Synthetic multivalent ligands in the exploration of cell-surface interactions
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Processes such as cell–cell recognition and the initiation of signal transduction often depend on the formation of multiple receptor-ligand complexes at the cell surface. Synthetic multivalent ligands are unique probes of these complex cell-surface-binding events. Multivalent ligands can be used as inhibitors of receptor-ligand interactions or as activators of signal transduction pathways. Emerging from these complementary applications is insight into how cells exploit multivalent interactions to bind with increased avidity and specificity and how cell-surface receptor organization influences signaling and the cellular responses that result.

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Abbreviations
LT-1 heat labile enterotoxin
MHC major histocompatibility complex
NeuAc N-acetyl neuraminic acid
SLT-1 Shiga-like toxin I
TCR T-cell receptor

Introduction
The surface of a cell is a complex and dynamic milieu. Protruding from the lipid bilayer are proteins and saccharides of different shapes and sizes. The arrangement of these diverse molecules also is heterogeneous: they can be distributed evenly across a cell surface or localized within microdomains. This complex ensemble of molecules acts as a reporter for the cell; it can convey changes in the external environment such as the presence of nutrients, toxins, attractants, or foreign invaders. In addition, the cell surface serves as a site for the docking of other cells, the extracellular matrix, or pathogens. All of these functions depend on the binding of specific ligands to cell-surface receptors.

The ability of cell-surface receptors to recognize and respond to ligands provides impetus for the discovery of natural or non-natural ligands that interfere with these binding events. Most research on receptor–ligand interactions focuses on the interaction of molecules with single receptor binding sites. Ligands that display multiple copies of recognition elements are termed multivalent. Multivalent ligands are critical probes of cell-surface recognition, as cells often encounter naturally occurring multivalent arrays [1]. In cell–cell recognition events, for example, multiple copies of a ligand on one cell can be recognized by receptors on the opposing cell. Multivalent arrays can also elicit a cellular response. The chemotactant chemokines, for example, interact with glycosaminoglycans to form multivalent arrays that recruit leukocytes through their interaction with receptors on the cell surface. The biologically important activities of these naturally occurring multivalent displays provide impetus for the chemist. Chemical synthesis can provide access to ligands that mimic endogenous multivalent arrays. Alternatively, it can yield new types of multivalent ligands that alter cellular function by mechanisms inaccessible to natural substances. In this review, we focus on recent examples in which synthetic multivalent ligands have been used to illuminate and manipulate cellular responses.

The advantages of using synthetic ligands to probe multivalent cell-surface binding events are numerous. For example, they can be used to dissect the contributions of various ligand features to a binding interaction that is naturally multivalent. Physiological multivalent ligands are often too scarce, structurally heterogeneous, or complex (such as a cell or viral surface) to identify the relevant underlying molecular mechanisms. Many features of these ligands, such as number (valency) and orientation of receptor binding sites and the flexibility, size, and shape of the scaffold from which these binding sites are displayed may contribute to binding [1,2*,3,4]. Synthetic multivalent ligands can be generated such that the scaffold structure, identity of binding elements, number of binding elements, and the spacing of binding elements can be varied systematically. Arrays that mimic natural multivalent displays and those that are unrelated have been generated; both can have intriguing biological activities.

Synthetic multivalent ligands as inhibitors and effectors
Synthetic multivalent ligands have been used to explore two primarily distinct cell-surface interactions: those in which the multivalent ligand prevents receptor–ligand binding and those in which binding of the multivalent ligand induces a cellular response. Ligands that have the former activity are inhibitors, and those with the latter are effectors. Multivalent inhibitors can interfere with a wide range of interactions including those involving cell–cell, cell–extracellular-matrix, cell–virus, and cell–toxin binding. Multivalent effectors can be applied to understand, dissect, and manipulate signal transduction pathways, especially those initiated by multiple receptor–ligand contacts at the cell surface. A range of responses can be elicited by multivalent ligand binding, in principle, and
these include cellular activation, migration, and differentiation. To provide a context for interpreting how multivalent ligands function, a brief overview of the mechanisms by which these ligands interact with their target receptors is provided (Figure 1).

