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Solid-Phase Synthesis for the Identification of High-Affinity Bivalent Lectin Ligands

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The development of carbohydrate-based therapeutics has been frustrated by the low affinities that characterize protein-carbohydrate complexation. Because of the oligomeric nature of most lectins, the use of multivalency may offer a successful strategy for the creation of high-affinity ligands. The solid-phase evaluation of libraries of peptide-linked multivalent ligands facilitates rapid examination of a large fraction of linker structure space. If such solid-phase assays are to replicate solution binding behavior, the potential for intermolecular bivalent binding on bead surfaces must be eliminated. Here we report the solid-phase synthesis and analysis of peptide-linked, spatially segregated mono- and bivalent ligands for the legume lectin concanavalin A. Bead shaving protocols were used for the creation of beads displaying spatially segregated binding sequences on the surface of Tentagel resins. The same ligands were also synthesized on PEGA resin to determine the effect of ligand presentation on solid-phase binding. While we set out to determine the lower limit of assay sensitivity, the unexpected observation that intermolecular bivalent ligand binding is enhanced for bivalent ligands relative to monovalent ligands allowed direct observation of the level of surface blocking required to prevent intermolecular bivalent ligand binding. For a protein with binding sites separated by 65 Å, approximately 99.9% of Tentagel[1] surface sites and 99.99% of the total sites on a PEGA bead must be blocked to prevent intermolecular bivalent binding. We also report agglutination and calorimetric solution-phase binding studies of mono- and bivalent peptide-linked ligands.

Introduction

Protein−carbohydrate interaction is integral to human disease. In the earliest phases of myriad viral, parasitic, mycoplasmal, and bacterial infections, pathogens recognize and adhere to host organisms through lectin-mediated oligosaccharide-directed binding events.[2] Such recognition phenomena are also implicated in both random and nonrandom metastatic processes during the progression of human cancers.[3] Cell-surface glycolipids provide recognition epitopes for the toxins secreted by enterotoxic bacteria, including the heat labile toxin from *E. coli*, the shiga and shiga-like toxins from *Shigella dysenteria* and *E. coli* O157, and the toxin from *Vibrio cholerae*.[4] Protein−carbohydrate interaction has also been implicated in neutrophil capture and inflammation processes.[5]

Because of the important roles played by protein−carbohydrate interaction in human disease, considerable effort has been devoted to the development of high-affinity ligands for carbohydrate binding proteins.[6] Such ligands would disrupt pathological carbohydrate-mediated recognition and act as a fundamentally new class of noncytotoxic therapeutic agents with broad applicability to a wide range of human diseases. The development of carbohydrate-based therapeutics has been frustrated

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by the weak affinities of carbohydrate binding proteins for their native saccharide ligands: typically, protein−carbohydrate complexation proceeds with dissociation constants ranging from millimolar to micromolar. With such weak affinities, simple carbohydrate derivatives are unsuitable for development as therapeutic compounds.

In an attempt to overcome this limitation, much research in contemporary carbohydrate chemistry is devoted toward the development of general strategies for enhancing protein−carbohydrate interaction energies. Two broad approaches have been explored in this regard. The first modifies monovalent carbohydrate ligands to either create new favorable interactions or improve the strength of existing interactions.[7] With a few notable exceptions, these efforts have failed to produce high-affinity ligands. Rather, enthalpy entropy compensation as a function of ligand structure is typically observed. While the underlying molecular origin of this phenomenon is unclear, the small desolvation enthalpies resulting from sequestration of carbohydrate ligands likely places fundamental limits on individual protein−carbohydrate binding free energies.[8]

Lectins typically exist in vivo as multivalent aggregates; the suggestion has therefore been made that Nature surmounts the "tight-binding" problem through multivalency.[9] A second strategy for the preparation of high-affinity saccharide therapeutics thus involves the development of multivalent carbohydrate ligands. A wide variety of such ligands have been prepared and, in at least some assays, many show large valence-corrected enhancements in affinity.[10] Such observations are typically termed "cluster glycoside" effects, which we define here as an enhancement in affinity on a valence-corrected basis, or values of β greater than one in the nomenclature of Whitesides and co-workers.[11] In some instances the effects are remarkable, and cluster glycoside effects as large as 10^9 have been reported. Despite the phenomenological observation of enhanced affinity—typically in agglutination assays—the molecular origin of the effect is obscure. Several processes could in principle provide enhancements in apparent affinity, including the stabilization of ordered or semiordered macromolecular aggregates, entropically assisted or chelate binding, and the presentation of water-swollen polymeric barriers to macromolecule−macromolecule interaction.

Another set of effects that could contribute to overall ligand affinities are favorable contacts between a linker domain and the protein surface outside of the saccharide binding site.[12] A potentially attractive methodology for scanning a wide variety of linker functionalities is the use of solid-phase libraries of peptide-linked multivalent ligands on beads. Even limiting functionality to that found in the 20 proteinaceous amino acids provides a considerable range of interactions; the incorporation of nonproteinaceous side chains greatly expands this range.

A major limitation to the use of resin-bound peptide ligands is the close spatial proximity of individual sequences on the surface of commercial peptide synthesis beads. If binding interactions on beads are to be representative of those in solution, binding must be limited to intramolecular events: the binding of multivalent lectins at the surface of a bead will almost certainly proceed in an intermolecular fashion. The trivial solution to this problem involves blocking a sufficient fraction of sites on the bead surfaces to prevent intermolecular binding. On the other hand, total bead loads must remain high enough to allow identification of the high-affinity ligands or "hits".[13] A solution to this problem was provided by the "bead-shaving" methodologies of Vágner et al.[14] In this protocol a large fraction of the *surface* residues of a cross-linked Tentagel resin, but a small fraction of the *total* residues, are blocked, facilitating both analysis of the biophysical properties of spatially segregated *exterior* residues and sequence analysis of the dense *interior* residues. We report here the applicability of this technique for screening libraries of peptide-linked bivalent ligands for the tetravalent lectin concanavalin A, in which the binding sites are separated by 65 Å.

