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# The Lectin-Binding Properties of Six Generations of Mannose-Functionalized Dendrimers

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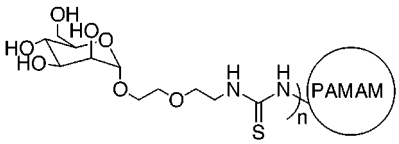
## ABSTRACT




Mannose functionalized
G(1)- to G(6)-PAMAM

**First- through sixth-generation PAMAM dendrimers have been functionalized with mannose residues. Characterization with MALDI-TOF MS and $^1$H NMR is reported. Different binding enhancements consistent with monovalent interaction, glycoside clustering, and multivalent binding are observed for different generations of dendrimers.**

Polymeric biomaterials that are designed to investigate and control specific cell behavior are becoming increasingly desirable for applications in drug delivery and tissue engineering.[1] Because protein−carbohydrate interactions have been implicated in a wide variety of intercellular recognition events, a clear understanding of the details of the requirements for this interaction is intensely sought.[2] Under physiologically relevant conditions, it is generally accepted that adhesion of lectins to saccharides on the surface of a cell involves multipoint attachment.[3] To mimic this motif, a variety of glycopolymers have been developed.[4] Saccharide-functionalized dendrimers capable of glycoside clustering have been previously reported, but until recently studies with dendrimers large enough to span multiple lectin binding sites (i.e., to bind multivalently) had not been reported.[5,6] In a recent example, generation one (G(1)-) through generation five (G(5)-) lactose-functionalized dendrimers were studied to evaluate how the topology of binding site presentation and ligand display effect binding selectivity.[7]

Here, we have optimized our model system so that multivalent binding (the ability of one dendrimer to bind to multiple lectin binding sites) should be facile for large saccharide-functionalized dendrimers but unlikely for small dendrimers. We have chosen concanavalin A (Con A) as our reference lectin because two mannose binding sites are located about 65 Å apart on one side of the protein, such that a large dendrimer should be able to bind simultaneously to two binding sites.[8] In this paper, we report a systematic

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study of mannose-functionalized G(1)- through G(6)-PAMAM dendrimers with Con A.

We observe significant differences in affinity on the basis of the size of the polymer, which we suggest is due to monovalent binding, glycoside clustering, and multivalent binding motifs. Glycoside clustering has been previously defined as "affinity enhancement achieved by multivalent ligands over monovalent ones that is greater than would be expected from a simple effect of concentration increase".[9] For the discussion in this paper, we adopt this definition of glycoside clustering but apply it in a narrower context than it is sometimes used in the carbohydrate literature.[10] Namely, we define multivalent binding (the ability of one dendrimer to bind to multiple lectin binding sites) and glycoside clustering (a ligand concentration effect) as two related but distinct terms. These definitions are shown pictorially in Figure 1. To our knowledge, this is the first time that studies with saccharide-functionalized sixth-generation dendrimers have been reported.

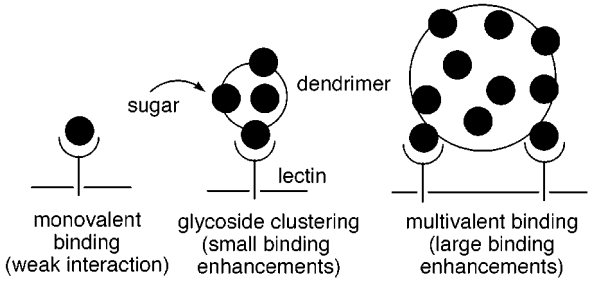
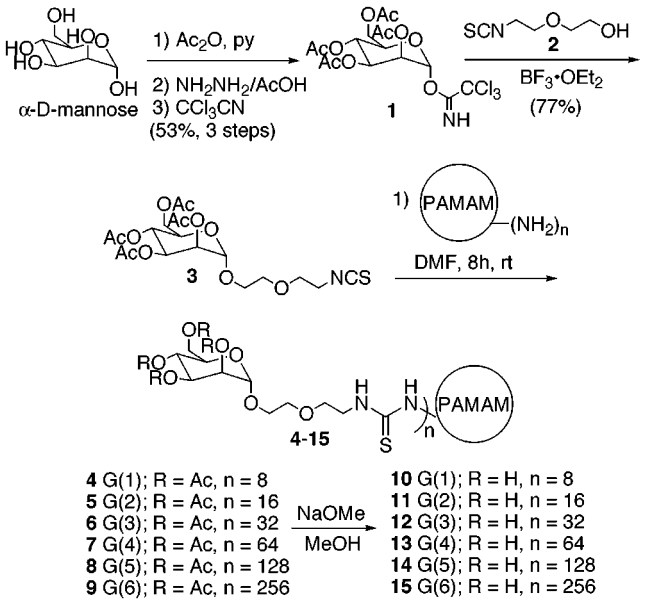



**Figure 1.** The three likely binding motifs and their expected relative activities for the interaction of glycodendrimer with Con A.