**Mechanisms of multivalent receptor–ligand interactions**
Multivalent ligands often possess increased functional affinity (the apparent affinity for the interaction) for their targets compared with that of monovalent ligands [1,5,6]. Several different mechanisms can contribute to their potency. For example, multivalent ligands can bind oligomeric receptors on the cell surface. In this case, the translational entropy cost is paid with the first receptor–multivalent-ligand contact; subsequent binding interactions proceed without additional translational entropy penalties, although there is a conformational entropy cost (Figure 1a). Even with receptors that are not oligomeric, multivalent ligands can bind avidly to multiple receptors, a process that is facilitated by the two-dimensional diffusion of receptors in the fluid bilayer (Figure 1b). Some proteins possess binding subsites in addition to the primary binding site, and these can be occupied by a multivalent ligand (Figure 1c). In addition, because multivalent ligands can display a higher local concentration of binding elements, they can have higher functional affinities even when only one receptor is engaged (Figure 1d). Another effect of multivalent-ligand binding that is relevant for inhibition of cell-surface interactions is steric stabilization. In steric stabilization, the size and hydration shell of the multivalent ligand precludes the engagement of a cell surface with an opposing viral particle or cell [7]. Together, these different modes of recognition impart unique activities to multivalent ligands.

**Multivalent inhibitors of cell-surface-receptor–ligand interactions**
The high functional affinities of multivalent ligands have prompted efforts to develop potent inhibitors of cell-surface binding events. Examples of success using this approach range from the generation of inhibitors of virus binding to host cells, to compounds devised to block bacterial cell binding, to materials that inhibit the binding of leukocytes to endothelial cells. Advances in chemical synthesis are fueling these developments by providing access to a variety of platforms for binding-element presentation.

Some of the first applications of multivalent inhibitors were focused on preventing the binding of influenza virus hemagglutinin to host cells [8–12], and this area of research remains active [13,14*]. The system is an excellent testing ground because the interaction of the virus with the cell surface naturally relies on multivalent binding. The virus contacts host cells through the interaction of the trimeric influenza virus hemagglutinin with N-acetyl neuraminic acid (NeuAc; a type of sialic acid) residues on the host-cell surface. Although viral strains arise each year with different antigenic epitopes, all invade their hosts through NeuAc binding. High concentrations of monovalent sialic acid derivatives are required to inhibit binding (ca 10⁻³ M) [15]; consequently, multivalent inhibitors with high functional affinities have been sought. A variety of different types of multivalent ligand have been identified as effective inhibitors of influenza virus hemagglutinin, including NeuAc-substituted acrylamide polymers [8–10], liposomes [16,17], and divalent ligands [11,12]. High molecular weight polyacrylamide polymers were found to be extremely effective inhibitors of the virus [18], yet other scaffolds continue to be investigated because of the toxicity of polyacrylamide-based materials.

The effects of a ligand’s architecture on its inhibitory activity and specificity were explored recently with a series of synthetic inhibitors of hemagglutination. Reuter et al. [14*] generated a number of structurally distinct ligands bearing NeuAc residues, and these were tested for their ability to...
Model systems prevent hemagglutinin binding and viral infection *in vitro* (Figure 2a; [14•]). The investigations revealed that the inhibitory potency of the multivalent ligand depends on its three-dimensional structure, and the different ligands are effective against various viral strains. In one striking example, branched polymers displaying NeuAc-substituted dendrimers were 50,000-fold more potent than monovalent NeuAc against sendai virus, but their increase in activity for influenza virus strain H2N2 was only twofold. This discrepancy may be due to differences in the three-dimensional structure of the viral hemagglutinin or to the distribution of viral hemagglutinin on the surface of the virus. Both of these variables may play important physiological roles in determining host–pathogen specificity *in vivo*.