Results and Discussion

The goal of this work was to determine the feasibility of evaluating intramolecular bivalent protein−carbohydrate binding on bead surfaces. Our strategy toward this goal was to prepare spatially segregated mono- and bivalent glycopeptide ligands by solid-phase synthesis and to evaluate their binding properties in both solid and solution phase. As a model lectin for these investigations, we chose the glucose/mannose specific lectin from *Canavalia ensiformis*, concanavalin A. Concanavalin A is a 26 KDa protein that aggregates into dimers at low (<5.2) pH and tetramers at high (>7) pH. Despite intense investigation during 60 years, concanavalin A continues to find utility in the study of protein−carbohydrate interaction.[10a−c,e] More recently concanavalin A has been utilized for the calorimetric study of protein−carbohydrate interaction.[15] A variety of crystal structures of both

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(13) The total volume of a 130 μM bead is approximately 1.15×10^{18} Å^3. To preclude intermolecular binding, each ligand requires *at least* a 100 Å radius, or 4.2×10^6 Å^3, free from contact with neighboring ligands. Each 130 μM bead contains approximately 3×10^{13} molecules; simple division provides that 99% capping is necessary to prevent intermolecular interactions. This calculation likely provides an estimate of the extent of blockage required since the physical construction of the bead provides additional steric bulk.

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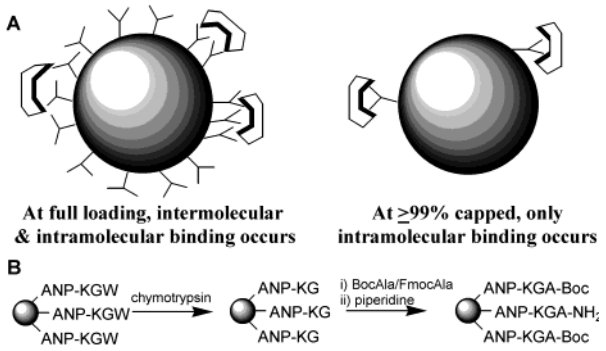

FIGURE 1. (A) The majority of resin loading sites must be blocked to preclude intermolecular binding. (B) Bead shaving of Vagner et al.[11] After incorporation of a photocleavable linker (ANP), a short peptide containing a chymotrypsin cleavage site is introduced with use of standard Fmoc chemistry. Beads are then treated with chymotrypsin, which cleaves the terminal tryptophan only from surface residues of a PEGA resin; in this fashion the interior and exterior volumes of the bead are differentiated. Reactive surface sequences are then elaborated with a mixture of Boc- and Fmoc-protected Ala. Standard piperidine deprotection reveals reactive amino groups on a fraction of the surface residues and on all of the interior residues. The protocol thus provides sufficient blocking of surface residues to preclude intermolecular binding but sufficient interior sequence to facilitate mass spectrometric sequencing.

native and saccharide-bound proteins have also been reported.[16] Crystal structures of concanavalin A and the closely related lectin from *Dioclea grandiflora* aggregated by multivalent ligands have also been reported.[17] The nominal through space distance between monosaccharide binding sites in either aggregation state is roughly 65 Å, although a ligand designed to span two sites must span an arc to this chord, a distance significantly greater than 65 Å.

For solid-phase peptide synthesis we required 100-mg quantities of glycosylated amino acids. Because of difficulties associated with both the preparation and stability of *O*-glycosyl serine derivatives, we chose to utilize the corresponding *C*-glycosyl analogue (**5**) (Figure 1).[18] We have previously reported an efficient preparation of *C*-glycosyl serines based on catalytic asymmetric hydrogenation of glycosylated enamide precursors.[19] In our original report the product amino acids were N-protected as the *tert*-butyl carbamates (Boc) and C-protected as the methyl esters. We have expanded the utility of the methodology by modifying the carboxyl protecting group such that the product amino acids are suitably protected for solid-phase synthesis.[20] During such synthesis, the carboxy terminus of the amino acid building blocks must be deprotected in the presence of the acetate and Boc protecting groups on the hydroxyl and amino functionalities, respectively. For this purpose, we chose the trimethylsilylethyl ester (TMSE), a group stable to Boc deprotection, but readily removed by treatment with TBAF.

The synthesis of mannosyl serine **5** begins with Horner−Emmons olefination of the aldehyde **2** with phosphonate ester **1**.[20] The required aldehyde **2** was prepared via radical allylation of acetobromomannose followed by ozonolysis to generate the glycosyl aldehyde **2**.[21] Catalytic asymmetric hydrogenation of the enamide **3** as an E/Z mixture was effected according to our previously reported protocols by using the Rh-DuPHOS catalyst system providing mannosylated CH_2-serine **4** in excellent yield and diastereoselectivity (>95% de).[20] Cleavage of the TMSE ester with TBAF provided **5** in quantitative yield.

