alkene was dihydroxylated, the diol intermediate was oxidatively cleaved, and the resulting





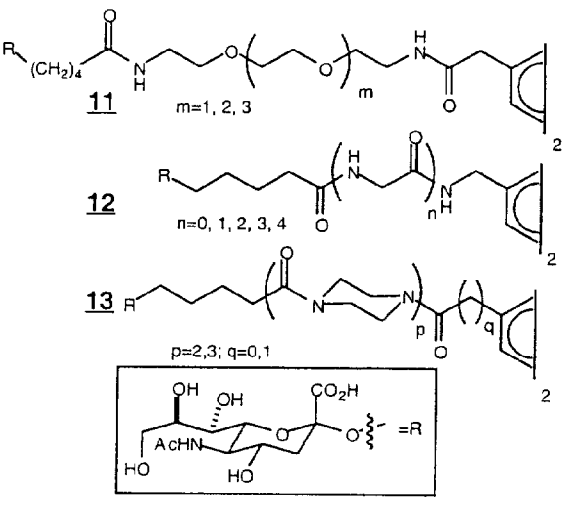


aldehyde was reduced to a primary alcohol (7). The alcohol was elaborated to afford a group of FK506 dimers, referred to as FK1012s, carbamates **5**-**7**. Dimers also were assembled by cross metathesis of the terminal methylene group with the Grubbs catalyst (13), a strategy that produced **8**-**10**. All of the divalent ligands can mediate oligomerization of the FKBP12 fusion proteins. Their biological activities do not differ significantly; consequently, the specific structural features of the linker group (rigidity, hydrophobicity, and length) have little influence on ligand function. Thus, it is the point of linker attachment that is critical; the attachment preserves interactions with the target protein (FKBP) and minimizes unwanted interactions with calcineurin.

Linker elements, which are generally not relevant in the design of monovalent compounds, are often crucial to the successful generation of multivalent ligands. Although the identity of the linker in the FK506 strategy was not critical, the activity of many multivalent ligands depends on the nature of the linker. For example, both linker length and conformational flexibility play important roles in the interaction of a series of various divalent *N*-acetyl neuraminic acid (referred to here as sialic acid) derivatives with the influenza virus hemagglutinin (HA).

Divalent sialic acid derivatives were designed to bind to HA, a homotrimeric protein with three sialic acid binding sites (14). HA mediates the binding of virus to the cell surface sialic acids. Monovalent sialic acid derivatives are only weak inhibitors of HA-mediated cell adhesion (IC_{50} values ca. 3 mM). Divalent displays might have increased potency because they have the potential to occupy more than one binding site. Three spacers, ethylene glycol (**11**), glycine (**12**), and piperazine (**13**), were selected, and linkers of a variety of lengths were generated. The tethers were chosen because they differ in conformational flexibility. The ability of the sialic acid dimers to inhibit HA-mediated hemagglutination was measured (hemagglutination inhibition assay; HIA). None of the anomeric linkers interfered with HA binding, but alterations in linker structure had dramatic effects on biological activities. When ethylene glycol linkers are employed, the maximum increase in potency was only 20-fold greater than monovalent sialic acid, in contrast to the 100-fold increase observed for the derivative with the less flexible glycine spacer. Interestingly, series **13**, which possesses the most rigid piperazine linker, showed no enhanced potencies, even when the tether length, as estimated by molecular modeling, was similar to that of the linker of the most potent glycine dimer. Inter- and intra-receptor binding may contribute to a ligand's activity for a receptor with multiple ligand binding sites, such as HA. When the ability of the most potent glycine dimer to bind free trimeric HA in solution was measured, it was found that the dimer was no more potent than monomeric sialic acid. The potency of active **12**, therefore, is likely due to its ability to interact with two HA trimers on the viral surface.

Schematic Ligand Structure:



Both the FK506 and sialic acid dimers illustrate the importance of design in the construction of divalent ligands. The FK506 examples demonstrate that the point of covalent attachment is critical for biological activity; changing the site of modification can result in dramatically different biological results. The HA example highlights the necessity to design

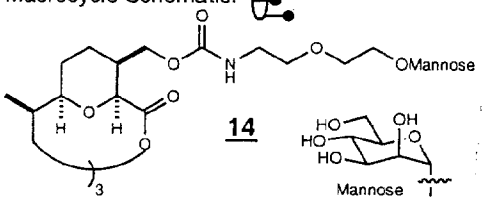
linkers based on the biological system where they will be studied or ultimately used. Changing the nature or length of the linker may allow different binding modes to be accessed, resulting in altered biological activities. The point of modification and the structure of the linking unit are the two key features involved in the synthesis of dimeric ligands, however there are other ligand features that must be considered in the design of compounds with greater valency.

