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Inhibition binding studies of glycodendrimer/lectin interactions using surface plasmon resonance

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ABSTRACT

Understanding protein/carbohydrate interactions is essential for elucidating biological pathways and cellular mechanisms but is often difficult due to the prevalence of multivalent interactions. Here, we evaluate the multivalent glycodendrimer framework as a means to describe the inhibition potency of multivalent mannose-functionalized dendrimers using surface plasmon resonance (SPR). Using highly robust, mannose-functionalized dithiol self-assembled monolayers on gold surfaces, we found that glycodendrimers were efficient inhibitors of protein/carbohydrate interactions. IC_{50} values ranging from 260 nM to 13 nM were obtained for mannose-functionalized dendrimers with Concanavalin A.

1. Introduction

Protein/carbohydrate interactions are the initiating event in a myriad of intercellular recognition events including cancer cell aggregation, the metastatic spread of cancer, bacterial invasion, and fungal and viral adhesion.[1–4] Because monovalent carbohydrate/lectin interactions are generally weak, nature often invokes multivalent and cross-linking processes to increase the overall avidity and specificity of the association.[5–8] The multivalent nature of the interaction often leads to cross-linking and aggregate formation. Agglutination of red blood cells, for example, is caused by lectin-red blood cell cross-linking and is the basis of the hemagglutination inhibition assay.[9]

Traditionally, hemagglutination and precipitation techniques have been used to determine relative interactions occurring between proteins and carbohydrates.[9,10] However, these assays are limited in that they do not provide either thermodynamic or kinetic data about the protein/carbohydrate interactions. Isothermal titration microcalorimetry (ITC)[11] directly measures thermodynamic binding parameters but requires large amounts of both the ligand and the receptor to obtain good signal. The requisite conditions of ITC have the potential to promote aggregation conditions, especially when using large, highly multivalent scaffolds, such as, dendrimers.

Surface plasmon resonance (SPR) technology provides a valuable technique for accurately analyzing protein/carbohydrate interactions. This method has gained a great deal of interest in recent years, and has been used to analyze many ligand/ligate complexes.[12] While many methods require the use of tags, such as radiolabels, SPR eliminates the need for labeled reagents. In addition, when qualitative binding comparisons across a series of compounds are needed, the need for extensive purification protocols is reduced when specific ligand/receptor binding is involved. The ligand of interest is passed through the system at a constant flowrate and non-participating compounds are quickly washed away. SPR allows one to monitor complex formations occurring in real time, offering insights into the kinetics and mechanism of a reaction. The assay is sensitive enough that low affinity interactions can be detected, since measurements can be made in the presence of excess, unbound protein. This is especially useful in protein/carbohydrate studies, where the interactions are known to be weak.

The use of SPR to elucidate binding events has seen a significant increase in recent years. Several noteworthy examples of the application of SPR to the study of protein/carbohydrate interactions have been reported; summaries of a few studies with direct relevance to this work follow. Keusgen and co-workers, for example, recently reported a lectin screen using immobilized oligosaccharides in SPR. The goal of this work was to devise a system that would ultimately allow for the identification of new lectins from natural sources.[13] Gabius and co-workers immobilized glycoproteins for sensitive detection of galectin-1 and *Ricinus communis* agglutinin in solutions containing mixtures of proteins,[14] and Nishimura and co-workers used SPR to evaluate how effectively sialyl Lewis X functionalized β-cyclodextrins inhibited the binding of *E*-selectin to a SLeXn-BSA functionalized sensor chip.[15] Suda and co-workers recently compared the binding of model heparin-binding proteins to immobilized monosaccharides and clusters of carbohydrates. They evaluated the

