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Binding of Mannose-Functionalized Dendrimers with Pea (*Pisum sativum*) Lectin

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Abstract: Lectins are invaluable tools for chemical biology because they recognize carbohydrate arrays. Multivalent carbohydrate binding by lectins is important for processes such as bacterial and viral adhesion and cancer metastasis. A better understanding of mammalian lectin binding to carbohydrate arrays is critical for controlling these and other cellular recognition processes. Plant lectins are excellent model systems for the study of multivalent protein−carbohydrate interactions because of their robustness and ready availability. Here, we describe binding studies of mannose-functionalized poly(amidoamine) (PAMAM) dendrimers to a mitogenic lectin from *Pisum sativum* (pea lectin). Hemagglutination and precipitation assays were performed, and results were compared to those obtained from concanavalin A (Con A), a lectin that has been studied in more detail. Isothermal titration calorimetry (ITC) experiments are also described.

Keywords: Dendrimer; pisum sativum; pea lectin; concanavalin A; multivalency; carbohydrate recognition

Introduction

Protein−carbohydrate interactions on the cell surface play key roles in many cellular processes. The recruitment and activation of cells for mechanisms of inflammation and for the mounting of an immune response, for example, are protein−carbohydrate mediated events. Protein−carbohydrate interactions are also critical in other processes such as the infection of host cells by viruses and bacteria, the adhesion and metastatic spread of cancer cells, and even cellular differentiation and growth.[1−4]

The interactions of monosaccharides with lectins, which are carbohydrate-binding proteins, are generally weak. Micromolar monovalent dissociation constants are common. In order to increase affinity and confer selectivity, multivalent protein−carbohydrate interactions are widely used in nature.[5,6] Many lectins have multiple sites for carbohydrate binding, which are shallow pockets relatively distant from one another (3−7 nm) on the protein surface.[7]

Among the lectins, legume lectins represent the best-characterized group of lectins. Legume lectins are typically

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homodimers or homotetramers that contain one highly conserved sugar-binding site on each monomeric unit.[8,9] Concanavalin A (Con A)[10,11] and a lectin isolated from *Pisum sativum* (pea lectin)[12,13] are two such lectins. In solution, Con A is a homotetramer at biological pH, while pea lectin is a homodimer. Both proteins bind methyl mannose with specificity, although Con A has 4-fold higher affinity than pea lectin for methyl mannose.[14] As shown in Figure 1, both Con A and pea lectin have binding sites about 65 Å apart.[11,13,15,16]

Because lectin−carbohydrate adhesion generally involves multivalent interactions, a variety of glycopolymers that can span multiple lectin binding sites have been developed to help study these processes.[17] Dendrimers, for example, can be functionalized with sugars to study protein−carbohydrate interactions.[18] Understanding multivalent biological systems on the molecular level can suggest strategies for novel drugs. Synthetic multivalent molecules can be designed to either inhibit or promote biological processes, greatly improving drug design.

Unlike most polymers, dendrimers have a regular branching pattern with predictable physical properties.[19] By controlling the number and nature of the tethered functional groups, the solubility and reactivity of the molecule can be customized to suit biological conditions. We have previously reported the synthesis and lectin-binding properties of generation one through generation six mannose-functionalized PAMAM dendrimers (compounds **1**−**6**, Figure 2) with Con A.[20] In these Con A−dendrimer interactions, multivalent binding motifs were observed for large dendrimers (generations four through six, compounds **4**−**6**), while smaller

