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## Probing Low Affinity and Multivalent Interactions with Surface Plasmon Resonance: Ligands for Concanavalin A

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**Abstract:** The affinities of the carbohydrate-binding protein concanavalin A (Con A) for mono- and multivalent ligands were measured by surface plasmon resonance (SPR) detection. Assessing protein-carbohydrate affinities is typically difficult due to weak affinities observed and the complications that arise from the importance of multivalency in these interactions. We describe a convenient method to rapidly evaluate the inhibitory constants for a panel of different ligands, both monovalent and multivalent, for low-affinity receptors, such as the carbohydrate-binding protein Con A. A nonnatural, mannose-substituted glycolipid was synthesized, and self-assembled monolayers of varying carbohydrate density were generated. The synthetic surfaces bind Con A. Competition experiments that employed monovalent ligands in solution yielded  $K_i$  values similar to equilibrium binding constants obtained in titration microcalorimetry experiments. In addition, this assay could be used to examine various polymeric ligands of defined lengths, generated by ring-opening metathesis polymerization (ROMP). This study demonstrates the utility of this method for rapidly screening ligands that engage in low affinity interactions with their target receptors. Our results emphasize that those molecules that can simultaneously occupy two or more saccharide binding sites within a lectin oligomer are effective inhibitors of protein-carbohydrate interactions.

### Introduction

Numerous cellular recognition processes depend on protein-carbohydrate interactions. These lectin-ligand attachments are critical in fertilization, cell signaling, pathogen identification, and the inflammatory response.<sup>1,2</sup> Lectin binding of carbohydrate ligands is enigmatic because the attractive forces at work are noncovalent and relatively weak (i.e.,  $K_a \approx 10^3\text{--}4\text{ M}^{-1}$ ), yet the strength and specificity required for proper cellular targeting is great.<sup>3-5</sup> The functional similarity of carbohydrates

as substrates for protein binding and the weak affinity observed in monovalent interactions have led to speculation that the clustering of binding events into multivalent arrays leads to a greater affinity and specificity than predicted from the sum of the constitutive one-on-one interactions.<sup>5-9</sup> Treating human diseases that involve protein-carbohydrate interactions is challenging since targeting a specific recognition event requires

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an intimate knowledge of factors leading to lectin–ligand specificity. Understanding these key elements will facilitate the development of new therapeutic strategies.

The recent automation of surface plasmon resonance (SPR) technology, which can be used to measure affinity rate constants, allows the convenient application of this valuable technique for assessing the rates of interaction. This method has been used to analyze a multitude of ligand–ligate complexes.<sup>10–12</sup> Using an optical biosensor, it is easy to determine the apparent rates of association and dissociation at a surface by monitoring free ligand binding to an immobilized binding partner. High affinity mono- and multivalent protein–carbohydrate interactions have been studied previously using SPR.<sup>13–19</sup> These assays, however, do not allow for quantitative, rapid screening of multiple low-affinity ligands. In each case, a new surface must be created for presentation and subsequent assessment of each ligand to be tested. Despite its potential, SPR has not been applied to the evaluation of multivalent inhibitors.

We have developed an SPR competition binding assay to garner quantitative binding data on monovalent and multivalent lectin–ligand complexes. Our interest is in understanding how weak, low-affinity interactions are used physiologically to achieve enhanced affinity and specificity. To this end, synthetic, multivalent glycoprotein mimics, termed neoglycopolymers, were devised to probe this issue.<sup>6,20–23</sup> In search of a rapid, sensitive, and reproducible assay requiring modest amounts of lectin and ligand, we evaluated SPR detection for these purposes.

The sensitivity limit for  $K_a$  determination using a commercially available surface plasmon resonance instrument

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(BIAcore 2000) is reported to be  $10^3$ – $10^6$  M<sup>-1</sup>.<sup>24</sup> Association constants in this range approximate the low affinities observed in many protein–carbohydrate complexes, as is seen for the jack bean lectin concanavalin A (Con A) binding to mono- and multivalent glycopyranosides.<sup>6,23,25</sup> Complexation of the low molecular weight (200 Daltons) monosaccharide ligands to immobilized protein would be difficult to detect by SPR; consequently, lectin binding to surface-bound carbohydrate was monitored.

Many investigations employing SPR have monitored the binding of an immobilized ligand to a target receptor in solution. One thorough study in which several receptor–ligand interactions were analyzed indicated that the off-rates for receptor dissociation from a surface are often unrelated to the dissociation rate in solution.<sup>26</sup> Thus, the precedents indicate that apparent affinities determined for ligand-modified surfaces do not correlate with solution binding constants. Moreover, the features of the synthetic surfaces can influence the observed binding interactions, complicating attempts to determine the binding of ligand in solution by comparing surface binding.

Several recent studies identify distinct advantages of competition assays in which the ability of a substrate to inhibit the interactions of a soluble receptor with an immobilized ligand, is explored.<sup>26–29</sup> In such assays, a ligand competes for a receptor in solution, thereby minimizing differences associated with surface composition. In addition to this advantage, only one surface is needed to measure binding to a variety of inhibitors. Consequently, competition assays were used to determine the efficacy of several inhibitors, using protocols similar to those reported by Karlsson and Morelock *et al.*<sup>26,29</sup> A mannose-derivatized glycolipid, **1**, was synthesized (Figures 1 and 2) and noncovalently bound to an optical sensor chip surface through lipid bilayer formation. By combining the synthetic glycolipid with phosphatidylcholine (POPC) in various molar ratios, it was possible to control the density of saccharide ligands presented.<sup>13,30</sup> Solution competition studies using the glycolipid surface proved an excellent means for rapid generation of inhibition data, both with small, low-affinity monovalent and larger, high-affinity multivalent Con A ligands.