Solid-Phase Glycopeptide Synthesis. We next set out to prepare spatially segregated glycopeptide ligands such that intermolecular bivalent binding on the surface of the bead was not feasible. Simple analysis of this problem—consideration of the density of sites on a bead, the spacing between binding sites on the lectin, the depth to which a 104 KDa protein can penetrate a Tentagel bead, and the volume such a protein occupies—suggests that greater than 99% of all sites must be blocked.[13] To achieve this separation while retaining sufficient ligand for sequencing, we utilized the bead-shaving protocol of Vágner et al.[11] This methodology takes advantage of the fact that only a small fraction of the total volume of a cross-linked Tentagel bead is accessible to even small macromolecules: the bead exterior can thus be differentiated from the interior by enzymatic digestion. Specifically, short Fmoc-protected peptide sequences containing a chymotrypsin cleavage site are built onto the cross-linked hydrophobic Tentagel resin. Treatment of the resin with chymotrypsin exposes a free amino terminus on only those sequences accessible to the 26 KDa protease; this fraction has been estimated at 2−2.5% of the total bead load.[11] Variable blockage of the exposed surface residues is accomplished by coupling the enzymatically released amino termini with a mixture of a Boc and Fmoc protected amino acid, typically Ala. Standard Fmoc deprotection next exposes a fraction of the surface sites, determined by the composition of the Boc/Fmoc protected mixture in the previous step, as well as all of the chymotrypsin inaccessible internal sites. Completion of the peptide synthesis provides a bead with spatially segregated surface peptides for binding studies, but dense internal loadings for MALDI MS peptide sequencing.

Our strategy for creating both mono- and bivalent ligands from a single precursor hinges on the use of a lysine residue differentially protected at the N^{α} and N^{ϵ} amino groups.[20] N^{α}-Fmoc/N^{ϵ}-Dde lysine was incorporated early in a sequence constructed with use of Fmoc chemistry. Selective deprotection of the amino terminus with 20% piperidine/DMF and addition of the *C*-mannosyl serine provides the monovalent ligand. Alternatively, simultaneous deprotection of the amino terminus and the ϵ-amino moiety of the interior lysine with 2% hydrazine hydrate/DMF revealed reactive amines at two sites; coupling with *C*-mannosyl serine produces the analogous bivalent ligand.

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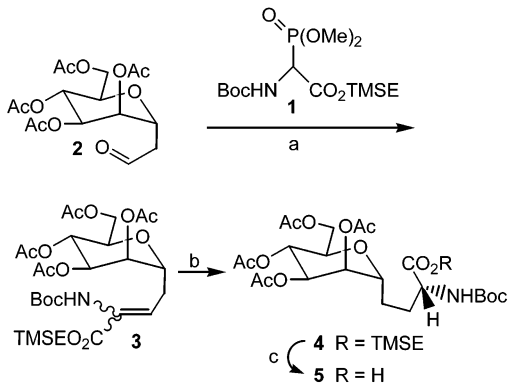
SCHEME 1. Synthesis of the Free Acid 5[a]

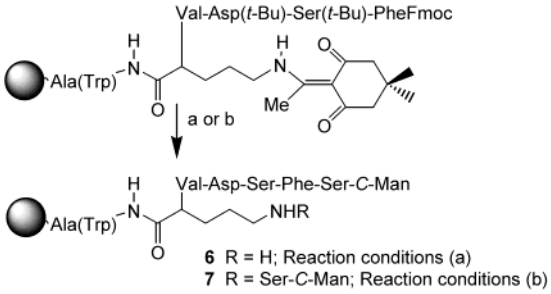


[a] Reagents and conditions: (a) TMG, THF, −78 °C, 65%; (b) [(COD)Rh-((*R*,*R*)-Et-DuPHOS)]$^+$OTF$^-$, THF, 90 psi H_2, >95% de, 99%; (c) TBAF, 99%.

Our goal here was to determine the sensitivity limit of the solid-phase binding assays with respect to the density of surface binding sites. Accordingly, we evaluated binding of concanavalin A to monovalent and bivalent peptide ligands with bead surfaces blocked at 99.0, 99.5, 99.9 and 99.99%. Amino Tentagel resin was derivatized sequentially with the photocleavable 3-amino-3-(2-nitrophenyl)propionyl (ANP) linker, Lys(N$^\epsilon$Boc), Gly, and Trp(Boc)NHFmoc.[22] The initial Boc-protected lysine was incorporated to ensure efficient ionization during MALDI MS/MS sequencing while the final two residues provide a chymotrypsin cleavage site. Three successive 8-h treatments with chymotrypsin followed by extensive washing completely removed the terminal Trp, exposing the amino termini of surface macromolecule-accessible glycine residues.

Enzymatically "shaved" beads were split into four pools and the exposed amino termini on the outside of the bead were capped with mixtures of 99% BocAla/1% FmocAla, 99.5% BocAla/0.5% FmocAla, 99.9% BocAla/0.1% FmocAla, or 99.99% BocAla/0.01% FmocAla. Fmoc deprotection then exposed 0.01 to 1% of surface alanine amino termini and tryptophan amino moieties of all internal sequences. Following coupling with N$^\alpha$-Fmoc/N$^\epsilon$-Dde lysine facilitated a convergent strategy for preparing both monovalent and bivalent ligands from a common set of beads.[23] The arbitrary tetrapeptide sequence VDSF was installed, and the beads from each synthesis split into two pools. The first fraction from each synthesis was Fmoc-deprotected with 20% piperidine and coupled with the R diastereomer of *C*-mannosyl serine (**5**) providing the monovalent peptide ligand (**6**). The second fraction was treated with 2% hydrazine hydrate, removing both the lysine N$^\epsilon$ Dde group and the terminal phenylalanine Fmoc amino protection (Scheme 2). Coupling of both amino residues with *C*-mannosyl serine provided the bivalent peptide linked ligand (**7**). In preparative scale syntheses on the solid phase, C-mannosyl serine showed reactivity identical to O-mannosyl serine in coupling reactions and no further attempts to analyze the yield of coupling reactions were made.[24]

SCHEME 2. Solid-Phase Synthesis of Monovalent and Bivalent Ligands[a]



[a] Reagents and conditions: (a) (i) 20% piperidine/DMF; (ii) **5**, DIC, HOBt; (iii) 2% hydrazine hydrate/DMF; (iv) 95% TFA, 2.5% H_2O, 2.5% TES; (v) 0.1 M NaOMe. (b) (i) 2% hydrazine hydrate/DMF; (ii) **5**, DIC, HOBt, (iii) 95% TFA, 2.5% H_2O, 2.5% TES; (iv) 0.1 M NaOMe.