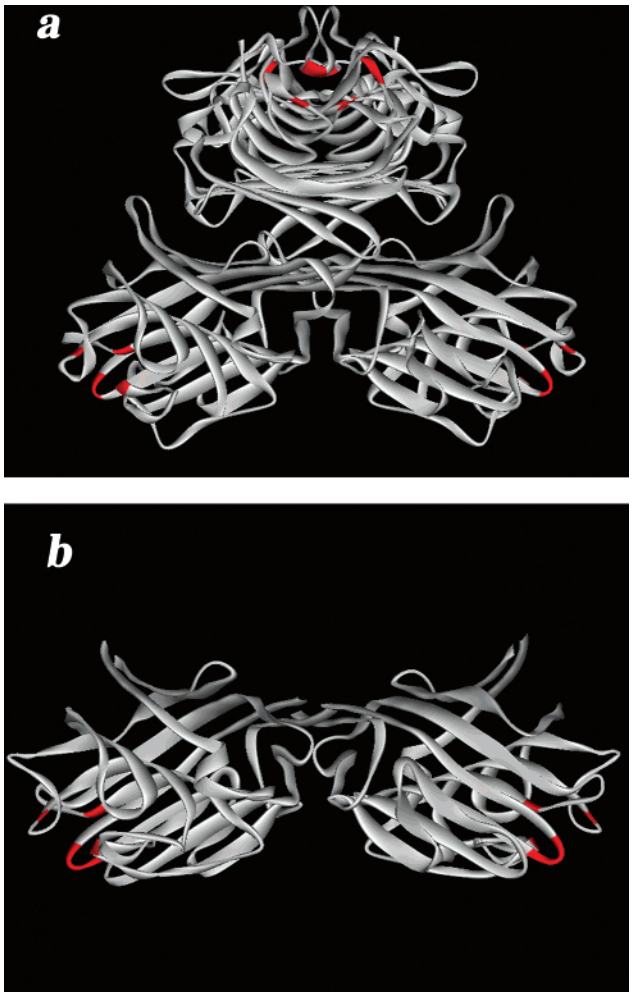

Figure 1. Structures of (a) Con A[13] and (b) pea lectin[15] bound to methyl mannose. The binding sites of both lectins[11,16] are about 65 Å apart; binding residues of both lectins are indicated in red.

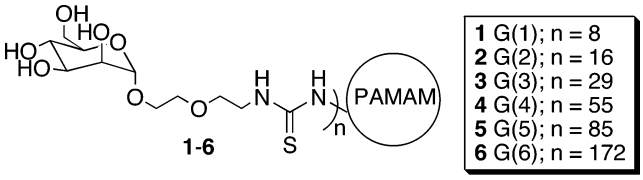

Figure 2. Mannose-functionalized PAMAM dendrimers **1**−**6**.

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statistical or proximity-type enhancements in binding were observed for **3**, and relative affinities comparable to methyl mannose were observed for dendrimers **1** and **2**.[21] Changing the size of the dendrimer framework (by changing the generation of PAMAM used) changed the type of interaction that was observed with Con A.

In order to test the generality of the results that we obtained with Con A, we studied the binding of mannose-functionalized dendrimers to pea lectin. The binding results for mannose-functionalized dendrimers with pea lectin are presented here and are compared to previously obtained results with Con A. Hemagglutination assays, precipitation assays, and isothermal titration microcalorimetry (ITC) studies are described.

Multivalent protein−carbohydrate interactions serve a critical function in many intercellular recognition events. A thorough understanding of their fundamental requirements is essential if therapeutic agents are to be developed that rely on protein−carbohydrate interactions. Through careful exploration of glycodendrimer interactions with legume lectins, our goal is to develop generally applicable parameters for development of therapeutic agents.

Experimental Section

General Methods. Mannose-functionalized dendrimers **1**−**6** were synthesized as previously described in ref 20. Pea lectin was isolated from commercial green split peas according to a procedure from Trowbridge.[12]

Hemagglutination Inhibition Assay. Hemagglutination assays were performed similarly to previously published procedures.[22] The concentration of pea lectin was kept at 2 μg/mL for all assays. Assay buffer consisted of 0.5% w/v BSA in 10 mM phosphate buffered saline (PBS), pH 7.2. The pea lectin was added to serial dilutions of a 20 mg/mL glycodendrimer stock solution, and the solutions were incubated for 3 h at room temperature. Rabbit erythrocytes (2% v/v in 0.5% w/v BSA) were added, and the lowest amount of dendrimer to cause inhibition was determined. Salt effects for monovalent binding of methyl mannose to pea lectin were determined for 0.15, 0.25, and 0.35 M NaCl solutions.

Isothermal Titration Calorimetry. ITC experiments were performed similarly to previously published procedures[23,24] using a microcalorimeter from Microcal, Inc. (Northhampton, MA). Injections of 6 μL of mannose-functionalized PAMAM dendrimer were added via a 250 μL syringe at an interval of 4 min into 1.435 mL of a pea lectin solution while the solution was stirred at 310 rpm. The concentration of the lectin ranged from 20 μM to 200 μM, and the sugar concentration ranged from 0.5 mM to 20 mM. Titrations were done at 27 °C in a 10 mM phosphate buffered saline solution (pH 7.2).