## Results

Initial attempts to develop an assay involved the use of a gold surface coated with dextran, a polymer composed of

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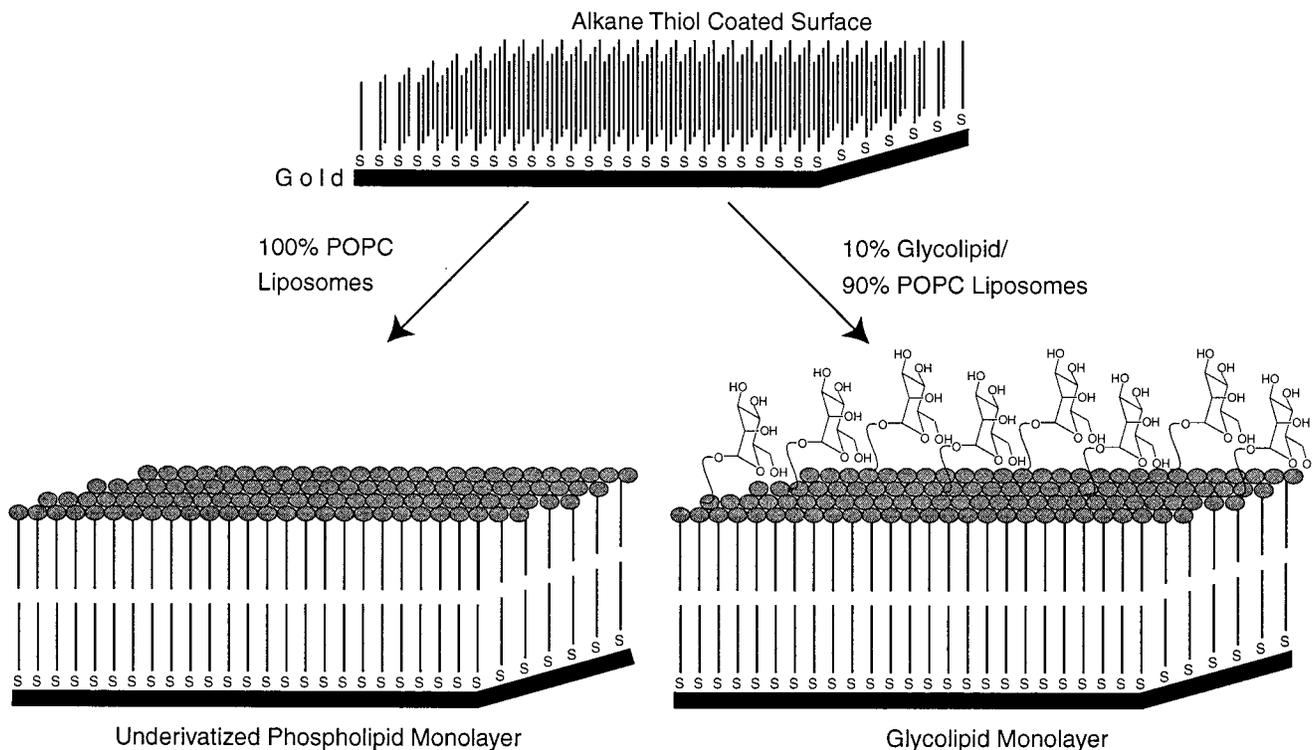
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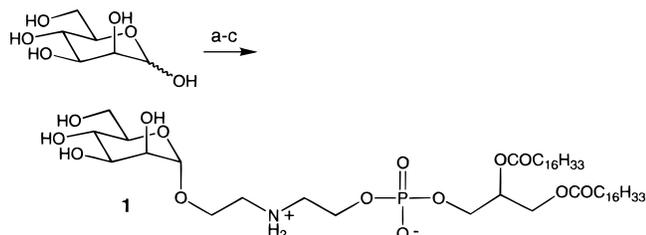
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**Figure 1.** Scheme illustrating control over ligand density on the SPR surface. Liposomes are generated by dissolving POPC and the glycolipid separately in 50:50 MeOH/CHCl<sub>3</sub>. Combining appropriate volumes of the pure solutions in the desired ratio followed by solvent evaporation and resuspension in aqueous buffers prepares liposomes for injection onto the hydrophobic surface where monolayers are generated.



**Figure 2.** Scheme for synthesis of the glycolipid (**1**). (a) Allyl alcohol, Dowex-H<sup>+</sup> (39%  $\alpha$ ). (b) (i) O<sub>3</sub>, 50:50 MeOH/CH<sub>2</sub>Cl<sub>2</sub>; (ii) PPh<sub>3</sub>. (c) NaCNBH<sub>3</sub>, PEt<sub>3</sub>N, 50:50 MeOH/CHCl<sub>3</sub> (53%, 2 steps).

glucose residues. This surface, which can be used for many applications, has been designed to minimize nonspecific protein binding in SPR experiments.<sup>31</sup> However, competition studies using the dextran matrix for immobilization of the carbohydrate ligand mannose were problematic. When the Con A tetramer was presented with a dextran surface modified by coupling with  $\alpha$ -C-(ethylamino)-mannose, it showed some increased binding relative to that obtained with the dextran matrix alone. As expected, the signal enhancement was small, relative to that obtained with the unmodified dextran surface. Specifically, with a mannose-substituted surface that gave rise to a signal of 288 response units (RU),<sup>32</sup> an injection of Con A (25  $\mu$ M) elicited a signal of 2799 RU compared to the 2615 RU obtained with the dextran surface alone. Although Con A has a 3–4-fold higher affinity for mannose over glucose,<sup>25</sup> background binding to the dextran matrix was difficult to dissect from specific Con A–mannose interactions. Ideally, a control surface, should demonstrate no affinity for the analyte and simply reflect bulk refractive index changes in solution. The error associated with

subtracting high background binding from relatively small, specific binding events complicated the analysis of the specific binding values obtained.