To determine the effect of ligand presentation on assay performance, an analogous synthesis was repeated on Amino PEGA support. This resin is permeable to macromolecules up to 35 kD molecular mass and "shaving" does not differentiate surface and interior residues. Since our goal here was to determine assay sensitivity and the effect of ligand presentation, "shaving" was not required, and the chymotrypsin cleavage site was not installed. Amino PEGA resin was derivatized with photocleavable ANP linker, Lys(N$^\epsilon$Boc), and Gly, then capped with mixtures of 99% BocAla/1% FmocAla, 99.5% BocAla/0.5% FmocAla, 99.9% BocAla/0.1% FmocAla, or 99.99% BocAla/0.01 %FmocAla. Fmoc deprotection exposed 0.01−1% of the amino termini to which were coupled N$^\alpha$-Fmoc/N$^\epsilon$-Dde lysine and the tetrapeptide sequence VDSF. Deprotection with 20% piperidine or 2% hydrazine followed by coupling with C-mannosyl serine afforded the monovalent and bivalent ligands, respectively.

Binding Studies of Peptide-Linked Ligands. The binding of concanavalin A to glycopeptide ligands was evaluated by using both solid and solution phase assays. Binding was measured on the Tentagel and PEGA resins with use of commercially available concanavalin A—horseradish peroxidase conjugate: this assay is essentially a variant of the enzyme-linked lectin assay (ELLA) developed by Roy and co-workers.[25] Nonspecific binding was blocked by treatment of the beads with 2% BSA prior to incubation with lectin. Following washing, the beads were incubated with peroxidase-labeled lectin, H_2O_2, and ABTS prodye. The extent of ligand binding is assessed qualitatively by the appearance of color (Table 1). As a control, beads containing peptide sequence but no saccharide were also examined: in no case did any such resin develop observable color.

Diminished density of surface binding epitopes was accompanied by a diminished rate of color formation following treatment with peroxidase-labeled concanavalin A, demonstrating the expected dependence of lectin binding on surface ligand concentration (Table 1). Unexpectedly, *mono- and bivalent ligands on Tentagel beads blocked to 99.0% and 99.5% displayed a marked difference in the rate of color development*. The only plausible explanation for this behavior lies in a more facile intermolecular bivalent binding of concanavalin A to

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TABLE 1. Qualitative Results from Modified ELLA[a]

ligand	99.0%	99.5%	99.9%	99.99%	control[b]
Tentagel					
monovalent **6**	++[b]	++	++	−	−
bivalent **7**	++++	+++	++	−	−
PEGA					
monovalent **6**	+++	+++	++	+	−
bivalent **7**	++++	+++	+++	+	−

[a] Control beads contained the peptide sequence KVDSF, but lacked carbohydrate residues. [b] ++++ = dark color change within less than 3 min; +++ = dark color change within 5 min; ++ = color change within 5 mins; + = slight color change within 10 minu; − = no color change within 10 min.

TABLE 2. Solution-Phase Binding of 6 and 7 to con A

ligand	K_{eq} (M^{-1})[a]	con A IC_{50} (mM)[b]
α-MeMan	7.6×10^3	0.67
R-Man-*C*-ser	8.9×10^3	3.75
monovalent **6**	2.2×10^3	2.10
bivalent **7**	2.5×10^3	1.54

[a] Titration microcalorimetry. [b] Hemeagglutination assay.

immobilized bivalent ligands than to the corresponding monovalent analogue at equivalent bead loadings above some threshold density of binding epitopes. The spacing between recognition domains on a single bivalent peptide ligand is too short to accommodate intramolecular bivalent binding: we thus exclude intramolecular bivalent binding as the origin of the observation. Similarly, the close spacing between recognition domains on a single bivalent peptide ligand coupled with the steric constraints imposed by the surface of the bead almost certainly rules out simple concentration effects as the origin of the difference in apparent affinity between mono- and bivalent ligands. At the surface of a bead only a single dimeric lectin could conceivably approach a single peptide-linked bivalent ligand: the relevant ligand concentration on the surface of the bead is thus that of the peptide, regardless of the saccharide substitution level. From this perspective the concentration of "ligands" is identical on beads displaying bivalent and monovalent ligands.

Alternatively, beads containing bivalent ligands apparently offer a higher concentration of ligands disposed appropriately for *intermolecular* bivalent binding than do monovalent ligands: we suggest this intermolecular bivalent binding results in higher apparent affinity and darker staining of the bivalent beads. The construction of the two classes of ligands, with the second mannose residue of a bivalent ligand located proximal to the surface of the bead relative to the first saccharide, would seem a *priori* to require that roughly equivalent distances could be spanned by mono- and bivalent ligands. Free of additional constraints, the N-terminal mannose residue—that present in monovalent ligands—should describe the limits of the volume swept by a peptide-tethered recognition epitope. Apparently, the physical constraints imposed by the heavily cross-linked polymeric resin are such that unrestricted mobility of the ends of the peptide chains is not feasible. With such limited mobility the addition of a second saccharide ligand, even proximal to the polymeric support, reduces the distance between saccharide epitopes on adjacent peptide sequences (or increases the effective volume swept by a recognition epitope belonging to an individual sequence) and facilitates intermolecular bivalent binding.

