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Control of Multivalent Interactions by Binding Epitope Density

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Abstract: Receptor clustering by multivalent ligands can activate signaling pathways. In principle, multivalent ligand features can control clustering and the downstream signals that result, but the influence of ligand structure on these processes is incompletely understood. Using a series of synthetic polymers that vary systematically, we studied the influence of multivalent ligand binding epitope density on the clustering of a model receptor, concanavalin A (Con A). We analyze three aspects of receptor clustering: the stoichiometry of the complex, rate of cluster formation, and receptor proximity. Our experiments reveal that the density of binding sites on a multivalent ligand strongly influences each of these parameters. In general, high binding epitope density results in greater numbers of receptors bound per polymer, faster rates of clustering, and reduced inter-receptor distances. Ligands with low binding epitope density, however, are the most efficient on a binding epitope basis. Our results provide insight into the design of ligands for controlling receptor−receptor interactions and can be used to illuminate mechanisms by which natural multivalent displays function.

Introduction

Multivalent ligands can cluster soluble and cell-surface receptors. Receptor clustering governs many biological processes, including immune responses and growth factor signaling.[1−3] Factors known to be important for receptor clustering include the stoichiometry of binding,[4] the rate of cluster formation,[5] and the proximity of receptors.[6] Understanding the molecular features of a multivalent ligand that influence these parameters would provide insight into how natural multivalent interactions are regulated. Additionally, the properties of synthetic ligands can be optimized so that they serve as regulators for systems of interest.

Many proteins that participate in multivalent interactions are oligomeric. One of the most studied members of this class is the tetrameric lectin, concanavalin A (Con A).[7] At neutral pH, the lectin exists as a homotetramer that presents four distinct binding sites for mannopyranosides ($K_d \sim 0.1$ mM[8]). Con A is a well-known activator of cellular signaling.[7] The oligomeric state of Con A can influence its signaling activity, and the formation of macromolecular Con A-ligand assemblies on the cell surface appears to be important for signal transduction.[9] Con A-induced signaling has been studied, but its physiological relevance is unknown. Con A is structurally similar to many animal and bacterial lectins implicated in signaling events.[10−12] One class of these, the galectins, are galactose-binding lectins that can regulate cell adhesion, cell proliferation, and cell survival.[13−15] As with Con A, the abilities of the galectins to cross-link cell surface receptors have been implicated in

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initiation of signal transduction.[15] Thus, both Con A and galectin-mediated signaling appear to depend on the formation of macromolecular assemblies on the cell surface.

Multivalent ligands that bind and cluster lectins can serve as scaffolds for the assembly of macromolecular displays. For example, multivalent arrays of mannose have been shown to promote the formation of higher order assemblies containing multiple copies of Con A. In the case of Con A, these macromolecular complexes can possess many unoccupied sugar binding sites poised for interaction. For example, we have found that scaffolded displays of Con A bind more avidly to surfaces presenting mannose residues.[16] Such macromolecular complexes are more effective than Con A itself at promoting cell−cell associations.[17] Thus, multivalent ligands that cluster lectins without saturating their carbohydrate-binding sites can be used to cross-link cell surface glycoproteins and thereby intiate signaling.[17−19] Multivalent interactions of saccharide ligands with Con A have been studied using a wide range of methods, including electron microscopy,[20] calorimetry,[21] and X-ray crystallography.[22] Because of the signaling properties of Con A and the abundance of information on its complexes, it is an excellent model for examining the features that impact receptor clustering.

The binding epitope density[23] of a multivalent ligand has been shown to influence its activity.[24] Often these increased activities have been attributed to increases in functional affinity.[1,2,25] We hypothesized that binding epitope density also could affect kinetic and structural parameters important in cluster formation. To test this hypothesis, we investigated a series of multivalent ligands varying in binding epitope density. Polymeric carbohydrate displays that vary in the density of binding epitopes presented are known.[26] The compounds employed in prior studies were synthesized by increasing the ratio of unfunctionalized to functionalized monomer to decrease binding site density. The unfunctionalized monomer units were typically smaller and of different hydrophobicity than the functionalized units; consequently, the resulting polymers did not present a uniform steric and electronic environment. To allow selective analysis of the impact of changes in binding epitope density on receptor clustering, we devised a method to synthesize multivalent ligands with monomer units that varied only in their binding activity. Moreover, we employed a battery of assays to characterize different parameters relevant to receptor clustering.