Given that Con A binds to dextran, competition studies could be performed using the underivatized dextran surface. Winzor and co-workers reported a method for determining the  $K_a$  and concentration of binding sites on the surface for multivalent interactions using a modified rectangular hyperbolic relationship.<sup>33</sup> Using these tools, we analyzed Con A binding to dextran. The number of available surface binding sites and the  $K_a$  of Con A for the dextran surface were evaluated. Subsequent competition experiments using monovalent saccharides yielded solution inhibition data. To derive the inhibition constants for various molecules, the SPR data were analyzed with the solution competition equation:

$$f = [I]/([I] + K_i(1 + F/K_d))$$

The parameters are defined as:  $f$  is fractional inhibition,  $I$  is the inhibitor concentration,  $K_i$  is the solution affinity of the inhibitor for the Con A,  $F$  is the concentration of free binding sites available on the surface, and  $K_d$  is the dissociation constant of Con A for the surface.<sup>34</sup>

The  $K_i$  values determined for monosaccharide inhibition of the Con A tetramer binding to the dextran surface were significantly different from the  $K_d$  values that had been accurately attained by titration microcalorimetry.<sup>25</sup> Specifically, the measured  $K_i$  values for  $\alpha$ -D-methyl-mannopyranoside ( $\alpha$ Me-Man, **2**) and  $\alpha$ -D-methyl-gluco-pyranoside ( $\alpha$ MeGlc, **3**) (Figure 6) inhibition of Con A binding to dextran were  $33 \pm 10$  and  $240 \pm 30 \mu$ M, respectively, data that suggest mannose is 7-fold more active than glucose. Microcalorimetry experiments indi-

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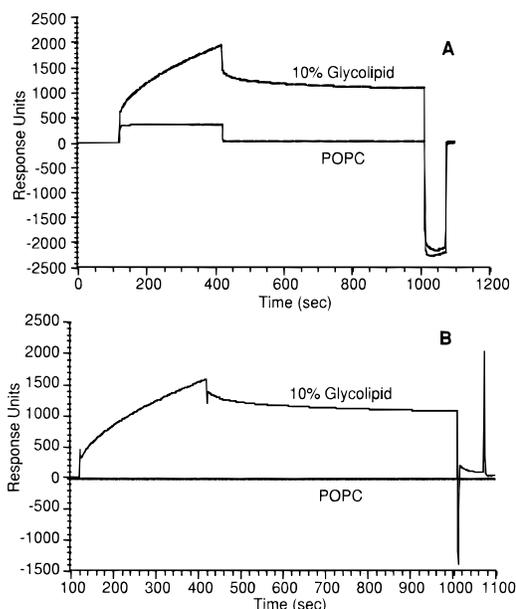
cated a less dramatic preference between the methyl pyranosides;  $K_d$  values of 130 and 423  $\mu\text{M}$  were obtained for  $\alpha\text{MeMan}$  and  $\alpha\text{MeGlc}$  (Figure 4), respectively, a 3.25-fold preference for mannose.<sup>25</sup> The distorted values observed with the dextran surface are likely due to the large number of binding sites available. If the ratio of  $F$ , the concentration of free binding sites, to  $K_d$  is greater than 0.1, one of the complexation partners will be tied up with little available in solution. This situation precludes the accurate determination of even relative affinities.<sup>35</sup> The  $F/K_d$  ratio observed with Con A and the dextran surface was 0.46, which clearly does not satisfy the experimental constraints.

**Incorporation of Glycolipid 1 To Generate Synthetic Surfaces.** Given the complexities encountered, we reasoned that a synthetic lipid monolayer could be generated to control the available ligand density on the surface and, thereby, the concentration of binding sites.<sup>30</sup> In this approach, a gold surface is modified with a long-chain alkane attached through a thiol group, upon which a new self-assembled monolayer (SAM) of defined composition can be generated.<sup>13,30</sup> We anticipated that this strategy would identify conditions in which the  $F$  to  $K_d$  ratio would be in the desired range, thereby affording a tractable system for solution competition studies.

A synthetic glycolipid was designed to bind to Con A. The target,  $\alpha$ -*O*-ethylaminomannosylphosphatidylethanolamine (**1**), displays a terminal mannose residue with an  $\alpha$ -anomeric linkage, the preferred configuration for Con A binding.<sup>36</sup> The glycolipid was readily constructed using standard transformations and then incorporated into SAMs. By varying the percentage of synthetic glycolipid **1** relative to phosphatidylcholine (POPC) in the injected liposomes, the ligand density was altered, and surface monolayers composed of POPC and 5, 10, 50, or 75% synthetic glycolipid were generated. A parallel flow cell, containing a surface composed solely of unmodified POPC, was used to assess background bulk refractive index changes in solution and at the surface (Figure 1). The cell containing POPC only was used to evaluate the amount and the magnitude of nonspecific protein binding to the lipid surface. For evaluating Con A–mannose interactions, responses derived from the POPC cell were subtracted from those obtained from the mannose-modified surface to yield values for specific binding of Con A to the glycolipid surface.

### SPR Experiments

The first objective was to determine the minimum concentration of glycolipid needed for selective binding of the tetrameric form of Con A.<sup>36,37</sup> As described above, surfaces composed of 5, 10, 50, and 75% glycolipid–POPC mixtures were analyzed. At the lowest glycolipid concentration tested (5%), a weak signal for binding of the Con A tetramer to the surface was detected. When the glycolipid density of the surface was augmented to 10%, very good signal-to-noise (1974 RU at 17  $\mu\text{M}$  Con A) and reproducible binding results were obtained. Under these conditions, the Con A tetramer showed considerably higher affinity for the surface and displayed a binding profile with characteristic association, equilibrium, and dissociation phases. In contrast, the control POPC surface showed only bulk refractive index changes in signal (Figure 3). Regeneration of the surface was accomplished by injection of 10  $\mu\text{L}$  0.1 M  $\text{H}_3\text{-PO}_4$  to remove bound Con A. As the glycolipid surface density



**Figure 3.** Specific binding of Con A tetramer to 10% glycolipid SPR surface. A. Raw data for injection of Con A over glycolipid and POPC monolayers. B. Bulk refractive index change subtraction yielding specific signal data for Con A binding to carbohydrate.

was altered from 10% to 75%, increased binding to the Con A tetramer was observed (data not shown). For the binding assays, the surface with 10% glycolipid was employed since it afforded the best sensitivity while minimizing the concentration of free surface binding sites.