This fortuitous result allows direct observation of the extent to which surface capping is required to prevent intermolecular bivalent binding. Beads containing mono- and bivalent ligand blocked at 99.9% developed color at approximately equal rates, suggesting that intermolecular bivalent binding is no longer significant for the bivalent ligand. The similarity in color development between beads blocked at 99.9% provides further evidence that differences between mono- and bivalent ligands observed earlier were not the result of concentration effects; such effects should persist regardless of the level of surface blocking. Finally, beads blocked at 99.99% failed to develop any color during 2 h of treatment with concanavalin A—peroxidase conjugate, regardless of the valence of the surface ligand, providing a lower limit to the sensitivity of the assay.

Broadly, PEGA beads blocked to various levels behaved identically to the corresponding Tentagel resins. Thus, mono- and bivalent ligands affixed to beads blocked at lower levels showed marked differences in the rate of color development, while at the highest level of blocking differences in color development disappear. Our interpretation of these observations is identical with that for Tentagel-bound ligands, and confirms that these observations are not pathological or a unique property of a particular matrix. In detail, the behavior of ligands on the two matrices differs. Specifically, the limit of sensitivity is enhanced on PEGA by roughly 1 order of magnitude compared to Tentagel and the concentration of ligand required to abolish differences between mono- and bivalent ligands is diminished by roughly 1 order of magnitude. We interpret these differences in terms of the accessibility of the interiors of the beads to macromolecules. While Tentagel excludes macromolecules with molecular masses above roughly 20 KDa, the interior of PEGA resins is completely accessible to species of at least 35 KDa. This cutoff, of course, depends more on molecular shape than molecular mass and is not a sharp demarcation. Presumably even complexes as large as the concanavalin A HRP conjugate penetrate the surface of the PEGA bead for at least some distance. To the extent this penetration proceeds, ligands are presented in a three-dimensional space, rather than in the two dimensions available on Tentagel beads. This higher concentration of available ligand presumably enhances sensitivity and diminishes the point at which individual macromolecules cannot access two unique ligands in an intermolecular sense.

To further examine the binding behavior of peptide-based mono- and bivalent ligands, each ligand was synthesized on a preparative scale and bound to concanavalin A. Binding was evaluated in both hemeagglutination and calorimetric assays (Table 2). Both mono- and bivalent peptide ligands as well as parent *C*- and *O*-mannosyl amino acids have essentially identical inhibitory potencies in agglutination assays. This observation again argues against concentration effects as the origin of the difference in apparent avidity between mono- and bivalent ligands on bead surfaces. Despite the fact that hemeagglutination assays rely on the formation of cross-linked aggregates, low-valent ligands seldom show cluster glycoside effects.

At the broadest level, calorimetric titration data mirror agglutination assays; both mono- and bivalent ligands

bind with roughly equal facility despite differences in valency (Table 2). Finally, we note again the weak correlation between binding data derived from agglutination and calorimetric assay, suggesting the techniques evaluate fundamentally different phenomena.[26]

In summary, we have demonstrated that spatially segregated solid-phase ligand synthesis allows identification of ligands for multimeric proteins with binding sites separated by 65 Å at 99.9% surface blockage, despite the fact this level of site blockage is close to the sensitivity limit of the method. Because most lectins have binding site spacings of less than 65 Å, solid-phase synthesis on enzymatically shaved beads should be an effective method for preparing large libraries of random peptide-linked ligands in the search for high-affinity bivalent ligands. We note parenthetically that the results reported here are of utility in the broader context of multivalency as a route to high affinity.[27] We are currently utilizing this strategy for the preparation of ligands for a range of lectins and will report our results in due course.

Experimental Section

General Methods. All reactions were conducted under an inert argon atmosphere. THF was distilled from sodium benzophenone ketyl. Dichloromethane and acetonitrile were distilled from calcium hydride. Methanol was distilled from magnesium. Water in all cases was purified with a Millipore purification system that involved passage through reverse osmosis, charcoal, and two ion-exchange filters. Solutions of compounds in organic solvents were dried over sodium sulfate prior to rotary evaporation. DMF was 99.5% pure and anhydrous. HOBt, Dhbt-OH, BSA, chymotrypsin, concanavalin A (Type IV), and DIC were purchased from Sigma. Benzyl bromide was filtered through alumina prior to use. TLC plates were Kieselgel 60 F254. Carbohydrate compounds were visualized on the TLC plate by charring with H_2SO_4/EtOH/H_2O (1:10:10). Flash column chromatography was performed on silica gel 60 (230−400 mesh). ^1H and ^{13}C NMR spectra were recorded on a 400-MHz spectrometer. All peaks are reported relative to 0.00 ppm (TMS). Although not technically correct, compounds are named as derivatives of the corresponding *O*-glycosides for ease of identification. Combustion analysis was carried out by Atlantic Microlab.

Solid-Phase Synthesis. All synthetic manipulations involving the resin were carried out in syringes fitted with PTFE filters (50 μm) with a second syringe used to evacuate waste fluids.

Ninhydrin Color Test. To ~5 beads was added 1 drop of ninhydrin (2.0 g in 20 mL EtOH), 40% phenol (w/v in EtOH), and KCN (1 mL of a 0.01 M KCN (aq) solution in 99 mL of pyridine). The mixture was heated to 100 °C for 1 min. A positive test for the presence of a primary amine resulted in blue resin. A negative test resulted in no color change.