The power of multivalent ligand design is illustrated by two studies describing the development of potent inhibitors of bacterial toxin binding to host cells [19••, 20••]. Heat labile enterotoxin (LT-1) from *Escherichia coli* and the Shiga-like toxin I (SLT-1) from *Shigella dysenteriae* type 1 are members of the class of bacterial AB₅ toxins, and many fatalities result from the diseases caused by this class of proteins. The toxins gain access to cells by multivalent binding to the saccharide portion of gangliosides on mammalian cell surfaces. The structures of LT-1 [21] and SLT-1 [22] bound to monovalent carbohydrate derivatives have been determined by X-ray crystallography. In the case of LT-1, five monovalent trisaccharides were found to bind to a single face through their interactions with the five B subunits. The structure of SLT-1 is similar, although it differs in that 15 saccharide residues interact, such that each B subunit binds three saccharides. The structural information regarding the location of the saccharide-binding sites was used to design multivalent inhibitors of the toxins.

A modular strategy for the assembly of pentavalent ligands that target LT-1 was devised by Fan *et al.* ([20••]; Figure 2c). A pentacyclen core was used to present galactose residues such that the connecting linker could readily be varied. By evaluating compounds possessing linkers that varied in length (variations ranging from 32 to 83 atoms), the authors identified an inhibitor of LT-1 that is 10⁵-fold more active than galactose. Dynamic light scattering experiments suggest that this inhibitor forms a 1:1 complex with the toxin. These results, along with the high potency of the ligand, suggest that the inhibitor acts by the chelate effect (see Figure 1a). This is an important example of a compound that appears to use chelation to block a
Communication. Synthetic divalent ligands that promote immune system function and neuronal cell processes, some of which include growth factor signaling clustering (Figure 1d) is important in many biological systems through this mode of binding. Ligand-promoted receptor signaling pathway [30,31]. Physiological multivalent ligands change in receptor proximity can initiate a particular signal transduction. Thus, the strategy for design of SLT-1 inhibitors focused initially on generating divalent derivatives of the Pn trisaccharide that would target two adjacent trisaccharide-binding sites of the toxin (Figure 2b). The divalent ligand target was 40-fold more active than the trisaccharide, but the authors reasoned this gain would not be sufficient to inhibit the binding of the toxin to cells. Consequently, they appended their lead to a glucose scaffold through a linker (31 atoms) to display five copies of the divalent binding element. The resulting compound is a powerful inhibitor. It is 10-fold more active than is the Pn trisaccharide at inhibiting SLT-1, and it was able to protect cells in culture from the toxin. To better understand the source of their ligand’s potent activity, the authors conducted X-ray crystallographic studies on the complex of the multivalent ligand and the toxin. Their data indicate that the ligand acts by a binding mode not anticipated in the ligand design. The solid-state structure reveals a complex containing two copies of the toxin to one of the ligand. All five subunits on each toxin are occupied with a single trisaccharide, and the two trisaccharide units within the divalent binding element act to dimerize the toxin. Thus, the multivalent ligand appears to act by the chelate effect but also by receptor clustering. This important study highlights the benefits of multivalent ligand design, and demonstrates the potential of incorporating different binding modes into ligand design.

The number of examples in which multivalent ligands have been found to be potent inhibitors is growing. The identification of efficacious inhibitors of bacterial toxins suggests blueprints for inhibitor design that perhaps can be generalized to create inhibitors of other toxins in this family. These advances and other recent results [23–29] underscore the interplay between chemistry and biology in the investigation and prevention of cell-surface interactions.