The affinity of Con A tetramer for the 10% glycolipid surface and the concentration of available surface binding sites ( $F$ ) was determined by titration with successive injections of 50  $\mu\text{L}$  of Con A. The concentrations of Con A employed ranged from 17  $\mu\text{M}$  to 0.14  $\mu\text{M}$ , and the solutions were generated by 2-fold serial dilution. From the raw SPR data, a plot of effective bound surface concentration versus the effective injected concentration of Con A was generated (Figure 4).<sup>26</sup> Using equilibrium binding and the rectangular hyperbolic equation to calculate  $F$  and  $K_a$  for the binding of the Con A tetramer to the surface, values of  $0.97 \pm 0.03 \mu\text{M}$  and  $(2.7 \pm 0.2) \times 10^4 \text{ M}^{-1}$ , respectively, were obtained. The  $F/K_d$  ratio for this system is 0.026, conditions under which the assumptions in the fractional inhibition equation are valid.

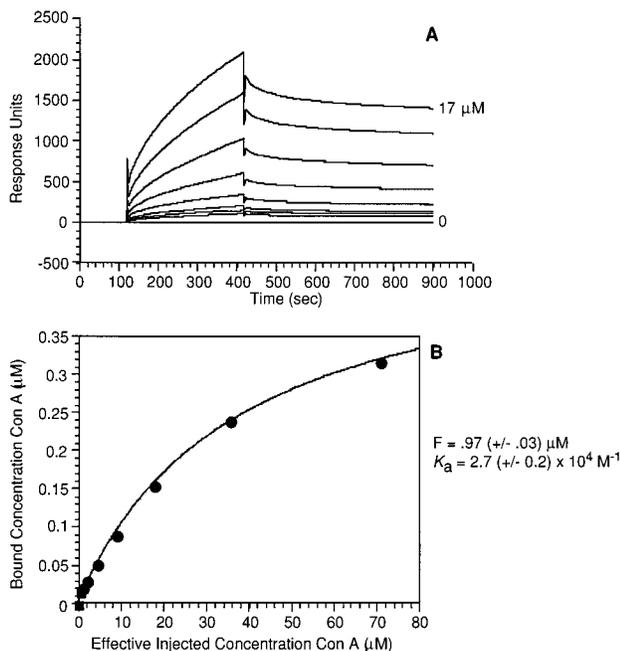
In the competition assays, inhibition curves were generated by measuring the binding responses for 500 nM Con A tetramer in the presence of increasing concentrations of inhibitor. Fractional inhibition constants were calculated using equilibrium values generated in the absence of inhibitor (Figure 5). The inhibition constants ( $K_i$ ) were generated for  $\alpha\text{MeMan}$  and  $\alpha\text{MeGlc}$  by generating a plot of the fractional inhibition values obtained versus inhibitor concentration employed. Although the  $K_i$  values that arise from this analysis for  $\alpha\text{MeMan}$  and  $\alpha\text{MeGlc}$ ,  $92 \pm 6$  and  $290 \pm 10 \mu\text{M}$  respectively, are not identical to  $K_d$  values generated by calorimetry, 130 and 423  $\mu\text{M}$ , the relative affinity values are comparable; a 3.2-fold ratio from SPR versus 3.25-fold from calorimetry. These results indicate that this competition assay can reproduce the binding constants determined by well-tested solution methods.<sup>25,36</sup>

Our success in determining monovalent inhibition constants with this assay system prompted us to investigate the utility of the method for comparing multivalent ligands. We have been developing the ring-opening metathesis polymerization (ROMP) as a method to generate carbohydrate-substituted polymers of

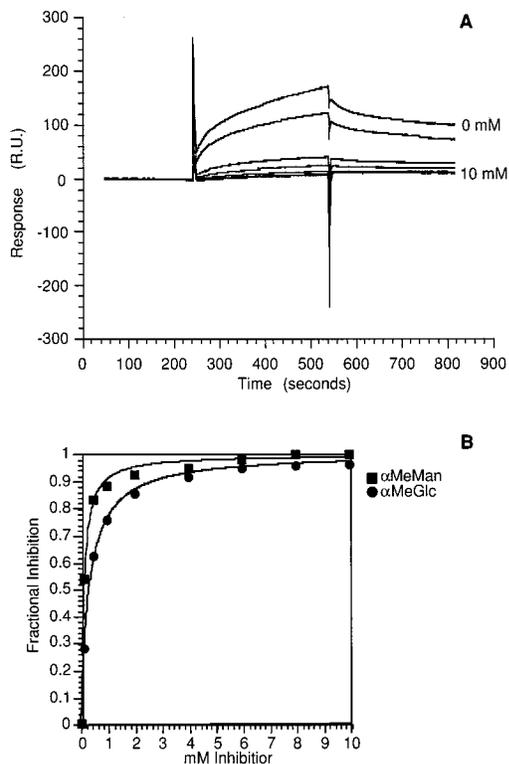
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**Figure 4.** Titration of Con A tetramer on glycolipid surface. A. Data used to determine the  $K_a$  of Con A binding to the surface, and  $F$ , the concentration of available binding sites. B. Evaluation of the results by the rectangular hyperbolic equation to determine  $K_a$  and  $F$ .

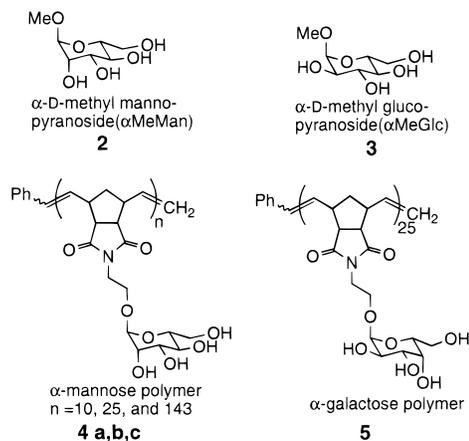


From SPR competition experiments:  $K_i = 92$  ( $\pm 6$ )  $\mu\text{M}$  for  $\alpha\text{MeMan}$   
 $K_i = 290$  ( $\pm 10$ )  $\mu\text{M}$  for  $\alpha\text{MeGlc}$   
 $K_{\text{rel}} = 3.2$