Resin Preparation. AminoTG resin (200 mg, 0.056 mmol) was washed with DMF (4 × 10 mL). DMF (4 mL) was then added to the resin and allowed to swell for 15 min. The resin was then rinsed with DMF (4 × 10 mL).

Installation of ANP Linker. 3-Amino-3-(2-nitrophenyl)-propionyl (2 equiv, 48 mg) was dissolved in DMF (1 mL). HOBT (3.5 equiv, 26.5 mg) and DIC (2.0 equiv, 8.8 μL) were added and the solution was mixed thoroughly. The combined solution was added to the resin and the mixture was allowed to stand at 25 °C with occasional agitation for 24 h. Additional ANP (1.5 equiv, 35 mg) and DIC (2.0 equiv, 8.8 μL) were added and the reaction was allowed to stand for 16 h. At this time the ninhydrin test was negative.

Fmoc Deprotection. Piperidine (2 mL, 20% v/v in DMF, 5 mL) was added to the resin and allowed to stand at ambient temperature with occasional agitation for 15 min. Reagent was removed and the beads were rinsed with DMF (4 × 10 mL). The process was repeated once. The resin was rinsed with DMF (5 × 10 mL) and assayed with ninhydrin.

Peptide Bond Formation. Each amino acid, activated as the Pfp ester (2−4 equiv), was dissolved in DMF (2 mL). HOBt (5 equiv), DIC (3 equiv), and Dhbt-OH (1.5 equiv) were added prior to the addition of the resin. The resin was added to the mixture and incubated for 4−24 h at 25 °C with occasional agitation until ninhydrin assay was negative. The reagents were removed and the resin rinsed with DMF (4 × 10 mL).

Chymotrypsin Shaving. The resin was rinsed with DMF (4 × 10 mL), MeOH (4 × 10 mL), CH_2Cl_2 (4 × 10 mL), AcOH (5% v/v in water, 4 × 10 mL), water (4 × 10 mL), and 0.1 M NH_4CO_3 pH7 (4 × 10 mL). The resin was incubated with a solution of chymotrypsin (1.5 mg in 2.0 mL buffer) for 8 h at 37 °C. The resin was washed with water, 1:1 *t*-BuOH/water (4 × 10 mL), water (4 × 10 mL), and buffer (4 × 10 mL), then incubated again with chymotrypsin for 8 h. The process was repeated twice. The resin was assayed for a positive ninhydrin test.

Dde Side Chain Protecting Group Removal. Resin was washed with DMF (2 × 10 mL) and hydrazine hydrate (2% v/v in DMF, 3 mL) was added. The solution was allowed to sit at 25 °C for 3 min before the reagents were removed and the resin rinsed with DMF (4 × 10 mL). The process was repeated twice. The resin was then rinsed with DMF (5 × 10 mL) and assayed for positive ninhydrin test.

Removal of Acid-Labile Side Chain Protecting Groups. Resin was washed with DMF (2 × 10 mL), CH_2Cl_2 (4 × 10 mL), and deionized water (4 × 10 mL). A solution of 95% TFA, 2.5% H_2O, and 2.5% Et_3SiH (total volume of 2 mL) was added to a syringe containing the resin and the mixture was allowed to stand at 25 °C for 16 h. The reagents were removed and the resin washed with water (4 × 10 mL) and DMF (4 × 10 mL).

Removal of Acetate Protecting Groups. Resin was washed with DMF (4 × 10 mL), deionized water (4 × 10 mL), and anhydrous MeOH (4 × 10 mL). NaOMe (2 mL, 0.1 M) was added to the resin and allowed to stand at 25 °C for 2.5 h. The resin was washed with anhydrous MeOH (4 × 10 mL), water (4 × 10 mL), and DMF (4 × 10 mL).

Cleavage of ANP Linker. Resin was washed with DMF (4 × 10 mL), deionized water (4 × 10 mL), and anhydrous MeOH (4 × 10 mL) then dried in vacuo for 7 h. The resin was re-swollen in 2:1 THF/H_2O (10 mL) and irradiated with mercury lamps for 24 h. The resin was removed by filtration and the filtrate lyophilized.

ELLA Assay. Resin was placed in a funnel with a 10 μm glass frit and washed with 8 M guanidine-HCl (20 mL) for 15 min, water (4 × 10 mL), DMF (4 × 10 mL), deionized water (6 × 10 mL), and PBS-T (4 × 10 mL) [PBS-T buffer: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.1% Tween 20, 1 mM $ZnCl_2$, 1 mM $CaCl_2$, pH 7.2]. Nonspecific binding was blocked by treatment with 3% BSA/PBS-T for 1 h at 25 °C. The beads were rinsed with 1% BSA/PBS-T and incubated with 1 mL of peroxidase labeled con A (Sigma) (1 μg, 100 μL PBS-T) for 3 h at 25 °C. The resin was washed with PBS-T (4 × 10 mL) and incubated with substrate (50 mg ABTS in 0.05 M citrate-phosphate buffer (100 mL), pH 5.0 with 0.014% H_2O_2). Beads were transferred to a Petri dish for color development and evaluation.