Compounds with higher valencies have been generated to explore systematically the effects of various multivalent ligand parameters on biological activity. The design of these materials requires consideration of additional ligand features. The parameters outlined in the examples discussed here include the three-dimensional shape and size of the multivalent scaffold, the length and nature of the linkers that connect the recognition epitope and the incorporation of ancillary functional groups.

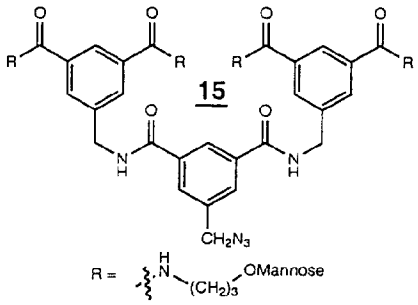
Size and three dimensional shape

Different synthetic approaches can be used to vary the size and shape of multivalent ligands. Even when the identity of the recognition epitope is held constant, variations in size and shape of the scaffold can result in ligands with dramatically different biological activities. A protein that has been used as a tool to study multivalent interactions is concanavalin A (Con A), a tetrameric, mannose-binding lectin. A number of multivalent ligands that display mannose have been generated in different shapes and sizes. For example, a trivalent mannose display based on a conformationally defined macrocycle, **14**, was synthesized and determined to have a 30- to 40-fold higher affinity for Con A than monomeric mannose in a surface plasmon resonance assay. Fluorescence resonance energy transfer (FRET) experiments revealed that one of the mechanisms responsible for the increase in potency is the ability of the ligand to form soluble Con A clusters (15). Dendrimers bearing mannose, **15**, also are ligands for Con A and these are more potent inhibitors of hemagglutination than monovalent mannose. Tetrameric **15** showed a 31-fold enhancement over mannose on a saccharide residue basis. The activity of the dendrimer was suggested by titration microcalorimetry and X-ray crystallographic studies to be due in part to aggregation and precipitation of Con A from solution (16). A cyclic scaffold, β-cyclodextrin, was used to generate a heptavalent display of mannose residues, **16**. Like the dendrimers, this ligand elicits efficient Con A precipitation (17). Mannose-substituted linear polymers, **17**, generated using ring-opening metathesis polymerization (ROMP) have shown increased potency in HIAs as well as in surface plasmon resonance assays. As the size of the polymer increases, the ligands exhibit

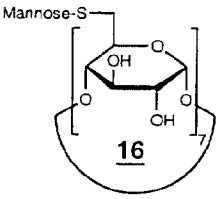
Macrocycle Schematic:



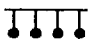
Dendrimer Schematic:

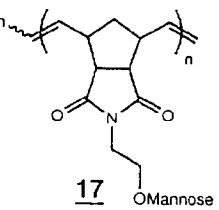


Cyclodextrin Schematic:



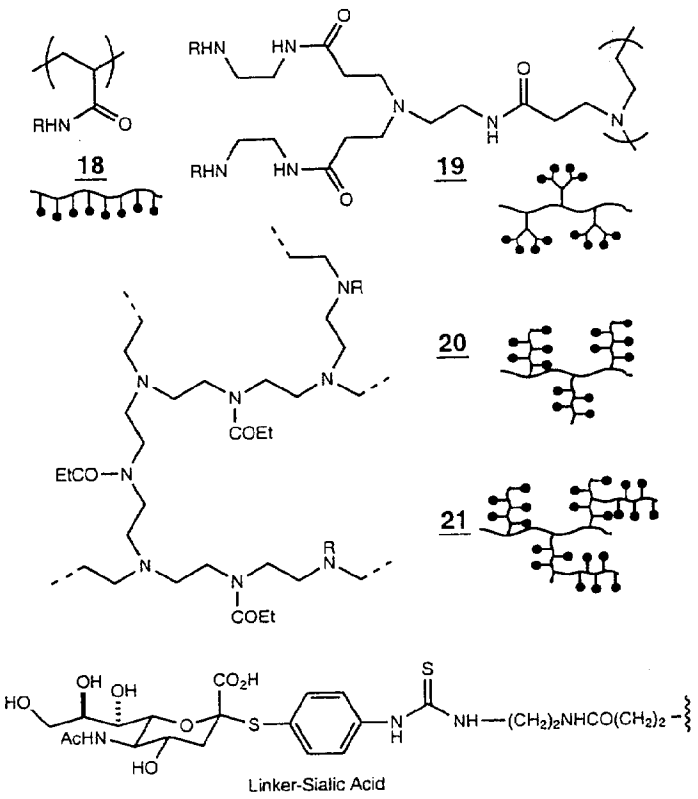
ROMP-Derived Polymer Structure:





potencies significantly greater than many of the smaller templates described above (18-20). The use of different assays for each of these ligand types, however, makes direct comparisons of their activities difficult. Still, based on the degree of potency enhancement and the mechanistic information from compounds 14 and 15, the differences in potency of these ligands can likely be attributed to changes in their mechanisms of action. The modes of binding available to these ligands are dictated by their sizes and shapes.

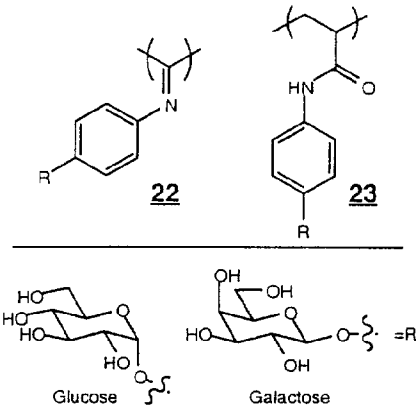
Two commonly used multivalent scaffolds are linear polymers, such as polyacrylamide polymers, and wedge-shaped or spherical dendrimers, such as PAMAM dendrimers. Baker and coworkers have combined polymer and dendrimer chemistry to generate different ligand architectures (21). The products displayed carbohydrate recognition epitopes and were tested against four different viral strains in a hemagglutination assay. Sialic acid-substituted, linear acrylamide polymers 18 were compared to linear polymers displaying sialic acid-bearing dendrimers along the polymer backbone (dendron-displaying linear polymer, 19). The biological activity of the linear polymer and dendron-displaying linear polymer was target dependent. Specifically, the linear polymers 18 were more effective against one viral strain (influenza A H2N2 mouse) than was the equivalent dendron-displaying polymer 19. The activity of polymer 19, however, was greater against another strain (X31), and both materials had essentially the same activity towards a third virus (Sendai). Two additional polymer architectures, termed comb-branched (20) and dendrigraft (21) also were examined. Comb-branched refers to a linear polymer displaying sialic acid-bearing linear polymers, and dendrigraft refers to a similar, but more highly branched, structure. Unlike the previous comparison, there were no significant differences in the hemagglutination inhibition activities of 20 and 21 for any of the four viral strains tested. Thus, for high valency ligands, backbone structure can play a role in biological activity, although the trends are difficult to predict.



Flexibility of the Scaffold

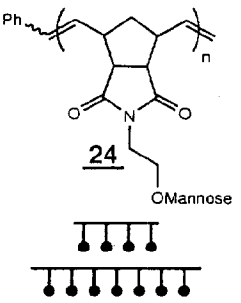
The flexibility of a scaffold also can affect the biological activity of a multivalent display, as observed by Kobayashi and coworkers (22). These researchers explored the relative potencies of two classes of linear saccharide substituted polymers, which vary in backbone flexibility. Saccharide-bearing poly(phenyl isocyanides) (PPI) 22 were generated, and these materials have a rigid,

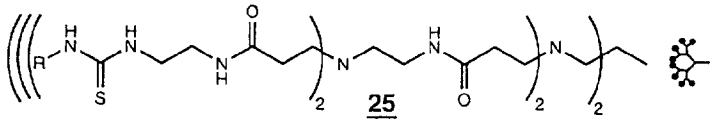
helical structure. For comparison, carbohydrate-substituted phenylacrylamide polymers (PAPs) **23** were synthesized because they are more flexible and can adopt a more extended conformation. The activities of the substituted PPI and PAP materials were assessed using two different lectins, ricin agglutinin (RCA_{120}) and Con A. When the activities of galactose-bearing PPI and PAP materials in an assay with RCA_{120}, were compared, the flexible PAP materials exhibited significantly higher potencies. Con A, which binds both glucose and mannose, was tested with glucose-bearing PAP and PPI displays. The backbone again affected the biological activity; the more flexible PAP materials had greater activity in assays with Con A. These data indicate that too rigid a scaffold can obstruct protein binding to recognition elements. It should be noted, however, that increasing backbone flexibility too much could diminish activity because of unfavorable contributions due to increased conformational entropy.