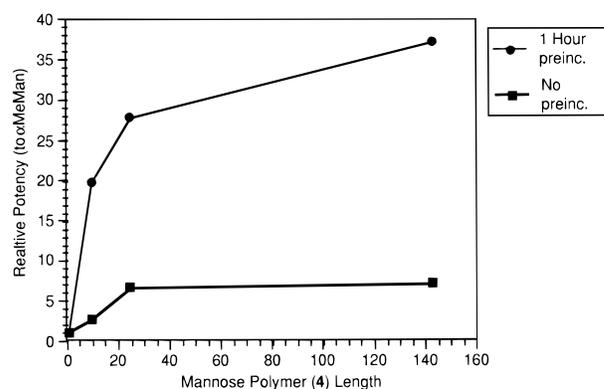
From microcalorimetry experiments:  $K_d = 130$   $\mu\text{M}$  for  $\alpha\text{MeMan}$   
 $K_d = 423$   $\mu\text{M}$  for  $\alpha\text{MeGlc}$   
 $K_{\text{rel}} = 3.25$

**Figure 5.** Inhibition of 500 nM Con A tetramer binding to the glycolipid surface. A. Inhibition curves for 0, 0.1, 0.2, 1, 2, 5, and 10 mM  $\alpha\text{MeGlc}$  (3). B. Fractional inhibition curves for  $\alpha\text{MeMan}$  (2) and  $\alpha\text{MeGlc}$  (3) inhibition of Con A binding.

defined length,<sup>20–23</sup> which have been termed neoglycopolymers. A series of mannose- and galactose-substituted materials (Figure



**Figure 6.** Inhibitors used in SPR experiments. 2 and 3: monovalent ligands for Con A. 4 and 5: neoglycopolymers of different lengths used to explore structure/function relationships in multivalent binding.



**Figure 7.** Polymer inhibition of Con A tetramer binding to glycolipid.  $K_i$  values are normalized to  $\alpha\text{MeMan}$  (2). Polymer concentrations were calculated on a per residue basis. Data from no preincubation or 1 h incubation times of Con A with polymers before injection onto the glycolipid surface are reported.

6) had been generated in conjunction with these studies.<sup>38</sup> To investigate the abilities of these agents to inhibit the binding of Con A to the glycolipid surface, solution competition experiments with the neoglycopolymers were carried out in a manner analogous to those performed with  $\alpha\text{MeMan}$  2 and  $\alpha\text{MeGlc}$  3. Polymer length was estimated by NMR integration analysis referencing internal double bond protons to the terminal aromatic protons.<sup>39</sup> For these substrates, the reported inhibitor concentrations were calculated on a saccharide residue basis. The resulting  $K_i$  values, therefore, do not report on the molar inhibitory constants of the polymers, but rather the average of the saccharide units within the polymer (Figure 7).

The inhibition data from the polymers reveal that the relative potencies of the mannose-substituted polymers increase with their length. For example, the 10mer 4a is 3-fold less active than the 25mer 4b, a trend that is not continued when the polymer length is extended to 143 residues, as in 4c. As expected, no inhibition was observed with the galactose 25mer 5 because Con A does not bind galactose. This result provides further evidence that the Con A interaction with the surface is specifically occurring via the mannose-substituted glycolipid; only saccharide ligands that bind Con A inhibit surface binding.

(38) Kanai, M.; Mortell, K. H.; Kiessling, L. L. *J. Am. Chem. Soc.* **1997**, *119*, 9931–2.

(39) Molecular masses calculated in this manner correspond well to those determined by gel permeation chromatography: Kanai, M., unpublished results.

These data also rule out nonspecific inhibition by the polymer backbone as a mechanism of action of the mannose derivatives.

Because the polymers are much larger than the low molecular weight monosaccharides investigated previously, the kinetics of binding might be a factor in their observed inhibitory potencies. To examine this issue, the polymers were incubated with Con A for 1 h, prior to their use in SPR competition studies. In these experiments, an increase in the relative potency, from 5-fold up to 40-fold, was observed when compared to their activities with no preincubation. Significantly, the same dependence on polymer length was obtained, with no dramatic increase in potency occurring for materials with lengths beyond the 25mer.

## Discussion

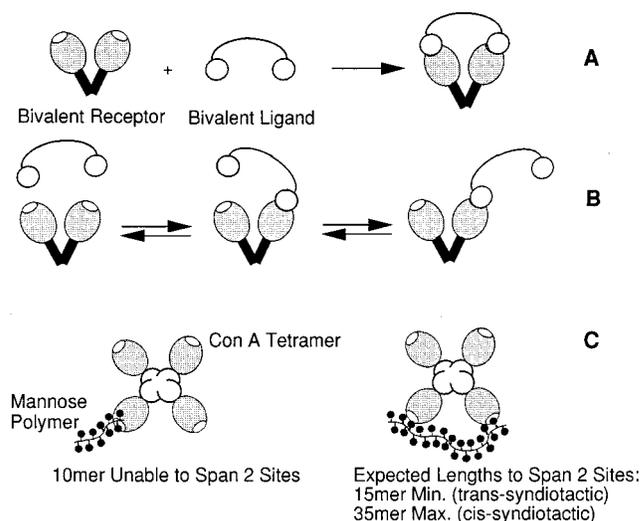
The self-assembled glycolipid monolayer provided an excellent surface for assessing the affinity of Con A for monovalent ligands in solution via competition experiments. The sensitivity achieved by this method enhances the detectable affinity range observable by SPR. Although Con A binds only weakly to monosaccharides, the relative solution affinities of Con A for these compounds could be measured by this method. This assay, therefore, provides a method for rapidly screening low molecular weight compounds for inhibition of protein-carbohydrate interactions. In addition, the relative activities of the multivalent inhibitors for the Con A tetramer could be assessed conveniently and rapidly.