**Effectors: multivalent ligands that activate biological systems**

The ligand-binding modes that result in the initiation of a biological signal can be similar to those that are involved in inhibiting binding interactions. Specifically, when a ligand induces the dimerization of a cell surface receptor, the change in receptor proximity can initiate a particular signaling pathway [30,31]. Physiological multivalent ligands for cell-surface receptors often cause cellular responses through this mode of binding. Ligand-promoted receptor clustering (Figure 1d) is important in many biological processes, some of which include growth factor signaling [31], immune system function [32,33], and neuronal cell communication. Synthetic divalent ligands that promote receptor clustering have been used to initiate cellular responses [34] and to explore the role of receptor proximity in signal transduction and the cellular responses that result. Recent chemical strategies have opened the possibilities of exploring higher valency ligands as effectors of biological function.

The most common synthetic multivalent effectors are those that provoke an immune response; partially or fully synthetic vaccines are examples. These materials display multiple copies of an antigen on a synthetic scaffold or carrier protein. In a poorly understood process, these arrays can induce potent immune responses. The generation of anti-cancer vaccines is one area in which chemical synthesis is playing a major role [35–38,39••]. The development of anti-cancer vaccines is challenging because tumor cells can differ from normal cells in their cell-surface composition, but normal immune responses can be inhibited or circumvented by the tumor. Thus, attempts to develop anticancer vaccines have focused on taking the tumor antigens out of the cellular context and presenting them as multivalent arrays on different scaffolds. Although peptide antigens are being investigated for generating anti-cancer vaccines [38,40], it is the attempts to generate immune responses to carbohydrate antigens that have prompted many recent synthetic efforts [35–37,39••,41].

The utility of the synthetic oligosaccharide and glycopeptide epitopes that are commonly found on tumor-cell surfaces to serve as anticancer vaccines is an active area of exploration (Figure 3). In recent investigations by Danishefsky, Lloyd and co-workers [42,43•], multivalent conjugates composed of synthetic oligosaccharides linked to a standard carrier protein were successful at eliciting both humoral and cellular responses (IgM and IgG antibodies). These synthetic vaccines are now in clinical trials, and the synthetic approach taken to generate these immunogens provides opportunities for optimizing their activities. For example, the clustering of antigens can influence the effectiveness of a vaccine [44•]. These issues also are under investigation in the development of vaccines based on dendrimeric scaffolds [45], which display multiple copies of T-cell and antigenic epitopes [46,47]. Together with studies that use synthetic multivalent ligands to explore the clustering of specific receptors in the immune system [48•,49], these investigations are illuminating the molecular requirements involved in immune-cell–antigen recognition.

Multivalent ligands can be used to ameliorate as well as generate an immune response [50•,51]. The production of a specific antibody can be diminished in the presence of a ligand that presents multiple copies of an antigen to the relevant B cells. The induction of B-cell tolerance to the specific antigen depends on the inability of the scaffold to activate T cells. A recent study explored the ability of tetravalent antigen displays to suppress the production of antibodies to a cyclic peptide antigen [50•]. A simple scaffold that displays four copies of the peptide antigen suppressed...
Chemical structures of multivalent ligands that elicit biological responses. (a) A glycoconjugate based on the carbohydrate tumor antigen Lewis Y. This synthetic vaccine elicits antibodies that bind Lewis-Y-displaying cells. (b) Structure of a tetravalent peptide derivative that results in a decrease in B cell production of anti-cyclic peptide antibodies. (c) Structure of a divalent conjugate of a peptide-bound MHC used to explore the importance of TCR clustering in T cell activation. The MHC complex was appended to the peptide backbone through conjugate addition of an MHC cysteine side chain to maleimide groups. (d) Multivalent galactose derivatives that act as chemoattractants for E. coli. (e) Multivalent 3',6-disulfo Lewis X derivatives that inhibit L-selectin and promote L-selectin downregulation from the surface of human neutrophils.
the production of 93% of the anti-cyclic peptide antibodies in mice (Figure 3b). Some of the conjugates tested were significantly less effective, and these results indicate that both the structure of the antigen and that of the scaffold can influence the tolerogenic activity of the display. A detailed understanding of how specific structural features influence activity awaits further elucidation of the molecular interactions that lead to a decrease in antibody production. This study provides an illustration of how a multivalent display can be used to down-regulate an immune response.