Mannose-functionalized dendrimers **10**−**15** were synthesized as shown in Scheme 1. Peracetylation of D-mannose followed by selective deprotection and activation at the anomeric position afforded trichloroacetimidate **1**.[11] Coupling of **1** with the isothiocyanato alcohol **2**[12] using $BF_3{\cdot}OEt_2$ gave the mannose monomer **3**. Addition of **3** to the dendrimer followed by global deacetylation gave dendrimers **10**−**15**. Dialysis (water/cellulose tube, MW cutoff 1000 g/mol) afforded **10**−**15** in purified form.

Although the acetyl protecting groups are not required during thiourea formation, we have chosen to deprotect the sugars after addition to the dendrimer. This is because the acetyl groups give diagnostic signals in the $^1$H NMR spectra and because we can check the molecular weight (MALDI-TOF MS) of our products both before and after deacetylation (vide supra).[13]

**Scheme 1.** Synthesis of Mannose-Functionalized Dendrimers




The $^1$H NMR (500 MHz) spectra of acetyl-protected mannose-functionalized dendrimers **4** and **9** (theoretical MWs 3,902 and 137,152 g/mol respectively) in $d_6$-DMSO are shown in Figure 2. For **4**−**9**, the amide protons from the interior of the dendrimer are the peaks that are farthest downfield, and the peak at 7.5 ppm is the thiourea NH signal. The relative integrations of these signals suggest that a high degree of surface functionalization has been achieved. For example, in **4** there are 12 amide and 16 thiourea protons, so a 1:1.33 ratio of peaks is expected. Since the observed ratio is 1:1.25, this suggests that 96% of the possible functionalization occurred. In all cases, ≥90% functionalization is indicated by NMR. Unfortunately, the MALDI-TOF MS results suggest a lower degree of surface loading.

The MALDI-TOF MS of mannose-functionalized dendrimers **10** and **15** and of the starting G(1)- and G(6)-PAMAMs are shown in Figure 3. For G(4)- to G(6)-PAMAMs, the measured molecular weights of the dendrimers were lower than the theoretical values.[14] Subtraction of the experimentally determined molecular weight of the PAMAM starting materials from the molecular weight of **4**−**15** and division of the remainder by 477 (molecular weight of **3**) or by 309 (molecular weight of deacetylated **3**) indicates that

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(10) For a broader definition of the glycoside cluster effect, see: Lee, Y. C.; Lee, R. T. *Acc. Chem. Res.* **1995**, *28*, 322−327.

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(12) Synthesized as described in the Supporting Information from 2-(2-aminoethoxy)ethanol with thiophosgene, 70% yield.

(13) Our synthesis procedure is similar to our previously reported route using *p*-isothiocyanatophenyl-α-D-mannopyranoside, except that the aromatic ring in the linkage to the dendrimer was eliminated to improve solubility in aqueous media: Woller, E. K.; Cloninger, M. J. *Biomacromolecules* **2001**, *2*, 1052−1054.

(14) Tomalia et al. have reported that the average molecular weights of the PAMAMs are actually smaller than the theoretical molecular weights; our MALDI results are consistent with Tomalia's electrospray MS results: Tolic, L. P.; Anderson, G. A.; Smith, R. D.; Brothers, H. M.; Spindler, R.; Tomalia, D. A. *Int. J. Mass Spec. Ion Processes* **1997**, *165/166*, 405−418.