The investigations involving the neoglycopolymers provide further insight into multivalent ligand binding to proteins, especially as it applies to the interplay between multivalent ligand length and inhibitory potency. The Con A tetramer presents two saccharide binding sites on each face, and the orientation of these two sites allows Con A to engage in multivalent interactions.<sup>40,41</sup> The distance between the two relevant saccharide binding sites within the Con A tetramer is approximately 65 Å, as determined from X-ray structural analysis.<sup>36,37</sup> The neoglycopolymer length needed to place saccharide residues in each of the binding sites can be approximated using molecular mechanics calculations. Polymer models of stereochemically homogeneous linkages were generated to provide insight into the geometries that can be adopted. The synthetic materials are composed of a mixture of *cis*- and *trans*-alkene isomers and isotactic and syndiotactic stereoisomers;<sup>20c</sup> however, by focusing on the limiting cases, estimates of polymer lengths could be generated. The least extended structure is the *all-cis*-syndiotactic isomer, in which the rise per residue is approximately 1.9 Å. The most extended structure is the *all-trans*-syndiotactic isomer, with an average separation of approximately 4.5 Å between residues. On the basis of these calculations, the minimum polymer length that could span the 65 Å gap between the two binding sites would be between a 35mer *cis*-syndiotactic polymer or a 15mer *trans*-syndiotactic polymer (Figure 8). These data suggest that neoglycopolymers of 25 or more residues can interact with Con A in a divalent manner.

The results from SPR detection indicate that the most potent neoglycopolymers are those that can simultaneously bridge two saccharide binding sites within the Con A tetramer.<sup>40,41</sup> Moreover, once multiple binding sites can be spanned by a single binding partner, polymers with increased length exhibit only

(40) For example, succinylated Con A which exists as a dimer with binding sites oriented in opposite directions does not engage in multivalent interactions.

(41) For a discussion of the chelate effect, see: Page, M. I.; Jencks, W. P. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 1678–83.



**Figure 8.** Multivalent modes of binding. A. Binding enhancement due to simultaneous spanning of two binding sites. B. Enhancement of binding due to increased local concentration of available ligand. C. Both modes of inhibition illustrated in the model system reported with Con A tetramer binding to neoglycopolymers.

minor increases in activity (Figure 7). Thus, these data suggest that the neoglycopolymers act most effectively when their length permits concurrent multiple binding site interactions. These overall findings with regard to multivalent ligand activity using the SPR competition assay are consistent with our studies of cell agglutination.<sup>38</sup>

Although the observed trends in inhibitory potency for various molecules are similar, the activities of compounds in the cell agglutination assay<sup>38</sup> span a dynamic range broader than those obtained from SPR. For example, in the SPR assay, the maximum increase in inhibitory potency for the most potent multivalent mannose derivative is approximately 40-fold over that of monovalent  $\alpha$ MeMan. Augmentations of approximately 1000-fold were found in the cell agglutination study. It is not surprising that large differences in relative potencies may occur between two distinctly different assays, especially when those assays involve multivalent binding interactions. The observed magnitude of enhancement when comparing a monovalent to a multivalent ligand will depend on the conditions under which the ligand must function. For example, the activities of compounds measured in static assays may be different than those measured under flow.<sup>46,47</sup>

Under the SPR assay conditions, the mechanism of action of the polymers may not involve simultaneous placement of saccharide epitopes in the Con A binding sites. Specifically, the activities of the polymers could be attributed to their abilities

(42) For studies exploring cross-linking of concanavalin A in the solid state, see: (a) Mandal, D. K.; Brewer, C. F. *Biochemistry* **1992**, *31*, 12602–9. (b) Mandal, D. K.; Brewer, C. F. *Biochemistry* **1993**, *32*, 5117–20.

(43) Several studies have identified multivalent ligands that are more potent inhibitors of lectin binding to cells than they are of lectin-saccharide binding in solution. The authors have proposed that the multivalent ligands can cross-link the lectins in the cellular environment. For some representative examples, see: (a) Lee, Y. C. In *Binding modes of mammalian hepatic Gal/GalNAc receptors*; Lee, Y. C., Ed.; John Wiley & Sons: Chichester, U.K., 1989; Vol. 145, pp 80–95. (b) Glick, G. D.; Toogood, P. L.; Wiley: D. C.; Skehel, J. J.; Knowles, J. R. *J. Biol. Chem.* **1991**, *266*, 23660–9. (44) Quesenberry, M. S.; Lee, R. T.; Lee, Y. C. *Biochemistry* **1997**, *36*, 2724–32.

(45) Lee, R. T.; Ichikawa, Y.; Kawasaki, T.; Drickamer, K.; Lee, Y. C. *Arch. Biochem. Biophys.* **1992**, *299*, 129–36.

(46) Alon, R.; Feizi, T.; Yuen, C.-T.; Fuhlbrigge, R. C.; Springer, T. A. *J. Immunol.* **1995**, *154*, 5356–5366.

(47) Sanders, W. J.; Gordon, E. J.; Alon, R.; Kiessling, L. L., unpublished results.

to noncovalently cross-link Con A tetramers, as has been observed for other multivalent ligands.<sup>42</sup> With this mechanism of action, however, it would be expected that the inhibitory potencies of the neoglycopolymers would continue to increase with molecular mass with longer polymers exhibiting ever increasing potencies. Such an effect is not observed (Figure 7). Moreover, the most active multivalent compounds in both assays are those with the ability to span the requisite distance to occupy two Con A saccharide binding sites.

The differences in the results of the two assay systems illuminate important features of multivalent binding partners.<sup>27,41–45</sup> For example, the kinetics of the relevant interactions may lead to large differences between the two assays. In the cell agglutination assay, the assay is conducted over a period of several hours. With the optical biosensor, equilibrium must be established in seconds to minutes for the competition results to be accurate. With the SPR assay, an inherent difficulty in performing competition experiments under flow conditions is that the rate of dissociation of the analyte, Con A, from the glycolipid surface depends on the surface composition and local structure. Because Con A is multivalent, it has a high probability of “rebinding” to the surface, which renders the rate of dissociation of the lectin slow on the time scale of the competition experiments (Figure 3). Thus, it is difficult to establish a binding equilibrium among the three components of the mixture, Con A, the glycolipid surface, and the inhibitor tested under the conditions of flow. Rapid equilibrium can be established with monosaccharides but is clearly not reached when the neoglycopolymers are tested in our assay system (Figure 7). Other studies of protein–carbohydrate interactions suggest that the evaluation of binding events under conditions of flow affords results distinct from those determined under static conditions.<sup>46,47</sup> Interestingly, the Whitesides group has shown that two different static assays afford similar results in the evaluation of the inhibitory potencies of polyvalent sialic acid-substituted ligands toward influenza virus hemagglutinin.<sup>48</sup> The assays employed occur under static binding conditions on time scales which allow for equilibrium between inhibitor and detectable binding partner to be reached. The dynamic nature of the SPR assay requires that equilibrium be rapidly attained, which could lead to the potency differences observed here.