Defined, synthetic multivalent displays have been used to explore the importance of T-cell receptor (TCR) clustering in T cell activation ([48*]; see also Update). T cells can become activated when they interact with cells displaying peptide-occupied major histocompatibility complexes (MHCs). TCR clustering has been implicated in the activation process, but different conclusions have been drawn about how many TCRs must be clustered for activation.

To explore the role of TCR clustering in activation, Cochran et al. [48*] synthesized divalent, trivalent and tetravalent arrays of peptide-bound MHCs as TCR ligands (Figure 3c). When these were assayed for their ability to stimulate T cells, the monovalent ligand failed to induce activation. Ligand-induced dimerization of the TCR was found to be sufficient for triggering, and the extent of activation did not appear to depend on ligand valency.

The clustering of cell-surface receptors is probably an important mechanism for controlling immune responses, as the above examples suggest. Moreover, ligand-induced receptor clustering can activate diverse signaling pathways that control critical cellular responses. For example, receptor clustering has been implicated in controlling the chemotaxis of bacteria toward attractants [52,53]. A recent study addresses whether cell-surface receptor responses can be not just initiated but also modulated by changes in receptor proximity.

Many studies of protein function focus on elucidating the function of a single protein, yet the amount of information available from genomics and proteomics is now leading to a shift in perspective: there is a focus on understanding how systems of interacting proteins control cellular responses. Despite this interest in systems, the design of ligands has focused traditionally on inhibiting or augmenting the function of a single protein. Multivalent ligands with their ability to organize, activate, or inhibit multiple receptors, are uniquely suited to address emerging questions in systems biology.

**Conclusions**

Multivalent ligands possess elements of structural complexity not present in their traditional monovalent counterparts. The identity of the binding elements, structure of the scaffold, number of binding groups, and density of binding elements are all parameters that influence the mechanisms by which a ligand acts. Given these diverse variables, it is clear that synthetic multivalent ligands have unique potential as inhibitors and effectors. Thus, chemists and biologists are now poised to ask new fundamental questions. What are the binding modes that give rise to the best inhibitors? How does ligand architecture influence ligand function? How does the extent of clustering of a receptor influence its function? Does the pattern of engaged receptors on a cell influence the output response? Synthetic multivalent ligands of high complexity can now be generated to address such questions.

**Update**

The use of synthetic scaffolds to display peptide-loaded MHCs has been explored further by Cochran and Stern [63]. The authors use different scaffolds to generate a variety of...
multivalent displays of MHC molecules loaded with HLA-DR1 peptide fragments. The resulting conjugates, which were isolated as monomer, dimers, trimers, and tetramers, were characterized by their sizes. These conjugates capable of dimerizing cell-surface T-cell receptors (having valencies of two or greater) were able to activate HAIL T cells.

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Papers of particular interest, published within the annual period of review, have been highlighted as:
• of special interest
**of outstanding interest
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Synthetic multivalent ligands in the exploration of cell-surface interactions

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A synthetic strategy for an affinity matrix displaying carbohydrates used in cancer vaccines was developed. This new matrix was then used to analyze the range and specificities of antibodies produced from vaccines bearing the relevant carbohydrates. This provides a new method for characterizing the immune response to cancer vaccine therapy.


A series of complex saccharides were synthesized and appended to a peptide display in varying densities. Immunochemical studies demonstrated that the extent of clustering had a direct impact on the ability of the antigen to stimulate antibody production.


This paper describes a useful approach to exploring the clustering of the TCR using defined multivalent ligands. Using a variety of analytical methods, the authors conclude that the dimerization of the TCR causes receptor activation and that the extent of activation does not depend on the size of the TCR cluster.


This paper provides a clear illustration of the value of multivalent ligands for inhibiting antibody production.


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