Ligand Valency

Dissecting the effects due to changes in valency from those due to changes in presentation is difficult. One investigation that addressed this issue took advantage of the features of ROMP. This synthetic approach was used to generate mannose-bearing materials **24** that varied in valency. A key feature of this strategy is that valency can be changed, while holding other ligand features, such as epitope spacing and backbone flexibility, constant. The inhibitory activities of the ligands in a Con A-mediated HIA depended on their valency (18). Higher valency ligands had increased potency. Recently, the synthesis of defined, linear displays via ROMP was generalized so that libraries of displays can be generated (20). The ability to access different mechanisms by varying valency may provide new opportunities for the development of multivalent scaffolds with a variety of biological effects.

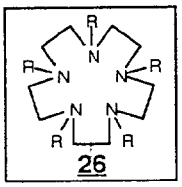




Several studies have explored simultaneous variations in scaffold size and the number of attachment sites for recognition epitopes (23). In one example, a series of dendrimers, with 2, 4, 8 (**25**), 16, 32, 64, and 128 lactose residues, was generated and the activities of these molecules in assays with a panel of proteins was measured. The proteins were chosen because they differ in their number of saccharide binding sites and their orientation of binding sites. The authors compared potency on a saccharide residue basis. In general, increasing the valency of the dendrimer increased protein-dendrimer binding for all four proteins. However, the magnitude of this effect is dependent on the structure of the target protein. This study demonstrates the importance of tailoring the structure of a multivalent ligand to the target protein; the design of ligands should account for the number, orientation, and spacing of protein binding sites.

Linkage to the Scaffold

Another critical design feature is the length of the linker or tether that connects the recognition element to the scaffold. This importance is highlighted in a search for multivalent inhibitors of the bacterial toxin enterotoxin (24). Enterotoxin is a pentameric protein with five saccharide-binding sites, one per monomer. Enterotoxin binds the GM1 ganglioside, which possesses a terminal galactose residue. A pentacyclen core was used to display five galactose residues, and these residues were tethered using linkers that range from 60 to 180 Å in length (**26**). The four ligands were tested for their ability to inhibit enterotoxin binding to its target ganglioside. The activity of each depended on the length of the linker employed. The compound with the longest linker was the most potent; it was 10^4-fold more effective than the monomeric galactose control. Dynamic light scattering experiments indicate that 1:1 protein-ligand complexes are formed, suggesting the efficacy of the ligand may be due to the chelate effect. In a related study, a decameric ligand was found to be a highly active inhibitor of Shiga toxin, which has 15 galactose binding sites (25). A structure of the complex reveals that this ligand dimerizes the toxin and each saccharide residue of the complex occupies a saccharide-binding site. Together, these studies demonstrate the importance of linker length and its impact on the biological activity and mechanism of action of the ligand. Moreover, they are outstanding examples of the importance and potential of multivalent ligand design.



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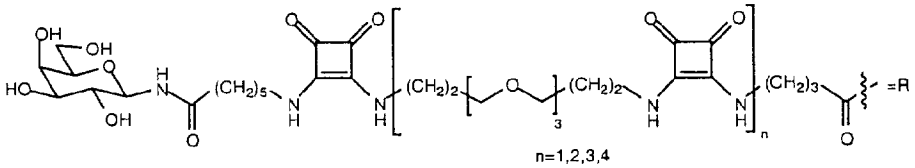
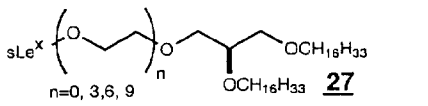


n=1,2,3,4

Schmidt and coworkers explored the importance of linker length in the context of a surface. Using sialyl Lewis x (sLe^x) glycolipid derivatives, they examined the structural requirements for selectin-mediated cell rolling (26). The selectins are a family of carbohydrate binding proteins that facilitate the rolling of leukocytes on the endothelial cell wall, the first step in the inflammatory response. SLe^x is a tetrasaccharide known to bind to the selectins, but is generally displayed on a protein backbone at the end of a complex carbohydrate chain. Determination of the optimal spacing between sLe^x and a multivalent scaffold may lead to ligands with greater biological activity. In this example, an ethylene glycol-based linker was installed between the recognition epitope, sLe^x, and the lipid necessary for incorporation on the surface, **27**. The structure of the linker was designed to mimic the "length" of a carbohydrate. The cell rolling assays suggest that there is a minimum requirement for accessibility of the recognition epitope; the incorporation of six or nine ethylene glycol units resulted in compounds with significantly higher activities than were derivatives with zero and three ethylene glycol units. These results highlight the importance of considering the physiological presentation of a ligand when designing the linker for synthetic macromolecular conjugates.