The organization and presentation of ligands on the very different surfaces, the artificial glycolipid bilayer, and the red blood cell used in the two assays may also be manifested in the divergent results. For example, the density of ligands displayed by each surface is likely to be very different. In the cell agglutination experiment, Con A binds to surface glycoproteins with mannose residues, while in the SPR assay, Con A binds to artificial glycolipid-containing bilayers. The rates of Con A dissociation from these surfaces should differ, and these rates can influence the outcome of the competition experiments, as described above. Another important feature that separates hemagglutination and artificial surface binding inhibition is the mechanism by which inhibition can be achieved. Since Con A is a tetramer, agglutination will be effectively inhibited by occupying two Con A subsites, but such a binding event will not necessarily inhibit surface binding to the other face of the tetramer (Figure 8). In addition, if polymer-bound Con A binds to the glycolipid surface, dissociation will also be slightly slowed by the increase in molecular mass.

Thus, the inherent differences in the time scales of the assays, the structures of the surfaces, and the nature of agglutination versus surface binding is likely to account for the discrepancies

in the magnitudes of the potencies observed. An important conclusion from our results is that both assays provide the same relative ranking of the multivalent ligands and reproduce the trends in their binding potencies.<sup>38</sup> Thus, the SPR assay is useful for measuring the relative affinities of molecules within a class of compounds (monovalent or multivalent). Our studies reveal that SPR is an effective method for screening the activities of a series of low molecular weight, monovalent ligands. Significantly, competition assays such as the one reported here can be used to rank inhibitors of weak protein–saccharide interactions. This information will illuminate the molecular features important for complexation and will provide a basis for optimizing inhibitor structure. Moreover, the SPR competition assay can also be used to order inhibitor potencies within series of multidentate ligands, providing a method to rapidly identify potent ligands from this class of compounds.

## Summary

Not all physiologically and medically relevant receptor–ligand interactions occur with high affinities. Saccharides constitute one class of ligands that often functions by associating weakly with their target proteins. Methods to rapidly and accurately monitor low affinity interactions can facilitate the development of structure and function relationships in such systems and lay the groundwork for the identification of inhibitors. To address this issue, an artificial ligand-containing bilayer was generated, and a surface plasmon resonance assay was developed to assay the ability of various inhibitors to block protein binding to the synthetic bilayer. The system investigated involved the binding of the lectin Con A to a bilayer containing synthetic mannose-substituted glycolipids. Results with known monovalent Con A ligands reveal the effectiveness of SPR for these purposes. Significantly, this method can be used to rapidly evaluate the inhibitory potencies of a series of low molecular weight ligands.

In addition to its effectiveness for investigating low affinity interactions, the SPR assay can provide insight into the relative ranking of a series of multivalent ligands. To investigate its utility for this purpose, the inhibitory potencies of a series of neoglycopolymers composed of various lengths were assessed. As was observed previously in cell agglutination studies, the most potent multivalent derivatives were those that could simultaneously occupy two saccharide binding sites within the Con A tetramer. The increases in potencies for the multivalent ligands that were observed in the cell agglutination assay were dramatic relative to those seen with SPR. These results underscore the differences that can arise when evaluating interactions with an artificial surface relative to those observed with cells.

## Experimental Materials and Methods

**General Methods.** All materials were obtained from commercial suppliers and used as provided. Solvents and allyl alcohol were distilled by standard protocols. All reactions were monitored by thin-layer chromatography (TLC) on 0.25-mm precoated Merck silica gel 60 F<sub>254</sub> and visualized with phosphomolybdic acid or *p*-anisaldehyde stains. Flash column chromatography was performed on Merck silica gel 60 (230–400 mesh). <sup>1</sup>H and <sup>13</sup>C spectra were recorded on a Bruker AC-300 or Varian Unity 500 spectrometer and are referenced solvent peaks (CDCl<sub>3</sub>, <sup>1</sup>H δ 7.24, <sup>13</sup>C δ 77.0). Mass spectrum data was collected on a Bruker MALDI-TOF instrument using α-cyano-4-hydroxysuccinamic acid matrix. SPR measurements were performed on a BIAcore 2000 operated using the Version 1.3 software. Buffers were sterile-filtered and deoxygenated. Con A was purchased from Vector Laboratories, Inc., Burlingame, CA.

(48) Sigal, G. B.; Mammen, M.; Dahmann, G.; Whitesides, G. M. *J. Am. Chem. Soc.* **1996**, *118*, 8(16), 3789–3800.

**Preparation of Mannosylated Dextran Matrix.** The CM5 chip carboxylated dextran was activated by injection of a mixture of *N*-ethyl-*N'*-(diethylaminopropyl)-carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) (120  $\mu$ L, 200 mM EDC, 50 mM NHS, 20  $\mu$ L/minute) dispensed by the BIAcore instrument.  $\alpha$ -*C*-Ethylaminomannose (0.100 mg/mL in 10 mM HEPES buffer, pH 6.0) was injected into flowcell 1 (120 s contact time) and flowcell 2 (30 s) and allowed an additional 30 s to react. Unreacted, activated carboxyl groups were capped by the injection of ethanolamine (120  $\mu$ L, 1 M pH 8.5) yielding an increase of 278 and 288 RU in flowcells 1 and 2, respectively.