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effect of ligand density and ligand clustering by measuring the binding of a binding domain of vWf to the SPR chip. They found that clusters of oligosaccharides were more effective ligands than monosaccharides, even when comparable (per carbohydrate) surface loadings were present.[16] In addition to using SPR to compare loading densities, Fieschi and co-workers used mannose-functionalized Boltorn hyperbranched dendrimers to inhibit the binding of DC-SIGN to a gp120-functionalized surface.[17] Kiessling and co-workers used SPR with mannose-functionalized surfaces to determine the concentrations of neoglycopolymers that inhibited the binding of Concanavalin A (Con A) to the surface. The neoglycopolymers varied in length, and the most potent compounds were those that could bind to multiple Con A binding sites as well as cross-link the Con A lectins.[18] Riguera and co-workers compared SPR results for mannose-functionalized dendrimers binding to surface bound Con A with the inhibition of Con A binding to mannose-functionalized gold surfaces.[19] Schengrund and co-workers used SPR to study the binding of HIV-1 gp120 to potential glycodendrimer inhibitors. They found that binding affinities of the glycodendrimers with rgp120 (as measured by SPR) and the ability of the glycodendrimers to inhibit HIV infectivity (as measured by viral inhibition assays) correlated well for sulfated glycodendrimers.[20] Imberty, Matthews, Vidal and co-workers used SPR inhibition assays to test the potential of galactose-functionalized calyx[4]arenes to block adhesion of PA-IL to galactosylated surfaces.[21] These examples indicate that applying SPR technology to the investigation of protein/carbohydrate interactions shows great promise.

Because many lectins of current interest for biological processes are not surface bound, robust biosensors for measurement of multivalent binding interactions in the solution phase are needed. We determined that dithiol compounds are an ideal system with which to build a self-assembled monolayer on a gold surface. The dithiols form a bivalent attachment to the gold that is robust enough to undergo many inhibition/binding measurements. Here, we describe the effectiveness of different generations of mannose-functionalized dendrimers to inhibit binding of Con A to a mannose-functionalized surface through SPR.

2. Results

2.1. Surface functionalization with carbohydrates

We created a self-assembled monolayer (SAM)[22,23] of dithiols on an unmodified gold surface.[24,25] Alkanemonothiols are known to spontaneously desorb from gold surfaces under ambient conditions, resulting in patchy surfaces with significant areas of metal exposed.[26] The stability of the surface can be significantly improved through the use of multivalent dithiol compounds.[27–29] The carboxyl-terminated dithiol compounds were coupled to amine-functionalized mannose derivatives[30] through amide bond formation. This allowed for a stable mannose surface to be presented on the gold chip, which could be reused for multiple assays (Fig. 1).

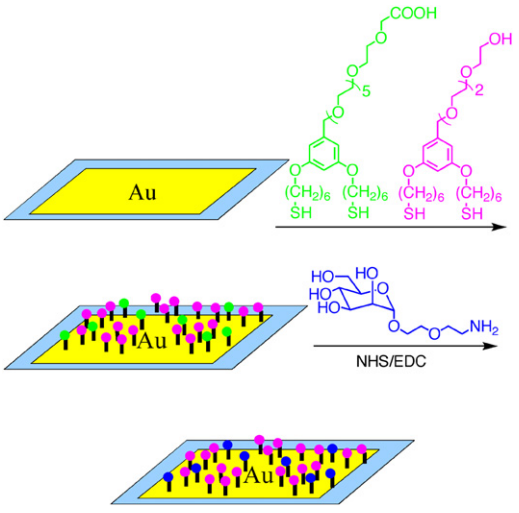

Figure 1. A schematic representation of mannose functionalization of a gold surface.

2.2. Surface plasmon resonance assays

Concanavalin A is tetrameric plant lectin that is able to specifically bind to mannose residues.[31] Serial dilutions of Con A were injected, and the SPR response was monitored to determine the affinity of Con A for the mannose-functionalized surface. The observed K_D for the binding of Con A to the mannose-functionalized surface was estimated at 78 nM (Fig. 2).