Precipitation Assay. Precipitation assays were performed using a modification of the procedure reported by Brewer and co-workers.[25] Serial dilutions of a 20 mM solution of mannose-functionalized G(5)-PAMAM were added to microcentrifuge tubes. A final volume of 500 μL was used. A pea lectin solution (500 μL, 60−71 mM as shown in Figure 5) was added to each tube and allowed to precipitate for ∼20 h at room temperature. The supernatant was removed after centrifugation at 5000 rpm for 5 min. The pellet was then washed three times with 500 μL of cold buffer and dissolved in 2 mM methyl α-D-mannopyranoside to a final volume of 1 mL. The solutions were then analyzed for protein content using $A_{280}^{1\%} = 15.0$ for pea lectin.[26]

Molecular Modeling. Commercially available air-drying clay was used to model both the dendrimers and the pea lectin using a scale of 1 Å = $^1/_{64}$ in. **5** was modeled as a sphere with a $^{95}/_{64}$ in. (=95 Å) diameter. Pea lectin was modeled as a cylinder with a diameter of $^{29}/_{32}$ in. (=58 Å) and a length of $^{41}/_{32}$ in. (=82 Å).

Results and Discussion

Hemagglutination Assays. Table 1 shows the relative activity of dendrimers **1**−**6** for pea lectin (concentration adjusted) compared to methyl mannose. For comparison, Con A−dendrimer affinities are also shown.[20,27] Although significant increases in affinity (on a per mannose basis) were observed with Con A as the generation of the dendrimer was increased, the affinity of the dendrimers for pea lectin remains roughly constant for the six generations of dendrimers.

Mannose-functionalized G(1)- and G(2)-PAMAMs **1** and **2** were bound to Con A with concentration-adjusted affinities comparable to that of methyl mannose, suggesting that

(21) Binding motifs are described in detail in ref 20. We define multivalent binding interactions as at least two sugars per dendrimer binding into two receptor sites on a lectin. Statistical or proximity enhancements upon affinity are smaller binding effects caused by the increased concentration of sugar near the binding site, are comparable to effective molarity phenomena of synthetic reactions, and are also discussed by Lee and Lee in ref 6.

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Table 1. Hemagglutination Inhibition Assays for Mannose-Functionalized Dendrimers[a]

		Con A		pea lectin	
	no. of sugars	inhibiting sugar concn[b] (mg/mL)	rel act./ mannose[b]	inhibiting sugar concn[c] (mg/mL)	rel act./ mannose[c]
methyl mannose	1	2.5	1	1.25	1
1	8	6.2	1	2.5	1.3
2	16	4.4 ± 0.29	1.5 ± 0.1	5	0.67
3	29	0.34 ± 0.081	20 ± 5	10	0.35
4	55	0.035 ± 0.015	200 ± 90	10	0.36
5	95	0.024 ± 0.016	300 ± 210	10	0.37
6	178	0.021 ± 0.0053	350 ± 90	10	0.38

[a] Each reported value represents at least three assays. [b] Values are taken from refs 20 (**1**, **2**) and 27 (**3**−**6**). [c] Values were consistent under the conditions used, so no ranges are reported.

monovalent binding between Con A and these dendrimers occurs. Higher generation mannose-functionalized PAMAMs, however, suggest glycoside clustering/proximity effects (G(3)-PAMAM **3**) and multivalent binding (G(4)- to G(6)-PAMAM **4**−**6**).[20,21] No increase in binding is observed with pea lectin, which suggests exclusive monovalent binding. On a per mannose basis, mannose-functionalized G(2)- to G(6)-PAMAMs **2**−**6** actually bind with lower affinity than G(1)-PAMAM **1** or methyl mannose does.

Hemagglutination assays with Con A were performed with 18 μg/mL Con A,[27] but assays with pea lectin were performed with only 2 μg/mL. The lower concentration of pea lectin was required since, for a low-affinity lectin, free lectin is always detectable in solution at higher concentrations. Using a low concentration of pea lectin eliminated the background agglutination. Inhibiting concentrations of dendrimers and methyl mannose are shown in Table 1. To allow for comparison of the results for dendrimers **1**−**6**, methyl mannose was assigned the relative activity value of 1 with each protein, even though the inhibiting concentrations of Con A with methyl mannose and of pea lectin with methyl mannose are different.