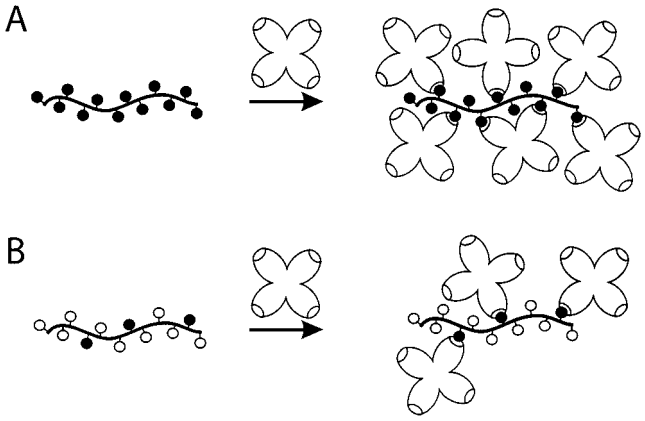

Figure 1. Schematic representation of Con A clustering by multivalent ligands. (A) High-density polymers can recruit many receptors to a single molecule; however, steric effects prevent binding of every residue. (B) Low-density polymers bind fewer total receptors per molecule. Increased spacing between residues allows for more efficient binding.

We used the ring-opening metathesis polymerization (ROMP) to synthesize polymers that vary only in the density of mannose residues (Table 1).[27] Con A binds mannose but does not recognize the sterically similar moiety galactose;[28] consequently, we altered the ratio of mannose- and galactose-substituted monomers in copolymerization reactions. Using this approach and a functional group-tolerant ruthenium initiator,[29] we could readily produce materials with different mannose epitope densities (Figure 2).[30] Differences in the mannose to galactose monomer ratios employed in the polymerization reactions were preserved in the final materials. Thus, ligands could be produced with similar length, polarity, and steric properties that differ only in their proportion of binding epitopes. With these compounds, we examined the influence of epitope density on the formation of Con A clusters. Our investigations reveal that the density of binding epitopes presented by a multivalent ligand influences critical features of receptor clustering.

Results

Stoichiometry of Con A within the Cluster. The number of Con A tetramers bound to ROMP-derived ligands has been found to depend on the degree of polymerization (length) of the display.[20] Steric effects preclude the binding of Con A tetramers to every mannose residue on a fully elaborated polymer generated by ROMP.[32] To determine the effect of binding epitope density on the stoichiometry of the Con

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Table 1. Composition of the Multivalent Displays

compound	length[a]	%mannose[b]	stoichiometry[c]	mannose/Con A[d]
1	143	100	16	9
2	145	71	15	7
3	115	45	9	6
4	86	31	7	4
5	102	18	6	3
6	116	10	3	3
7	129	2	1	2

[a] The degree of polymerization (DP) was determined by NMR integration of polymer end groups versus internal olefin resonances, to give the length (x). [b] Ratio between incorporated mannose and galactose ($m/(n + m) = \%$ Man). [c] Stoichiometry of the complex is calculated by using QP experiments.[31] The ratio is reported as the number of Con A tetramers complexed per polymer backbone. [d] The ratio of mannose residues to Con A tetramers is calculated as m/stoichiometry.

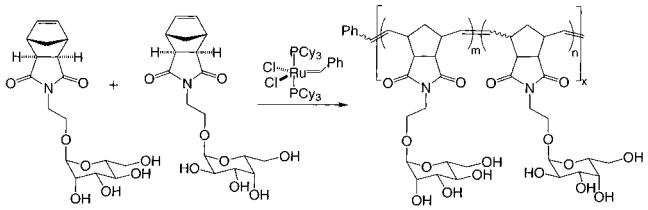

Figure 2. Structures of copolymers. The ratio of monomers used in the copolymerization determines the composition of the polymer (see Table 1). All polymers are of similar length (x), and vary only in proportion of mannose residues (m) to galactose residues (n).

A−ligand complex, compounds **1**−**7** were used in quantitative precipitation (QP) assays. These experiments assess the concentration of ligand required to completely precipitate the lectin from solution. Therefore, the results provide a determination of the stoichiometric composition of the precipitate.[31]

The initial aggregates formed in QP experiments likely consist of isolated complexes; however, cross-linked lattices may form in later stages.[22] The data from these experiments reveal that the number of tetramers bound per polymer increases with increasing epitope density (Table 1). The observed stoichiometry agrees well with our previous experiments using transmission electron microscopy, in which we examined the stoichiometry of isolated multivalent ligand-promoted clusters of Con A.[32] Because quantitative precipitation experiments measure the stoichiometry of a kinetically trapped species, the agreement with our results from transmission electron microscopy is notable. The fully elaborated polymer binds the greatest number of tetramers, but reducing the mannose epitope density to 70% does not significantly alter the stoichiometry of the precipitate. Polymers in which 2−20% of the residues are mannose bind the greatest number of receptors per residue. Thus, on a mannose residue basis, the most efficient compounds are those with the lowest density of binding epitopes. In contrast, the polymers that display the greatest number of binding sites generate the largest clusters.