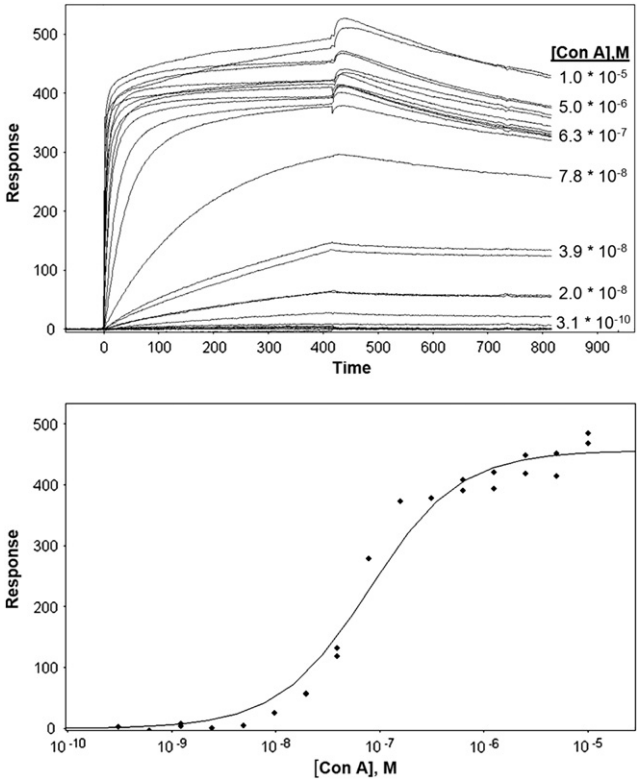

Figure 2. Top: doubly-referenced sensorgram of Con A binding to the mannose-functionalized surface. Bottom: affinity profile of Con A to the mannose-functionalized surface, fit using Scrubber 2.

The glycodendrimers that were used in this study are shown in Figure 3a, and the biosensor strategy is shown in Figure 3b. The values for the number of mannosides were determined from M_W (MALDI-TOF MS) and are the average number of mannosides per dendrimer. For a discussion of the homogeneity of the dendrimers, see Ref. 32. The affinity of the glycodendrimers for Con A was evaluated by studying their ability to inhibit binding of Con A to the mannose-functionalized surface. Serial dilutions of the inhibitor were pre-mixed with a constant amount of Con A, and the resulting equilibrium responses were used to determine IC_{50} values (Figs. 4 and 5). A key point is that glycodendrimer/lectin binding occurs in solution rather than at the monolayer surface, better mimicking many protein/carbohydrate interactions.

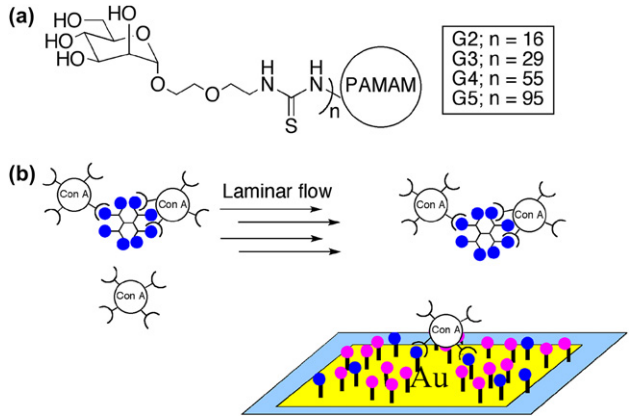

Figure 3. (a) Mannose-functionalized poly(amidoamine) (PAMAM) dendrimers. (b) A schematic representation of the inhibition binding experiment.