We surmise that unfavorable steric interactions between the dendrimer and pea lectin preclude bivalent binding. Con A has a concave surface that nicely compliments the roughly spherical shape of dendrimers **4**−**6**, but the area between the binding sites on pea lectin is flatter and therefore perhaps is less ideal for accommodating the dendrimer's bulk. Since pea lectin lacks the concave surface feature present in Con A, pea lectin may be unable to accommodate a multivalent binding motif for dendrimers **4**−**6**. Although they are clearly large enough to span the distance between mannose binding sites on pea lectin, **4**−**6** may be unable to bind in a bidentate fashion due to lack of shape complementarity.

A picture with the crystal structures of pea lectin and Con A superimposed upon one another reveals that the two proteins are quite similar (Figure 3). The only significant difference appears to be that Con A has larger loop regions that jut out from the protein, giving Con A a more curved shape than pea lectin between the binding sites. Although the difference seems relatively minor, such effects have previously been attributed to significant changes in binding motifs.[25] The affinity of pea lectin for methyl mannose is

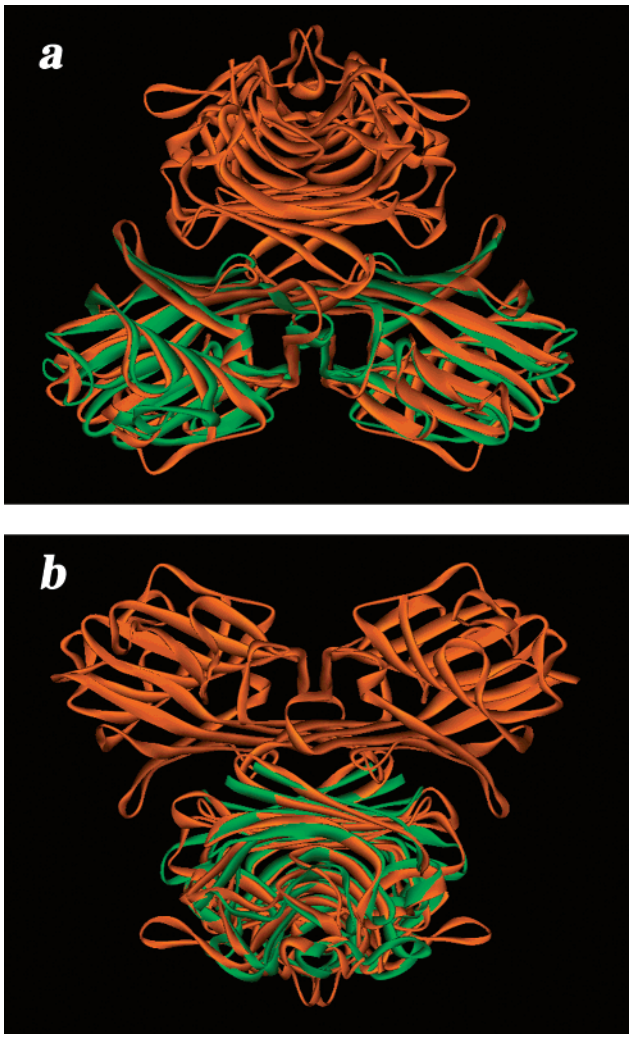

Figure 3. Pea lectin (green) and Con A (orange) structures superimposed. (a) Proteins are shown in the same orientation as in Figure 1 and (b) proteins are rotated 90°.

only about four times lower than the affinity of Con A for methyl mannose,[14] and we have previously shown that small differences in affinity do not change the binding motif from bivalent to monovalent binding for Con A−dendrimer systems.[27] Also, the pea lectin dimer and the Con A tetramers are both stable entities at neutral pH, so motifs where the pea lectin dissociates to form monomers (which are unable

to bind bivalently) seem highly unlikely.[28] Thus, it seems most likely that the monovalent binding pattern indicated by the hemagglutination assay results with pea lectin and dendrimers **4**−**6** is caused by the lack of shape complementarity between the two systems.