Rate of Cluster Formation. QP provides information about an endpoint of receptor clustering; yet, a critical parameter for biological systems is the rate of complex assembly. Cell-surface receptor clustering processes occur on time scales that range from seconds to hours.[5] To measure the effects of epitope density on the kinetics of receptor clustering, we employed a

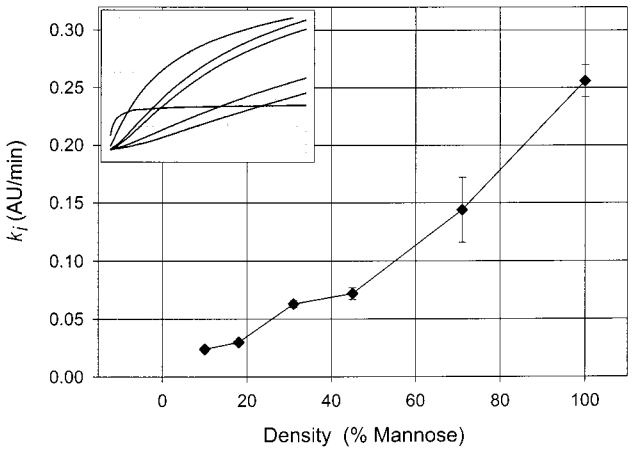

Figure 3. Initial rates of Con A precipitation using neoglycopolymers. Each point is the average of three replicates, and the error shown is the standard deviation. Some error bars are smaller than the symbols. Inset: Complexation is measured by monitoring A_{420} for 10 min. The initial rate is determined by a linear fit to the steepest part of the curve.

turbidimetric assay to monitor the rate of formation of lectin clusters in solution.[33,34]

In the turbidity assay, Con A is mixed with a 10-fold excess of multivalent ligand, a process that induces rapid precipitation (see Figure 3, inset). The initial rate of change in turbidity is related directly to the rate of receptor−receptor association mediated by the polymer backbone.[35,36] By determining the initial slope of the curve a measurement of the rate of Con A clustering is obtained. These initial values should relate to the formation of isolated complexes; however, at later time points secondary interactions between complexes obscure analysis. For example, the highest density ligand **1** rapidly aggregates, and then reaches a plateau value (see Figure 3, inset). Alternatively, the absorbance for assemblies formed in the presence of ligands **2**−**6** continues to increase over time. This result is consistent with the formation of higher order, cross-linked complexes in the presence of ligands **2**−**6**, the cross-linked complexes are expected to be more prevalent when precipitation is slower. We, therefore, have restricted our analysis to the initial rates of complexation.

The rate of Con A complexation depends on the epitope density of the multivalent ligand. The highest density polymer induces clustering at a rate approximately 10-fold faster than that of the lowest density polymers. Subtle decreases in binding epitope density (e.g. from 100% to 70%) can decrease the rate of clustering by 2-fold. In contrast, this change in epitope density produced little or no change in the stoichiometry of the clusters. Thus, the binding epitope density of a multivalent ligand has a considerable effect on the rate at which it mediates receptor clustering.

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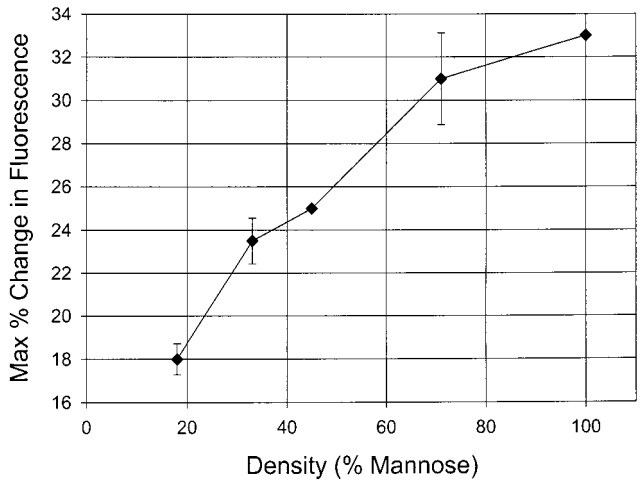

Figure 4. FRET induced by neoglycopolymer-promoted clustering of Con A. Fluorescein- and TMR-labeled Con A derivatives were mixed to afford a final concentration of 80 nM. Individual titrations consist of 3 repetitions in which 10 concentrations of polymer were tested over the concentration range of 0.001−100 μM mannose residues. Error bars represent the standard deviation, and some are smaller than the symbols.