2-Trimethylsilylethyl 4-(2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl)-2-ene-2-(*N*-*tert*-butyloxycarbonyl)butenoate (3). *C*-Mannosyl aldehyde **2** and phosphonate ester **(1)** were azeotroped with toluene (3 × 25 mL) and dried under high vacuum for 15 h. **1** (445.4 mg, 1.1 equiv, 1.15 mmol) and

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THF (8 mL) were cooled to −78 °C. Tetramethylguanidine (1.1 equiv, 0.145 mL) was added. The reaction was stirred for 10 min before the addition of **2** (390.3 mg, 1.04 mmol) in THF (8 mL) via cannula. After the addition was complete, the solution was warmed to 25 °C and stirred for 45 min. The reaction mixture was diluted with EtOAc (100 mL) and washed with 1 N HCl (1 × 75 mL) and saturated $NaHCO_3$ (1 × 75 mL), dried, and concentrated. Purification via flash chromatography (2:1 to 1:1 petroleum ether/ EtOAc) afforded **3** (472.6 mg, 68% yield) as a white foam. R_f 0.40 (1:1 EtOAc/petroleum ether); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.50−6.47 (t, J = 7.2 Hz, 1H), 6.24 (br s, 1H), 5.24−5.21 (dd, J = 3.2, 8.0 Hz, 1H), 5.11−5.08 (m, 2H), 4.42−4.38 (dd, J = 6.8, 12.2 Hz, 1H), 4.24−4.20 (m, 2H), 4.09−4.01 (m, 2H), 3.92−3.88 (dt, J = 3.2, 10.4 Hz, 1H), 2.67−2.59 (m, 1H), 2.50−2.43 (m, 1H), 2.06 (s, 3H), 2.05 (s, 3H), 2.04 (s, 3H), 2.01 (s, 3H), 1.43 (s, 9H), 1.02−0.972 (m, 2H), 0.01 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.6, 169.98, 169.69, 169.57, 164.45, 128.68, 80.74, 72.35, 71.17, 69.88, 68.36, 67.09, 63.90, 61.77, 28.54, 28.11, 20.81, 20.73, 20.65, 17.32, −1.58. Anal. Calcd for $C_{28}H_{45}NO_{13}Si$: C, 53.23; H, 7.18; N, 2.22. Found: C, 53.14; H, 7.17; N, 2.34.

2-Trimethylsilylethyl 4-(2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl)-2-(*N*-*tert*-butyloxycarbonyl)butanoate (4). In a drybox, [(COD)Rh-((*R*,*R*)-Et-DuPHOS)]$^+$OTf$^-$ catalyst (5 mg, 0.007 mmol) and enamide **3** (472.6 mg) were dissolved in deoxygenated THF (6 mL) in a Fischer−Porter tube. The reaction vessel was brought outside the drybox and pressurized with 90 psi of H_2 after five vacuum/H_2 cycles. The reaction was stirred at 25 °C for 48 h. The vessel was then depressurized and the mixture filtered through a short plug of silica gel to remove the catalyst and concentrated. **4** (470.1 mg, 99%) was isolated as a syrup. R_f 0.40 (1:1 EtOAc/petroleum ether); $[\alpha]^{20}_D$ −2.2° (*c* 1.03, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.12−5.07 (dd, J = 3.2, 8.6 Hz, 1H), 5.06−5.03 (m, 3H), 4.29−4.24 (dd, J = 6.5, 12.0 Hz, 1H), 4.21−4.20 (m, 1H), 4.16−4.12 (m, 2H), 4.03−4.00 (m, 2H), 3.88−3.81 (m, 1H), 3.78−3.73 (dt, J = 3.3, 10.9 Hz, 1H), 2.03 (s, 3H), 2.01 (s, 3H), 1.98 (s, 3H), 1.94 (s, 3H), 1.36 (s, 9H), 0.948−0.905 (m, 2H), −0.037 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.24, 170.42, 169.95, 169.62, 169.42, 155.19, 79.75, 73.53, 70.33, 70.27, 68.56, 66.88, 63.73, 62.08, 52.78, 28.42, 28.12, 24.40, 20.74, 20.56, 20.50, 17.31, 14.02, −1.71. Anal. Calcd for $C_{28}H_{47}NO_{13}Si$: C, 53.07; H, 7.48; N, 2.21. Found: C, 52.94; H, 7.42; N, 2.34.

4-(2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl)-2-(*N*-*tert*-butyloxycarbonyl)butanoic Acid (5). 4 (363.1 mg, 0.597 mmol) was dissolved in THF (2.4 mL) and 1 M TBAF in THF (2.4 mL, 4 equiv). The reaction was stirred at 25 °C for 5 h. The solution was diluted with 1 N HCl (2 × 75 mL), back extracted with EtOAc (1 × 75 mL), dried, and concentrated. **5** was used without further purification. R_f 0.10 (5% MeOH/ 95% CH_2Cl_2); $[\alpha]^{20}_D$ −6.5° (*c* 1.01, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.40 (br s, 1H), 5.21−5.18 (m, 2H), 5.13−5.04 (m, 2H), 4.34−4.29 (m, 2H), 4.10−4.05 (m, 2H), 3.95−3.92 (m, 1H), 3.83−3.73 (m, 1H), 2.07 (s, 3H, Ac), 2.05 (s, 3H, Ac), 2.03 (s, 3H, Ac), 2.00 (s, 3H, Ac), 1.99−1.84 (m, 1H), 1.82−1.66 (m, 1H), 1.40 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.49, 170.93, 170.21, 169.94, 169.66, 155.51, 108.92, 80.20, 73.51, 68.71, 67.80, 67.06, 62.25, 52.54, 28.19, 24.34, 20.84, 20.67, 20.64. Anal. Calcd for $C_{23}H_{35}NO_{13}\cdot 2H_2O$: C, 48.50; H, 6.90; N, 2.46. Found: C, 48.52; H, 6.45; N, 2.52.