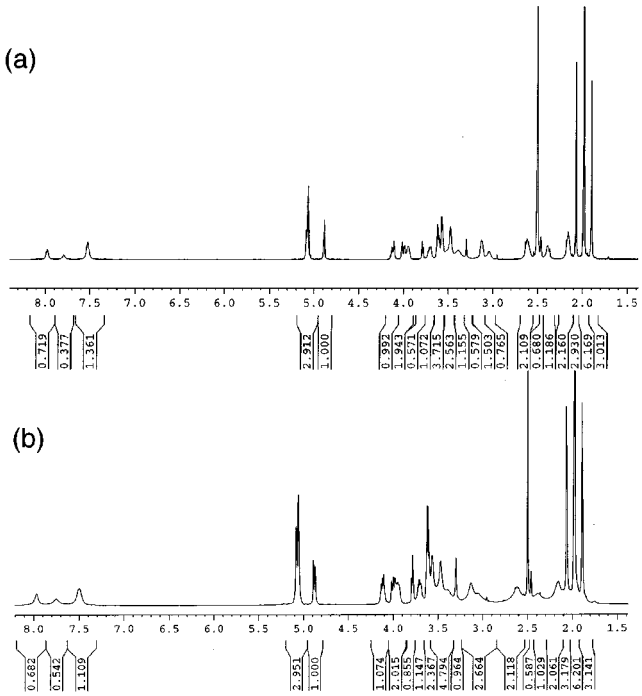


**Figure 2.** $^1$H NMR spectra (500 MHz) of (a) **4** and (b) **9**.

the percent incorporation of mannose residues is 100% for G(1)- and G(2)-PAMAMs, 92% for G(3)-PAMAM, and 84%, 73%, and 67% for G(4)-, G(5)-, and G(6)-PAMAM, respectively (Table 1).

The simplest explanation for the discrepancy between the NMR and the MALDI-TOF MS determinations of percent

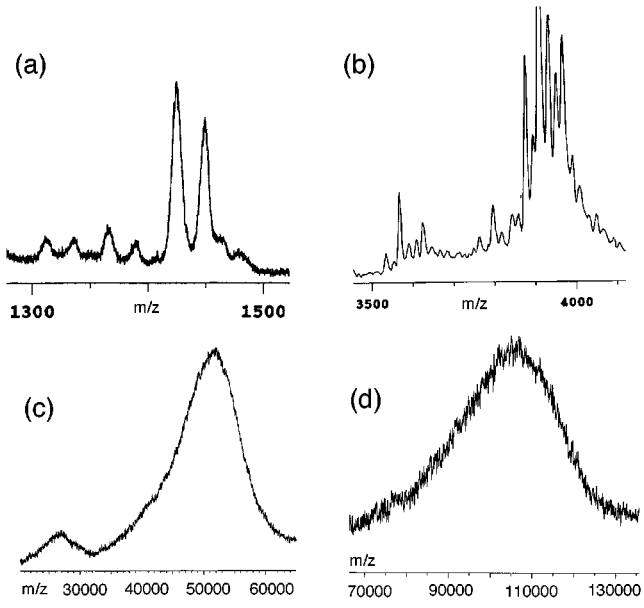


**Figure 3.** MALDI-TOF MS of (a) G(1)-PAMAM, (b) **10**, (c) G(6)-PAMAM, (d) **15**.

**Table 1.** MALDI-TOF MS Determination of Mannose Functionalization of G(1)- to G(6)-PAMAMs. Experimentally Determined $M_W$ Values Are Given in Parentheses

| gen. | theor. no. amines[a] (MALDI MW) | no. sugars[b] (MALDI of **4**−**9**) | no. sugars[c] (MALDI of **10**−**15**) | ave % loading |
|---|---|---|---|---|
| 1 | 8 (1430)[d] | 8 (5280)[d] | 8 (3877)[d] | 100 |
| 2 | 16 (3260) | 16 (10960) | 16 (8250) | 100 |
| 3 | 32 (6910) | 30 (21000) | 29 (15930) | 92 |
| 4 | 64 (13500) | 54 (39300) | 55 (30620) | 84 |
| 5 | 128 (25500) | 92 (69600) | 95 (55000) | 73 |
| 6 | 256 (50800) | 173 (133500) | 172 (103800) | 67 |

[a] PAMAM starting material. [b] no. sugars$_{\mathbf{4-9}}$ = (MW$_{\mathbf{4-9}}$ − MW$_{G(1)-G(6)}$)/477. [c] no. sugars$_{\mathbf{10-15}}$ = (MW$_{\mathbf{10-15}}$ − MW$_{G(1)-G(6)}$)/309. [d] g/mol.

loading is that, because of the high degree of symmetry present in PAMAMs, NMR does not identify structural defects as well as MALDI-TOF MS. If we assume loss of units of 114 (the mass of one missing terminal $CH_2CH_2$-$CONHCH_2CH_2NH_2$ unit) as the predominant defect present in the starting materials,[12] then our results indicate that at least 90% of the amines are functionalized. We suggest that the NMR and MALDI-TOF results indicate that a high degree of surface functionalization is occurring, but that defects in the PAMAMs preclude higher sugar loading.