Receptor Proximity. Multivalent ligands can alter the proximity of receptors on the cell surface.[19] Changes in receptor proximity can dramatically influence biological processes. To explore the influence of binding epitope density on multivalent ligand-induced receptor clustering, we exploited fluorescence resonance energy transfer (FRET) as a method to assess changes in receptor proximity.[37,38] This experiment relies on the distance-dependent transfer of energy between two differentially labeled receptors. Here, fluorescein- and tetramethylrhodamine (TMR)-labeled Con A were used as donor and acceptor, respectively.[16]

Decreases in the fluorescence emission of the fluorescein donor are an indication of the proximity of Con A tetramers in the assembly. Compounds **1**−**5** were added to a solution containing differentially labeled Con A, and the fluorescein emission was monitored (Figure 4). As the binding epitope density of the ligand increased, the maximum percent change in fluorescence emission it elicited also increased. FRET efficiency varies as the sixth power of the separation distance.[38] Thus, this change is anticipated if the average distance between Con A tetramers in the polymer−lectin complex decreases. Alternatively, the change may also reflect an increase in the population of Con A tetramers in close proximity. Although these data do not distinguish between the two scenarios, both represent relevant changes in receptor clustering. Applying the average distance analysis, the changes in FRET for compounds **1**−**5** indicate that altering the binding epitope density from 20% to 100% results in a decrease of the average inter-Con A distances of approximately 15%. This change is less pronounced for small reductions in binding epitope density; comparison of the highest mannose density ligands **1** and **2** (100% and 70% mannose residue incorporation) shows that little to no change in FRET is observed. This result is consistent with studies of the stoichiometry determined from quantitative precipitation experiments, in which the stoichiometry of complexes formed from compounds **1** and **2** was similar.

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Discussion

Multivalent ligands that influence receptor clustering can be used to illuminate and manipulate diverse physiological processes.[18,19] We hypothesized that the binding epitope density of a multivalent display influences parameters beyond its functional affinity. These include the stoichiometry of complexation, rate of cluster formation, and receptor proximity. Little is known about the influence of multivalent ligand structure on these parameters. Using a series of multivalent ligands that differ only in their binding epitope density, we explored mechanistic parameters relevant for receptor clustering events.

Applying assays that report on the assembly of Con A complexes, we investigated the effects of changing the binding epitope density of multivalent ligands. The stoichiometric composition of the multivalent ligand−receptor complexes was determined by QP assays. These results indicate that lower density ligands bind fewer receptors. Surprisingly, they also reveal that these ligands provide the most efficient interactions per recognition element. As anticipated, the ligands that display the most binding epitopes clustered the most receptors per polymer. Interestingly, increasing the binding epitope density beyond 70% mannose residues did not increase the number of receptors bound per multivalent ligand. FRET experiments showed that changes in binding epitope density afforded similar trends with regard to the proximity of receptors within the cluster. Increases of binding epitope density from 20 to 70% resulted in decreases in average inter-receptor distances. Again, increasing binding epitope density from 70% to 100% produced no significant changes in FRET.

Turbidimetric experiments allow quantitation of the relative rates of clustering induced by the polymers. We observed that the rate of clustering is directly related to binding epitope density; the highest density ligands showed the fastest rates of assembly. In contrast to both QP and FRET experiments, the rates of clustering for ligands with binding epitope densities of 70% and 100% were different. The highest density ligand **1** exhibited a 2-fold increase in rate of clustering of Con A over ligand **2**. This result suggests that the rate of clustering is more sensitive to small changes in the multivalent ligand than the other parameters measured.

Conclusions

The results presented here demonstrate that binding epitope density is an important parameter that influences ligand-induced clustering. In addition, there is an interplay between epitope density and cluster formation. Traditionally, research on multivalent interactions has focused on optimizing activity on a binding epitope basis. Our results reveal other parameters may be critically influenced by changes in ligand structure. We found that epitope density can be decreased to complex a greater number of receptors per binding element. In contrast, increasing the mannose density of a multivalent ligand increased the number of receptors bound per polymer, increased initial rates of complexation, and decreased the inter-Con A distances in the complex. Thus, when receptor proximity is essential, density should be increased to reduce inter-receptor distances. For interactions requiring fast kinetics, the highest possible binding epitope density may be optimal. Our studies indicate that ligand microstructure plays a key role in controlling the outcome of

multivalent binding events. Thus, it is a critical design parameter in creating multivalent ligands with tailored biological activities.[39,40]

Multivalent interactions govern many important biological responses, yet there is little known about the influence of multivalent ligand structure on these processes. It has been suggested that natural polyvalent ligands (e.g. mucins, heparan sulfate) may use variations in binding site density to modulate biological interactions and the responses that result.[41] Our results suggest mechanisms by which binding epitope density could influence the regulation of natural multivalent interactions to yield the range of kinetic parameters and structural features that these processes require.