**SPR on Dextran Matrix.** Con A (2.5 mg, 96 nmol) was dissolved in sample buffer (1.0 mL, 20 mM HEPES, pH 7.0, 90 mM NaCl) overnight at 4 °C and syringe-filtered (0.22  $\mu$ m). Final concentration was determined by measuring the absorbance at 280 nm ( $A_{280} = 1.37 \times [\text{mg/mL Con A}]$ ). Surface density titration was carried out by successive injections of Con A (50  $\mu$ L), allowing 500 s for dissociation, followed by regeneration (10  $\mu$ L, 0.1 M  $\text{H}_3\text{PO}_4$ ). The titration range covered 12.5 to 0.39  $\mu$ M by 2-fold dilutions.  $F$  and  $K_a$  were determined as reported by Kalinin.<sup>33</sup>

Competition experiments with  $\alpha$ MeMan (**5**) and  $\alpha$ MeGlc (**6**) were carried out by running a method supplied by the BIAcore software, version 1.3. Con A tetramer (100 nM) was mixed with inhibitor and this mixture (50  $\mu$ L) was injected (10  $\mu$ L/minute). Inhibitor concentrations of 10.0, 8.00, 6.00, 4.00, 2.00, 1.00, 0.500, and 0.100 mM were tested. Bound Con A response values were assessed during the equilibrium binding portion of the curve (295 s after injection).  $K_i$  values were determined by fitting the data to the fractional inhibition equation:  $f = [I]/([I] + K_i(1 + F/K_a))$ .

**Synthesis of Glycolipid 1.** D-Mannose (1.00 g, 5.10 mmol) was dissolved in allyl alcohol (10.0 mL, 160 mmol). Freshly activated acidic Dowex resin (0.504 g, 50  $\times$  8–200 mesh) was added and the mixture refluxed under nitrogen for 1.5 h. Column chromatography purification (silica, 30% 9:4:2 ethyl acetate/*n*-propyl alcohol/water in ether) yielded the  $\alpha$ -anomer (0.446 g, 39%). The allylated pyranoside (7.0 mg, 0.032 mmol) was dissolved in methanol (0.50 mL) and dichloromethane (0.50 mL) and cooled to  $-78$  °C under a nitrogen atmosphere. Ozone was bubbled through the mixture for 5 min. Polymer-bound triphenyl phosphine (20.0 mg, 0.0636 mmol) was added, and the reaction was allowed to warm to room temperature and stir for 3 h. The triphenylphosphine oxide was removed by filtration over a small plug of Celite, followed by washing with methanol (3.0 mL), to yield the desired aldehyde (7.0 mg, 0.032 mmol, 100%). The solvent was evaporated, and the resultant solid was dissolved in dimethyl sulfoxide (100  $\mu$ L). Methanol (500  $\mu$ L) was added, and this mixture was added to phosphatidylethanolamine (22.0 mg, 0.0319 mmol) dissolved in chloroform (0.50 mL) and methanol (0.25 mL). The reducing mixture (1% (w/v) sodium cyanoborohydride, 0.1% (v/v) acetic acid) in chloroform (0.25 mL) and methanol (0.25 mL) was then added. The resulting mixture was allowed to stir at room temperature for 30 h. Purification by flash chromatography (silica, 65:35 chloroform/methanol

containing 0.2% (w/v) calcium chloride) yielded **1** (15.0 mg, 53%):  $R_f = 0.10$  (2:1 chloroform/methanol);  $^1\text{H}$  (500 MHz, 50:50  $\text{CD}_3\text{OD}/\text{CDCl}_3$ )  $\delta$  5.26–5.20 (1H, m),  $\delta$  4.42 (2H, dd,  $J = 12.0, 3.0$  Hz),  $\delta$  4.17 (1H, dd,  $J = 12.0, 6.5$ ),  $\delta$  4.80–3.46 (14H, m),  $\delta$  3.30–2.70 (4H, m),  $\delta$  2.31 (4H, q,  $J = 7.5$ ),  $\delta$  1.70–1.55 (4H, m),  $\delta$  1.40–1.20 (52H, m),  $\delta$  0.869 (6H, t,  $J = 7.0$ );  $^{13}\text{C}$  (500 MHz, 50:50  $\text{CD}_3\text{OD}/\text{CDCl}_3$ )  $\delta$  180.4, 174.5, 147.1, 101.0, 100.8, 74.0, 73.8, 73.6, 71.7, 71.5, 71.3, 71.1, 68.1, 68.1, 67.9, 66.2, 65.3, 65.1, 64.2, 63.6, 63.2, 62.2, 62.1, 57.6, 57.2, 55.9, 34.7, 34.6, 32.5, 30.2, 30.1, 29.9, 29.7, 25.5, 23.2, 14.2. MALDI  $m/z$  M calcd for  $\text{C}_{45}\text{H}_{87}\text{NO}_{14}\text{P}$  896.6 g/mol, observed 896.5.

**SPR on Glycolipid.** An HPA chip was perfused overnight with degassed water (5  $\mu$ L/min), the hydrophobic surface was washed with octyl glucoside (25  $\mu$ L, 40 mM). Liposome mixtures of defined glycolipid/phosphatidylcholine (POPC) concentration were generated by mixing pure samples dissolved in 50:50 chloroform/methanol in the desired molar ratios. The solvent was evaporated under an argon stream, and liposomes were generated by resuspension in sample buffer (0.5 M in 20 mM HEPES, pH 7.0, 90 mM NaCl). The liposome suspension was injected (300  $\mu$ L) onto the lipid surface leading to an increase of approximately 1500 Response Units over initial background. After 30 min of washing at 5  $\mu$ L/min, the surface was purged at 1000  $\mu$ L/min for 5 min to remove weakly attached lipid vesicles.

After changing the solvent to degassed sample buffer, a titration was carried out. Injected concentrations ranged from 17  $\mu$ M to 0.14  $\mu$ M Con A tetramer by differing 2-fold dilutions (50  $\mu$ L KINJECT, 10  $\mu$ L/minute).  $F$  and  $K_a$  for Con A tetramer on the glycolipid surface were evaluated as reported for the dextran matrix. Solution inhibition experiments were carried out by mixing equal volumes of Con A tetramer (1  $\mu$ M) and double the concentration of inhibitor to be tested. 50  $\mu$ L of the resultant sample mixture (0.5  $\mu$ M Con A tetramer/1  $\times$  inhibitor) was injected (10  $\mu$ L/minute) and equilibrium binding response values were taken at equilibrium binding (260 s postinjection). The inhibitory potencies of the polymers were generated using either a direct injection or a 1 h preincubation after mixing the Con A and inhibitor prior to injection of the competition mixture. Bulk refractive index data was generated on a parallel 100% POPC coated flow cell.

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