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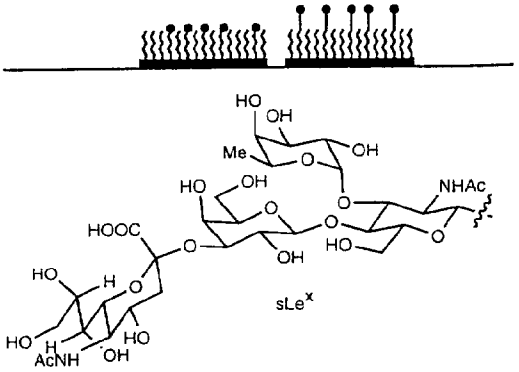
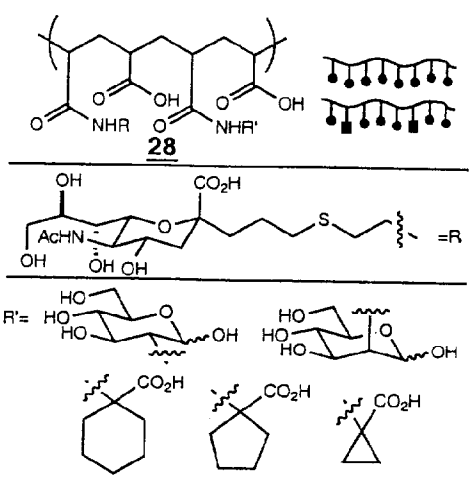


Incorporation of Ancillary Functional Groups



Most examples of multivalent ligand design focus on increasing the specific interactions between a recognition epitope and the target receptor. As with small molecules, however, incorporation of a variety of functional groups may contribute to biological activity. Applying this concept to multivalent displays, Whitesides and coworkers incorporated a range of functional groups into sialic acid-bearing polyacrylamide displays, **28**, and tested the ability of the resulting materials to inhibit viral agglutination (27). The inhibitory concentrations were determined based on the amount of sialic acid appended onto polymer backbone; the concentration of each ancillary functional group incorporated was kept constant. Inclusion of many of these groups resulted in materials with increased inhibitory potencies. Two types of ancillary groups were of particular interest due to their similar structures yet distinct biological activities. Incorporation of D-2-amino-2-deoxymannose resulted in a 40-fold greater activity than D-2-amino-2-deoxyglucose. In addition, the use of 1-amino-1-cyclopropanecarboxylic acid, 1-amino-1-cyclopentanecarboxylic acid, and 1-amino-1-cyclohexanecarboxylic acid, resulted in materials with increased potencies of 5- to 20-fold. These results suggest that ancillary groups can exploit unknown yet specific secondary binding interactions. This strategy could be useful in increasing the affinity of multivalent ligands for their targets.

Conclusion: Design of Multivalent Scaffolds

By assessing the effects of structural variations on multivalent ligand activities, structure-activity relationships (SAR) can be established, in analogy to SAR studies with small molecules. In both cases, the final three-dimensional display of recognition epitopes, determined by the features of the scaffold and linking groups, is critical for proper recognition by the appropriate target. Ligand activity can be modulated by incorporation of functional groups that may participate in specific or non-specific interactions. There are, however, critical differences that distinguish multivalent ligand design. Because multivalent ligands can generally bind multiple binding sites, there are a wide variety of mechanisms multivalent ligands can exploit. In addition, the size of ligands needed to bind discrete, spatially separated targets often is significantly greater than that needed for ligands that bind a single site. The examples described here emphasize the importance of controlling multivalent ligand structure with synthesis. We have focused on design features, because we anticipate that an understanding of the issues associated with these unconventional ligands will facilitate the development of new general approaches for their synthesis Advances in chemical synthesis undoubtedly will continue to facilitate systematic studies of ligand parameters. Such ligands could be used to elucidate and manipulate a diversity of cellular processes.

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4. IgG (PDB accession number 1IGT) and enterotoxin (PDB accession number 1EEF) are structures generated from X-ray crystallographic studies. Images were generated in RasMol 2.7.1. See www.rcsb.org/pdb. The FK1012 structure is based on PDB accession number 1A7X. The dendrimer is an eighth generation PAMAM dendrimer modeled by the Caltech Molecular and Process Simulation Center. The linear polymer is a mannose-substituted 10mer generated by ROMP and modeled by C.W. Cairo (UW-Madison). The enterotoxin protein is shown on the left and was removed from the right hand image to clarify the location of the saccharides. The scale bars indicate 2.5 nm.

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