Experimental Materials and Methods

General Methods. Multivalent ligands were synthesized by ROMP using procedures similar to those described previously.[27,30] Con A and tetramethylrhodamine-labeled Con A (6.3 mol/mol average loading density) were obtained from Vector Laboratories, Inc. Burlingame, CA. Fluorescein-labeled Con A (7.5 mol/mol average loading density) was purchased from Sigma, St. Louis, MO. All other reagents were obtained from Sigma unless otherwise noted. All experiments were conducted with HEPES-buffered saline (HBS) unless otherwise noted (HEPES 10 mM, NaCl 150 mM, and $CaCl_2$ 1 mM adjusted to pH 7.4 and filtered with 0.2 μm nylon filters). Concentrations of polymer stock solutions were estimated by gravimetric analysis and confirmed by the hexose assay.[42,43]

Quantitative Precipitation. Quantitative precipitations and analysis were carried out by a method modified from that previously described by Khan et al.[31] Briefly, Con A and ligand were prepared in precipitation buffer (0.1 M Tris-HCl pH 7.5, 90 μM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$), vortexed briefly to mix, and then incubated for 5 h at 22 °C. The final concentration of Con A was 30 μM (assuming Con A tetramers with a molecular mass of 104 000 Da). White precipitates were pelleted by centrifugation at 5000 × *g* for 2 min. Supernatants were removed by pipet and pellets were gently washed twice with cold buffer. Pellets were then resuspended in 600 μL of 100 mM methyl α-D-mannopyranoside, and were completely dissolved after a 10 min incubation at room temperature. Protein content was determined by measuring the absorbance at 280 nm by UV−vis spectroscopy on a Varian Cary 50 Bio using a 100 μL volume quartz cuvette with a 1 cm path length. Measurements are the average of three independent experiments with 2 scans performed in each experiment.

Turbidimetric Assay. Con A was diluted fresh for all experiments. The lectin was dissolved at ~1 mg/mL in HBS buffer, and the resulting solution was mixed and sterile filtered (2 μm). The concentration of the Con A stock solution was determined by using UV absorbance at 280 nm (A_{280} = 1.37 × [mg/mL Con A]). The solution was then diluted to 1 μM (based on Con A tetramer at 104 000 Da). Turbidity measurements were performed by adding 100 μL of the diluted Con A solution to a dry quartz micro-cuvette (100 μL volume, 1 cm path length). A solution of the ligand of interest in HBS buffer was then added (10 μL at 500 μM, final concentration was 50 μM *per mannose residue*). Upon addition, the solution was mixed vigorously for 5 s using a micropipet and then placed in the spectrometer. Absorbance data were recorded at 420 nm for 10 min at 1 Hz. Each sample was run three times, the steepest portion of the initial aggregation was then fit to determine the rate of aggregation. Error was determined as the standard deviation of the three rates. Curves shown are the average of all three runs.

Fluorescence Resonance Energy Transfer. Stock solutions of rhodamine-Con A and fluorescein-Con A were resuspended in HBS to 400 μg/mL. Ligand was suspended in distilled H_2O to 10× of the final concentration by serial dilution from a 20 mM stock. Ligand and Con A solutions were mixed in 96-well plates (Costar black with clear bottom cluster plate, Corning Inc., Corning NY). The final concentrations of rhodamine-Con A and fluorescein-Con A were each 4 μg/mL. The final concentration of ligand is varied over the concentration range of 0.001−100 μM mannose residues. The final volume was brought to 100 μL with HBS. Solutions were mixed by gentle tapping and then incubated at 22 °C for 30 min in the dark. Fluorescein emission was measured on a BioLumin plate reader using 5 nm slit widths, a PMT voltage of 850 V, excitation wavelength of 485 nm, and an emission wavelength of 520 nm. Results are the average of two experiments performed in duplicate. Error represents the standard deviation.

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Supporting Information Available: Characterization of compounds **1**−**7** and results for QP, turbidity, and FRET measurements (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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