Titration Calorimetry Measurements. Although hemagglutination assays provide helpful information about the relative activity of the mannose-functionalized dendrimers for Con A and pea lectin, they do not provide association constants or energetics of binding. Isothermal titration calorimetry (ITC) can provide valuable information about the number of binding sites (n), the enthalpy of binding (ΔH), and the association constant (K_a), from which the free energy of binding (ΔG) and the entropy of binding (ΔS) can be calculated. The binding of monosaccharides[14,24] and small mannose-functionalized multivalent frameworks[29] to Con A and pea lectin has been previously reported, which led us to hypothesize that ITC might be used for binding studies with our dendrimers **1**−**6** as well.

Unfortunately, aggregation was repeatedly observed upon addition of the mannose-functionalized dendrimers **1**−**6** to Con A (results not shown). Although pea lectin has a significantly lower affinity for methyl mannose and causes less aggregation than Con A, precipitation was still observed. Figure 4 shows a representative experiment during which minimal aggregation was observed. While an overall sigmoidal curve was obtained, a significant amount of noise is present. This is partially due to the relatively low amounts of protein used. In ITC measurements, the quantity $c = K_a M_t(0)$, where $M_t(0)$ is the initial macromolecule concentration, is important for optimum curve fitting to be achieved.[23] However, accurate ITC measurements require the presence of soluble complexes for the duration of the experiment. In order to minimize aggregation, a lower than optimal macromolecule concentration was required, which resulted in a low signal. The larger amount of variability at the beginning of the titration is most likely due to the formation of an aggregate. Because of the formation of an aggregate, the thermodynamic data obtained from the titration cannot be analyzed in detail.

Precipitation Assays. To further investigate aggregation behavior, precipitation assays were performed with pea lectin and dendrimers **1**−**6**. By treating varying concentrations of dendrimers with a constant amount of protein, the dendrimer−lectin binding stoichiometry is obtained.[25] If the concentration of pea lectin is high enough, the dendrimer will precipitate the protein. The ratio of dendrimer to protein at the point of maximum precipitation is considered the maximum number of pea lectins that are recruited by each dendrimer.

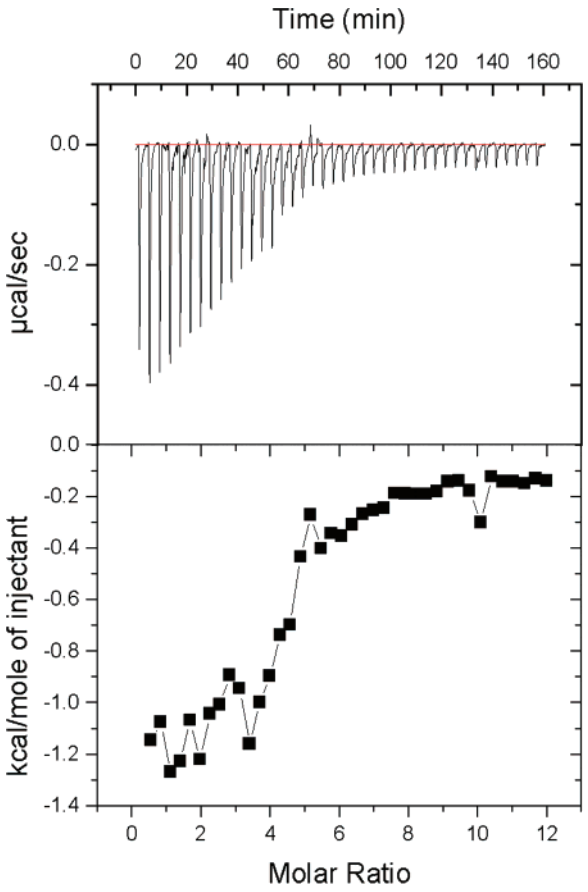

Figure 4. ITC profile of pea lectin (0.030 mM) with **5** (2 mM in sugar) at 27 °C. Top: Data obtained for 40 automatic injections. Bottom: Integrated curve showing experimental points and the best fit.

The ratio of mannose-functionalized dendrimer to pea lectin in the precipitates quickly increased with increasing amount of dendrimer until a maximum was reached, and then the ratio steadily decreased (Figure 5). This behavior was different from our previously obtained results with Con A, where the ratio of protein to dendrimer remained fairly constant after a maximum had been reached.[27] When the assay was performed in water, as opposed to buffer, results resembled those for Con A. This change in behavior was likely caused by a change in the ionic strength of the solution.