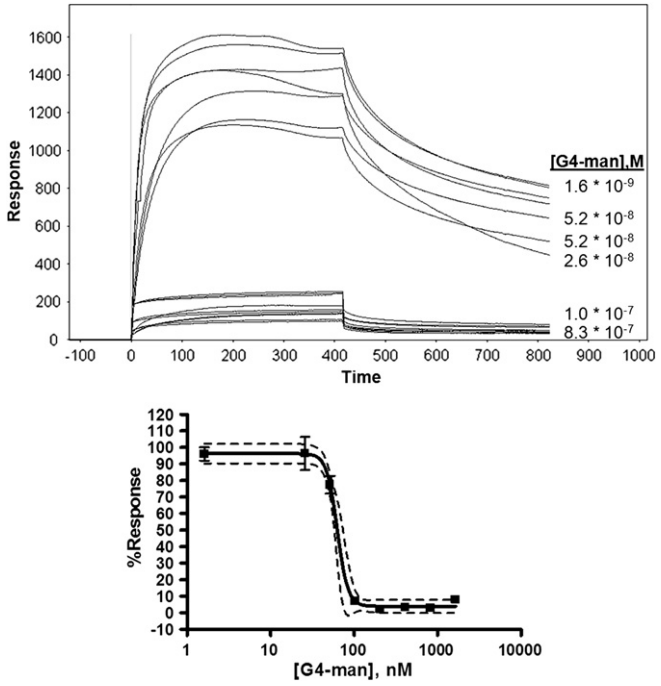

Figure 4. Inhibition of 2 μM Con A injected over the mannose-functionalized gold surface, normalized to 100% response from Con A without addition of inhibitor. Top: sensorgram of Con A binding to the mannose-functionalized surface, inhibited by mannose-functionalized G4 dendrimer. Bottom: inhibition by mannose-functionalized G4 dendrimer. Dotted lines show 95% confidence levels, fit using GraphPad Prism 4.

Whereas a marked increase in activity with increasing glycodendrimer generation was previously observed by hemagglutination assays,[33,34] the IC_{50} values obtained by SPR suggest that the ability to inhibit Con A binding to the surface is largely independent of dendrimer generation. While the glycodendrimers offer a definite advantage over methyl-mannose, higher generation dendrimers exhibit similar abilities to inhibit binding when compared to lower generations. Binding data are summarized in Table 1.

3. Discussion

3.1. Surface functionalization with carbohydrates

Although a commercially available gold surface coated with dextran, a polymer composed of glucose residues, is commonly used in SPR assays, competition experiments involving mannose-functionalized dendrimers and Con A proved to be problematic with this chip. The change in instrument response elicited by the addition of mannose-functionalized dendrimer was small compared to the binding of Con A to the dextran matrix in the absence of dendrimer, even though Con A has a 3- to 4 fold higher affinity for mannose than for glucose.[35] The minimal response change likely occurred because of the large excess of glucose residues on the dextran-coated gold surface relative to the number of mannose residues on the glycodendrimers.

Furthermore, using a dextran surface with large multivalent glycodendrimers may induce sugar/sugar binding events, complicating the analysis of the specifically targeted binding events. The mannose-functionalized monolayers used for this research were extremely robust because of the use of dithiols. The same chip was used repeatedly for at least six months with high precision in the acquisition of the data.

3.2. Surface plasmon resonance assays

SPR assays in which Con A was bound to a mannose-functionalized gold surface indicated that the observed K_D for the binding of Con A to the mannose-functionalized surface was 78 nM. This value indicates a significantly stronger interaction compared to the reported K_A of $7.6\times10^3\ M^{-1}$ (K_D of 130 μM) obtained for monovalent methyl-mannose from microcalorimetry.[36] This disparity almost certainly occurs because Con A is able to bind multiple surface sugars, increasing the overall affinity of Con A for the self-assembled monolayer.[5]

The gold surface used was functionalized with a rather high percentage (25%) of a carboxyl-terminated dithiol compound that was subsequently coupled to a tethered mannose derivative. This places the mannosides close enough to undergo bivalent binding with the tetrameric Con A, preventing an accurate determination of monovalent association and dissociation rate constants but allowing equilibrium constants of highly multivalent systems to be determined. The affinity profile for Con A, shown in Figure 2, does not significantly fit a 1:1 kinetic binding model (data not shown). Heterogeneous binding, which is a more effective mimic of relevant biological surfaces is suggested and allows us to study the ability of the dendrimers to inhibit multivalent binding modes to a surface. Because we compared the inhibitory propensity of different generations of glycodendrimers for Con A binding to the monolayer, determination of the exact degree of mannose incorporation into the monolayer was unnecessary. Although other researchers have used attenuated total reflection FT/IR spectroscopy to determine the degree of SPR chip functionalization,[16] this was not required for our studies because the same chip was used in all of the experiments, eliminating variability due to different chips.