Lys-Val-Asp-Ser-Phe-Ser-*CH*$_2$-mannose (6). The large-scale preparation of **6** was carried out with Rink amide MBHA resin. The amino acids were installed by using standard Fmoc peptide chemistry with HOBt (1.0 equiv), Dhbt-OH (0.5 equiv), and DIC (2−4 equiv) as the nucleophiles and DMF (100 mL) as the solvent. All amino acids were obtained as the activated Pfp ester. Qualitative ninhydrin assay was used to determine the extent of reaction after every reaction step. After the final coupling, the peptide was rinsed with DCM (3 × 40 mL), AcOH (3 × 40 mL), DCM (3 × 40 mL), and MeOH (3 × 40 mL). The resin was then transferred to a flask and dried under high vacuum for 15 h. The peptide **6** was cleaved with use of a solution of 95% TFA, 2.5% water, and 2.5% triethylsilane (100 mL total volume). The solution was added to the resin and allowed to stand at room temperature with occasional swirling for 1 h. At this time, the resin was filtered and rinsed with fresh TFA. The filtrate was concentrated in vacuo. The residue was treated with K_2CO_3 (20 mol %) in anhydrous MeOH (4.0 mL) for 4 h. The mixture was filtered, concentrated, and purified via C_{18} reverse-phase chromatography eluting with 100% H_2O, 3:1 H_2O/MeCN, and 1:1 H_2O/MeCN. The peptide eluted in the 3:1 solvent system as a white powder. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.26−7.08 (m, 5H), 4.70−4.54 (m, 2H), 4.34−4.31 (t, J = 6.0 Hz, 1H), 4.15−4.10 (m, 1H), 3.96−3.88 (m, 1H), 3.77−3.65 (m, 4H), 3.56−3.42 (m, 5H), 3.22−3.19 (br t, J = 7.2 Hz, 1H), 3.17−3.12 (dd, J = 5.2, 14.0 Hz, 1H), 2.99−2.70 (m, 5H), 1.95−1.90 (m, 2H), 1.79−1.59 (m, 3H), 1.56−1.48 (m, 2H), 1.47−1.36 (m, 2H), 1.33−1.24 (m, 3H), 0.87−0.77 (m, 7H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.27, 175.02, 174.29, 174.08, 173.09, 172.31, 170.23, 137.10, 129.94, 129.82, 128.37, 78.02, 74.62, 71.89, 71.56, 68.32, 62.16, 61.98, 60.69, 56.35, 55.62, 54.25, 53.83, 51.00, 40.16, 37.95, 36.21, 31.23, 31.00, 28.18, 27.15, 23.81, 23.05, 19.33, 18.68.

Ser-*CH*$_2$-mannose-N$^\epsilon$-Lys-Val-Asp-Ser-Phe-Ser-*CH*$_2$-mannose (7). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.23−7.14 (m, 5H), 4.68−4.60 (m, 1H), 4.33−4.30 (br t, J = 5.6 Hz, 1H), 4.10−4.06 (dd, J = 6.4, 8.6 Hz, 1H), 3.95−3.92 (m, 1H), 3.83−3.80 (m, 1H), 3.75−3.60 (m, 6H), 3.59−3.40 (m, 6H), 3.30−3.27 (m, 2H), 3.21−3.08 (m, 4H), 3.06−2.99 (m, 5H), 2.81−2.66 (m, 5H), 1.94−1.82 (m, 5H), 1.78−1.55 (m, 7H), 1.39−1.35 (m, 7H), 1.28−1.19 (m, 3H), 1.12−1.08 (t, J = 7.6 Hz, 2H), 0.77−0.75 (d, J = 6.8 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.44, 175.03, 174.22, 174.08, 173.08, 172.31, 170.74, 170.21, 137.11, 129.95, 129.82, 128.37, 77.97, 74.93, 74.61, 72.09, 71.89, 71.67, 71.57, 68.29, 62.16, 61.99, 60.63, 56.37, 55.62, 54.44, 53.85, 51.03, 47.65, 40.20, 37.96, 36.23, 31.34, 31.05, 28.75, 28.44, 28.18, 23.96, 23.81, 23.45, 19.35, 18.63, 9.20.

Calorimetry. Calorimetric measurements were made with the Microcal Omega titration microcalorimeter; details of the instrument design and data analysis are described elsewhere.[28] Lectin concentrations ranged from 0.54 to 1.324 mM. Titrations of concanavalin A were carried out at pH 7.15 in 50 mM Na_2HPO_4, 250 mM NaCl, 1 mM $CaCl_2$, and 1 mM $MnCl_2$ at protein concentrations ranging from 0.54 to 1.32 mM. Protein concentration was measured by the method of Edelhoch.[29] Titrations at pH 5.2 were carried out in 50 mM glutaric acid, 250 mM NaCl, 1 mM $CaCl_2$, and 1 mM $MnCl_2$. Ligand in identical buffer was added in 2.2-μL injections, 4.4 s in duration separated by 4-min intervals. The data were integrated to provide a titration curve, and by use of a nonlinear least-squares fit, the binding constant, K, the heat of binding, ΔH, and the stoichiometry of binding were extracted from the curve. Ligand concentrations of **x** were determined quantitatively in ninhydrin according to the method of Cocking et al.[30]

Hemagglutination Assay. The assay was performed with use of standard techniques. Briefly, 50 μL of buffer (50 mM PBS, 2%BSA) was added to microtiter plates followed by serial 2-fold dilutions of inhibitor. The initial concentrations of **6** were 16.8 and 49.2 mM for **7**. Concanavalin A (50 μL of 1 mM) in PBS was added to each well. After incubation at 37 °C for 2 h, 50 μL of 2% porcine erythrocytes anticoagulated with EDTA solution was added to each well. Plates were read following incubation for 1 h at 37 °C.

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