Similar effects were observed when Okada and co-workers studied the behavior of dendrimer−ion complexes.[30] Although Okada's study did not directly involve protein−carbohydrate interactions, parallels can be drawn for multivalent receptor−ligand interactions. Presumably, the dendrimer−protein complex became more soluble at high ionic strength due to the screening effect of microsalts weakening the binding interaction.[31] Such a screening effect

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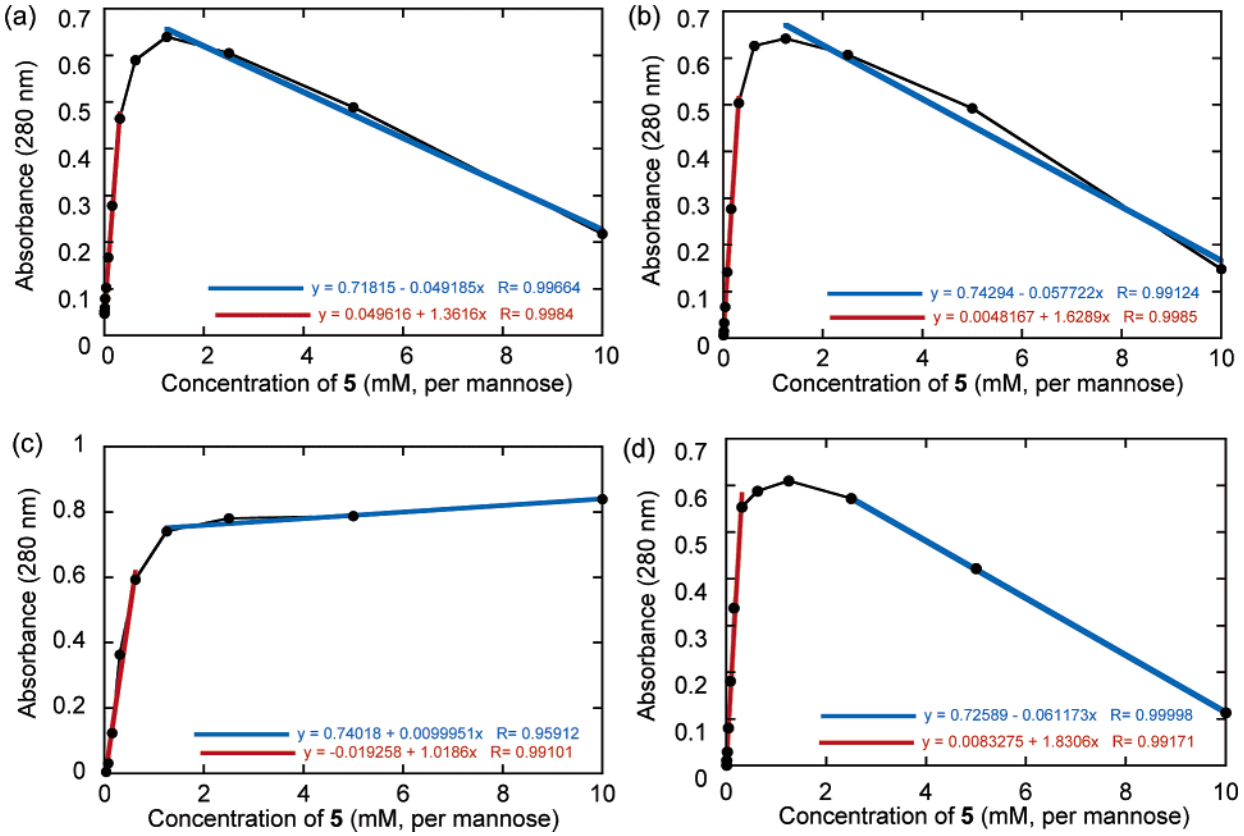

Figure 5. Precipitation assays in (a) 10 mM PBS, pH 7.2, with addition of 67 μM pea lectin; (b) 0.1 M TBS, pH 7.4, with addition of 71 μM pea lectin; (c) water, with addition of 71 μM pea lectin; and (d) 0.15 M NaCl, with addition of 60 μM pea lectin.

could occur if salts interfere with electrostatic interactions and hydrogen bonding in the binding pocket, which would increase the off-rate for complex dissociation and would disrupt the larger cross-linked complexes. The formation of soluble protein−dendrimer complexes in the presence of high concentrations of dendrimer, as well as at low ligand concentrations, is possible if the ionic strength of the solution is sufficiently high, because only small aggregates would form under these conditions. Near the equivalence point, insoluble complexes are formed in extensive lattices.[5]