While the results obtained by SPR show that the glycodendrimers exhibit a definite advantage over the monovalent sugar, the increased activity observed with higher generations of dendrimers in hemagglutination inhibition assays with Con A is not evident in SPR assays. In fact, the mannose-functionalized dendrimers showed only small affinity differences (on a per mannose basis), compared to remarkable activity increases observed in hemagglutination assays.

In contrast to the hemagglutination assay, SPR offers a method to more directly measure the interactions occurring between Con A and the sugar. In the hemagglutination inhibition assay, red blood cells are added as sources of surface sugars, which are bound by Con A. Inhibition of this interaction by glycodendrimers provides an entry-level comparison of our system to known systems, although the assay is not able to provide information regarding binding affinity. Rather, hemagglutination indicates the ability of

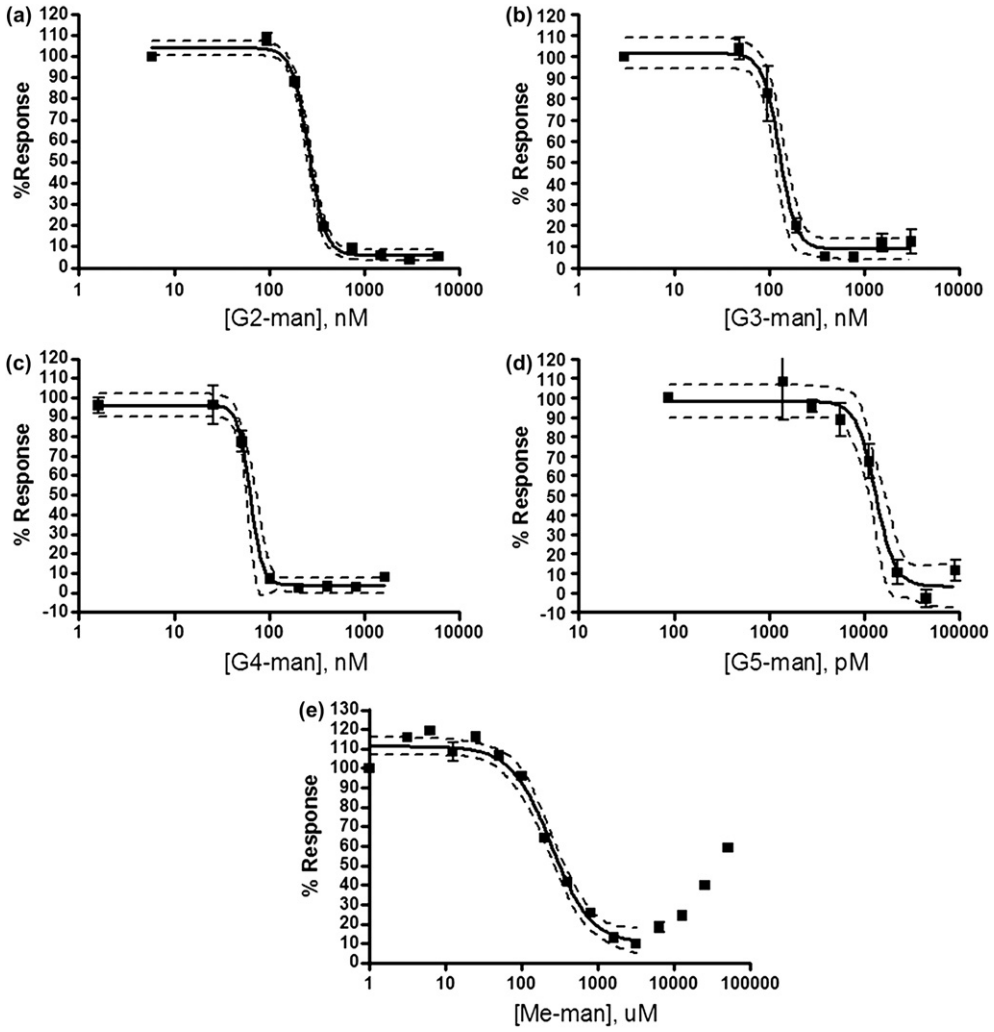

Figure 5. Inhibition by mannose-functionalized PAMAM dendrimers of 2 μM Con A injected over the mannose-functionalized gold surface, normalized to 100% response from Con A without addition of inhibitor. Dotted lines show 95% confidence levels, fit using GraphPad Prism 4. (a) Mannose-functionalized G(2)-PAMAM, (b) mannose-functionalized G(3)-PAMAM, (c) mannose-functionalized G(4)-PAMAM, (d) mannose-functionalized G(5)-PAMAM, (e) methyl-mannoside.

Table 1
Comparison of hemagglutination inhibition assays with SPR results for mannose-functionalized dendrimers with Concanavalin A

Hemagglutination inhibition assay results[a,b]		SPR inhibition assay results[a]	
Dendrimer generation (number of sugars)	Relative activity/ mannose (MIC)[c]	IC_{50} values/ dendrimer)[d]	IC_{50} values/ mannoside)[d]
Methyl mannose (1)	1 (6400 μM)		240 μM
G2 (16)	~1 (9700 μM)	260 nM	4.2 μM