After demonstrating the feasibility of synthesizing water-soluble mannose-functionalized G(1)- through G(6)-dendrimers, we evaluated their relative activities with Con A by performing hemagglutination assays. Although hemagglutination assays do not provide information regarding binding affinity,[15] their use to measure inhibition of protein−carbohydrate interactions is well documented and gives us an essential entry-level comparison of our system to other glycopolymers.[16] In the article discussing activity vs affinity and the use of the hemagglutination assay, Toone and coworkers note that "in many respects hemagglutination assays are a far more relevant measure of activity than are assays designed exclusively to evaluate protein−carbohydrate binding".[15]

Erythrocytes (rabbit) were added to preincubated solutions of Con A and varying concentrations of dendrimer. The lowest amount of dendrimer that caused inhibition of agglutination was determined.[17] The results are shown in Table 2. Each value represents at least three assays. The large standard deviation occurs because, while the overall trends

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(17) A detailed experimental protocol is provided in the Supporting Information. Although precipitation has been observed in previous studies of Con A binding to mannose-functionalized dendrimers (ref 5b), our assays were performed at 30-fold lower concentrations, and no precipitation is observed.

**Table 2.** Hemagglutination Assays for **10**−**15** with Con A

| cmpd | no. sugars | rel activity/sugar |
|---|---|---|
| methyl mannose | 1 | 1 |
| **10** | 8 | 1 |
| **11** | 16 | 1.5 ± 0.1 |
| **12** | 29 | 45 ± 25 |
| **13** | 55 | 275 ± 95 |
| **14** | 95 | 510 ± 295 |
| **15** | 172 | 660 ± 230 |

are always the same, different blood sources (different rabbits on different days) give different numbers.

When compared to the control monomer methyl mannose, dendrimers **10** and **11** did not show any increase in activity toward Con A. Thus, we surmise that **10** and **11** bind monovalently. Either clustering or monovalent binding was expected, since the lower generation dendrimers are too small to span multiple binding sites (i.e., to bind multivalently). In addition, our findings indicate that **12** (G(3)-PAMAM core) binds roughly 1 order of magnitude better than **10**, **11**, or methyl mannose. This is suggestive of a glycoside clustering motif. As with **10** and **11**, **12** is too small for multivalent binding to occur.[18] Dendrimers **13**−**15** all show increases in activity toward Con A (relative to methyl mannose) of 2 orders of magnitude, indicating that multivalent binding (Figure 1) is occurring.[19]

Molecular mechanics calculations (Macromodel V. 6.5, MM2*) suggest that reaction of **3** with the dendrimer will add a maximum of about 13 Å to the radius of the molecule. Thus, using published radii for the original PAMAMs (G(4)-PAMAM radius ∼22.5 Å, G(5)-PAMAM radius ∼27 Å, G(6)-PAMAM radius ∼ 33.5 Å),[20] we can calculate the expected approximate area that each endgroup would occupy on the dendrimer surface. Assuming **13**−**15** are spherical,[6] the area available to the endgroups on **15** is considerably smaller than the area available to endgroups on **13**. We postulate that, although all the larger dendrimers apparently bind multivalently, some may be better at glycoside clustering than others.[21]

Preliminarily, we suggest that the results with **13**−**15** indicate that the interplay between glycoside clustering and multivalent binding (Figure 1) may have important effects on lectin binding interactions. We are currently synthesizing dendrimers of the same generation with varying concentrations of sugars to further evaluate glycoside clustering vs multivalent binding motifs (to be published separately).

In summary, we have synthesized and characterized mannose-functionalized G(1)- to G(6)-PAMAMs **10**−**15**. Their relative activities toward Con A were evaluated using the hemagglutination assay. Depending on the size of the dendrimer framework, monovalent, glycoside clustering, and multivalent binding motifs were observed. The variety of binding motifs available for saccharide-functionalized dendrimers makes these compounds attractive polymeric biomaterials for the study of protein−carbohydrate interactions.

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**Supporting Information Available:** Experimental procedures and characterization data for **2**−**15** and experimental procedures for MALDI-TOF MS and hemagglutination. This material is available free of charge via the Internet at http://pubs.acs.org.

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(18) The relative activity results for **12** are same order of magnitude as those obtained with ELLA assays in ref 5b. Differences in binding activities for **10**−**12** (compared to similar compounds in ref 5b) may be caused by changes in the linker between mannose and the PAMAM.

(19) It is also possible that the change in shape from circular (G(1)- to G(3)-PAMAM) to spherical (G(4)- to G(6)-PAMAM) causes the observed binding enhancement. Studies with heterogeneously functionalized dendrimers are underway to address this issue.

(20) (a) Uppuluri, S.; Keinath, S. E.; Tomalia, D. A.; Dvornic, P. R. *Macromolecules* **1998**, *31*, 4498−4510. (b) Li, J.; Piehler, D.; Qin, J. R. B., Jr.; Tomalia, D. A. *Langmuir* **2000**, *16*, 5613−5616.

(21) Our dendrimer results (and our analysis) are in good agreement with a previous study of polymer length on binding affinity: Kanai, M.; Mortell, K. H.; Kiessling, L. L *J. Am. Chem. Soc.* **1997**, *119*, 9931−9932.