The precipitation assay results reported here are consistent with the aggregation behavior that was observed in ITC experiments, where the most noise occurs as the experiment approaches the maximum dendrimer to protein stoichiometry. That the salt content influences sugar binding by pea lectin was also shown by comparing hemagglutination assays with methyl mannose in buffers containing 0.15 and 0.35 M NaCl. Increasing the salt content from 0.15 to 0.35 M caused a 1.3-fold increase in the amount of methyl mannose required to inhibit agglutination. The results with methyl mannose suggest that a high salt content in the assay buffer can lower the affinity of the protein for mannose, which might disrupt the protein−dendrimer cross-links and reduce the amount of precipitate observed in the precipitation assays.

The precipitation profiles in Figure 5 show that, for a mannose-functionalized G(5)-PAMAM dendrimer **5**, precipitation steadily increases, reaches a maximum, and then steadily decreases in high ionic strength buffers (Figure 5a,b,d). In water, the precipitation profile levels off (Figure 5c). The points of intersection of the initial slope and the final slope are similar for all of the buffers tested, corresponding to an approximately 1:7 ratio of dendrimer **5** to pea lectin monomer.

Physical models suggest that the dendrimer could accommodate 11 pea lectin dimers, assuming bivalent binding. While the dendrimer:pea lectin ratio could theoretically arise from an average of bivalently binding three or four pea lectins, we suggest that extensive cross-linking is occurring, which is greatly weakened by the salting effects, thus forming a soluble complex at both high and low concentrations of dendrimer.[30,31]

Overall, the precipitation assays seem to suggest that even a large dendrimer such as **5**, which is theoretically able to span binding sites 65 Å apart and experimentally was shown to bind multivalently to Con A, probably binds pea lectin in a monodentate fashion in high ionic strength buffers. This is consistent with the results we obtained from the hemagglutination assays. The lack of shape complementarity between pea lectin and **5** may make multivalent binding less likely than with Con A; large cross-linking complexes probably occur more readily, which high ionic strength

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buffers can minimize. The shape complementarity between Con A and dendrimer is not present with pea lectin, and steric hindrance when binding dendrimer **5** to pea lectin may prevent multivalent binding motifs from occurring. Brewer and co-workers have previously presented a similar steric argument, in that the binding valency of a glycoprotein (which can be compared to our mannose-functionalized dendrimers) is influenced by the quarternary structures of the lectin and the interacting glycoprotein.[25]

Conclusions

The hemagglutination assays and precipitation assays described here suggest that mannose-functionalized PAMAM dendrimers **1**−**6** bind to pea lectin in a monovalent fashion, which contrasts with our previous results obtained using Con A. Hemagglutination assays for **1**−**6** with pea lectin showed no increase in binding affinity (on a per mannose basis) with increasing dendrimer generation. Previous experiments with Con A revealed significant increases in binding affinity as the dendrimer size increased, and the affinity changes were attributed to multivalent binding and glycoside clustering. Unfortunately, kinetic data for pea lectin−dendrimer binding could not be obtained via ITC due to aggregation. Precipitation profiles suggested monovalent binding of pea lectin by all dendrimers in high ionic strength buffers and in water.

Size and shape complementarity of the carbohydrate-functionalized dendrimers with the lectin appears to influence the multivalent (Con A−dendrimer) versus monovalent (pea lectin−dendrimer) binding motif. While we do not rule out the possibility that highly dynamic frameworks such as PAMAM dendrimers can undergo conformational shifts to overcome unfavorable steric repulsions and to achieve bivalent binding interactions, the unfavorable shape complementarity in the dendrimer−pea lectin system appears to induce monovalent association. The association is relatively weak (a micromolar dissociation constant is reported for pea lectin with methyl mannose),[14] and so higher affinity bivalent binding evidently cannot occur by overcoming the unoptimized shape complementarity in this case.

The comparative binding behaviors of the legume lectins, Con A and pea lectin, to carbohydrate-functionalized dendrimers **1**−**6** indicate that glycodendrimers may be very appropriate macromolecular therapeutic agents for targeted multivalent binding with some lectins but may be unsuccessful in other cases. The shape complementarity of the lectin with the carbohydrate-coated framework must be considered when dendrimer based systems are applied.

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