G3 (29)	20 (280 μM)	130 nM	3.8 μM
G4 (55)	200 (30 μM)	63 nM	3.5 μM
G5 (95)	300 (20 μM)	13 nM	1.2 μM

[a] Each reported value represents at least three assays.
[b] Values are taken from Ref. 33 (G2) and 34 (G3–G5).
[c] Minimum Inhibitory Concentration per mannoside for 0.7 μM Con A.
[d] 2 μM Con A.

a glycodendrimer to drive aggregation processes, and not the strength of protein/carbohydrate binding.[37,38] As a result, SPR provides a more accurate measurement of protein/carbohydrate affinity, independent of aggregation and precipitation events.

Although the mannose-functionalized surface is successfully bound by Con A, additional binding events can be observed in the inhibition sensorgrams. Non-specific binding arising from sugar/sugar interactions is prominently displayed at high concentrations of methyl-mannose (Fig. 5e). At sufficiently high concentrations of methyl-mannose, Con A is prevented from binding to the surface. Even higher concentrations of methyl-mannose, however, afford an increased response through nonspecific sugar/sugar interactions. This suggests significant sugar/sugar interactions; a pre-organization step between sugars may be a factor in cellular recognition, as opposed to relying solely on the specificity of a protein for a sugar residue.[39–41]

Because of the heterogeneous nature of the binding events, kinetic data cannot be accurately determined from this experiment. Several binding events are occurring, including multivalent binding motifs, which make it difficult to deconvolute individual binding events from the overall system. However, information can be obtained from equilibrium data, particularly about the efficiency of the tested inhibitors. Examining the response as a function of added glycodendrimer reveals the efficiency at which the glycodendrimers are able to inhibit Con A from binding to the surface of the self-assembled monolayer.

Of particular interest is the increased steepness of the curve when the inhibition data are fit to the four-parameter logistic equation (Fig. 4, bottom). Whereas, inhibition of the Con A-surface sugar interaction by methyl-mannose yields a rather shallow slope, inhibition

by glycodendrimers yield markedly steeper slopes. By contrast, inhibition by galactose-functionalized dendrimers could not be fit to the equation and yielded similar responses throughout the dilution series (data not shown). This suggests that addition of mannose-functionalized dendrimer to the system induces a pronounced effect on Con A binding, and that responses from the assay are more sensitive to changes in dendrimer concentration than in methyl-mannose concentration. As the amount of glycodendrimer, that is, added is increased, Con A sequestration (such that less Con A is available to be bound to the self-assembled monolayer) is enhanced (Fig. 5). This is indicative of the dendrimers' ability to effectively bind Con A and to isolate bound Con A from a sugar-functionalized surface, as expressed by IC_{50} values (Fig. 5 and Table 1).

4. Conclusion

Highly robust mannose-functionalized SAMs for inhibition binding studies using SPR were formed on gold surfaces using dithiols. SPR experiments with these dithiol SAMs demonstrated that glycodendrimers efficiently inhibit protein/carbohydrate interactions; the potencies reported here for glycodendrimers are considerably higher than those obtained for monovalent mannose (on a per mannose basis). However, the generation of the glycodendrimer had less of an impact in these SPR studies than was observed in previously reported hemagglutination inhibition assays.[33,34] That the amount of inhibitor needed to achieve complete inhibition of red blood cell aggregation is decidedly different from the IC_{50} values reported here for SPR assays is readily rationalized because of fundamental differences in what the assays measure. In a hemagglutination inhibition assay, glycodendrimers probably attenuate aggregation events between Con A and red blood cells by efficiently sequestering the Con A lectins from the red blood cells. In assays that do not rely on disruption of aggregation events, dendrimers exhibit solely sugar/protein interactions, with less pronounced increases in affinity due to glycoside clustering effects.[36–38] The SPR competition assays reported here reaffirm that affinity enhancements for multivalent ligands are present. These SPR competition assays are an effective tool for studying solution phase binding events.

Glycodendrimers are very potent frameworks for mediation of protein/carbohydrate interactions. They are capable of both forming and disrupting extensive cross-links. When designing therapeutic agents for human health, our results suggest that different multimeric glycosides can exhibit similar activities for inhibiting protein/carbohydrate interactions, but decidedly different activities for interacting with large existing complexes are likely.

5. Experimental section

5.1. General methods

Mannose-functionalized dendrimers were synthesized as previously described in Ref. 33. Solutions and running buffers were degassed before use in SPR experiments. Unless otherwise noted, 10 mM PBS, pH 7.4 was used as running buffer for all SPR experiments.

SPR experiments were performed using a Biacore 1000 upgrade equipped with a Sensor Chip Au (Biacore AB, Uppsala, Sweden). The instrument temperature was set to 25 °C. Dialkanethiols (SPT-0013 and SPT-0014) were purchased from SensoPath Technologies (Bozeman, MT) for functionalization of the gold surface. Concanavalin A was purchased from CalBioChem (Darmstadt, Germany). All other chemical reagents were purchased from Fisher Scientific (Waltham, MA).

5.1.1. Preparation of 2-(2-aminoethoxy)ethyl-α-D-mannopyranoside. 2-(2-Azidoethoxy)ethyl-2,3,4,6-tetraacetyl-α-D-mannopyranoside (330 mg, 0.74 mmol) was dissolved in 10 mL of MeOH and reduced over Pd/C and an excess of H_2. The catalyst was removed and the resulting mixture was filtered through a 0.45 μm filter. Removal of the solvent in vacuo yielded 217 mg (79% yield) of 2-(2-aminoethoxy) ethyl-2,3,4,6-tetraacetyl-α-D-mannopyranoside, which was used directly.

2-(2-Aminoethoxy)ethyl-2,3,4,6-tetraacetyl-α-D-mannopyranoside (217 mg) was dissolved in 10 mL of 1:1 MeOH/H_2O. The acetyl groups were then removed with 1 M NaOMe in MeOH (522 μL, 522 μmol), stirring overnight. The solvent was removed under vacuum. The resulting sugar and acetic acid salts were dissolved in Millipore water and were used without further purification. ^{1}H NMR (DMSO-d_6, 500 MHz, solvent peaks were visible but not recorded) δ 3.2–3.7 (m, 21H). ^{13}C NMR (DMSO-d_6, 125 MHz, solvent peaks were visible but not recorded) δ 30.1, 40.5, 61.7, 66.1, 67.5, 69.8, 70.7, 71.4, 74.4, 100.4. These results agree well with previously published data for this compound, which was synthesized via a slightly different route and for which NMR data are reported in D_2O.[30]

5.1.2. Preparation of carboxylated gold surface. 25% of a –COOH terminated aromatic dialkanethiol (SPT-0014, SensoPath Technologies) was mixed with 75% of a –OH terminated aromatic dialkanedithiol (SPT-0013, SensoPath Technologies) to provide a 1 mM dithiol solution in ethanol. A 1:10 dilution of this solution (v/v) in Millipore water afforded a 0.1 mM solution that was injected over a single flowcell area (flowcell 2) of a new gold chip several times at 1 μL/min for 60 min using Millipore water as running solution, until no appreciable signal increase was observed. This procedure was repeated using 100% –OH terminated aromatic dialkanethiol (SPT-0013) over flowcell 1 on the same gold chip for a reference cell.

5.1.3. Preparation of mannose-functionalized gold surface. The carboxylated gold surface was activated by injection of a mixture of *N*-ethyl-*N*′-(diethylaminopropyl)-carbodiimide (EDC) and *N*-hydroxysuccinamide (NHS) (30 μL, 200 mM EDC, 50 mM NHS, 5 μL/min) dispensed by the Biacore instrument. 2-(2-Aminoethoxy) ethyl-α-D-mannopyranoside (10 mg/mL in 10 mM PBS, pH 7.2, includes acetic acid salts from the deprotection step) was injected into flowcell 2 (12 min contact time) and allowed to react. This procedure was repeated three times to allow for maximum functionalization. Unreacted activated carboxyl groups were given extensive time to hydrolyze under running buffer; remaining activated carboxyl groups were then capped by injection of ethanolamine (20 μL, 1 mM pH 8.5).

5.1.4. Surface plasmon resonance. Concanavalin A (Con A) (100 μL in 10 mM PBS, pH 7.4) was injected over the mannose-functionalized surface at 10 μL/min, allowing 300 s for dissociation, followed by regeneration (5 μL, 10 mg/mL methyl-mannose in 0.1 M HCl). The titration range covered 10 μM–31 pM by 2-fold dilutions. K_D was estimated using Scrubber 2.0.

For competition experiments with inhibitors, Con A (2 μM monomer) was mixed with an equal volume of inhibitor and the solution was allowed to equilibrate for at least 1 h. The mixture (70 μL) was injected over the surface at 10 μL/min, allowing 300 s for dissociation, followed by regeneration (5 μL, 10 mg/mL methyl-mannose in 0.1 M HCl). The glycodendrimers were tested in triplicate, covering a range of 50 μg/mL–39 ng/mL by 2-fold dilutions. For competition experiments with methyl-mannose, the assay covered a range of 10 mg/mL–305 ng/mL by 2-fold serial dilutions. Equilibrium responses were determined using Scrubber 2.0, then analyzed in GraphPad Prism (version 4.0) using the four-parameter logistic equation to determine IC_{50} values.

Binding responses were obtained using the aforementioned conditions across a mannose-functionalized surface. The binding responses were referenced by subtracting the response generated from identical injection conditions over a flowcell containing only –OH terminated dialkanethiols (flowcell 1), and double-referenced by subtracting a response from buffer injections.

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Supplementary data

Supplementary data associated with this article can be found in online version at doi:10.1016/j.tet.2010.05.038.

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