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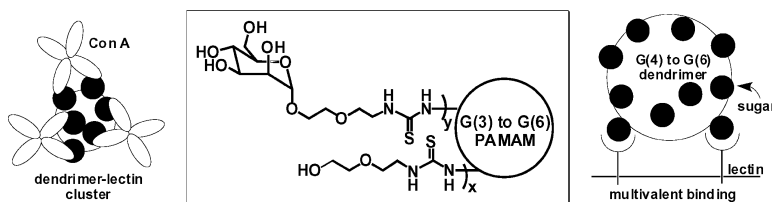
Altering the Strength of Lectin Binding Interactions and Controlling the Amount of Lectin Clustering Using Mannose/Hydroxyl-Functionalized Dendrimers

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Altering the Strength of Lectin Binding Interactions and Controlling the Amount of Lectin Clustering Using Mannose/Hydroxyl-Functionalized Dendrimers

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Abstract: Protein−carbohydrate interactions play a critical role in many biological recognition events. Multivalent therapeutic agents that utilize protein−carbohydrate interactions have proven difficult to design, primarily because the fundamental requirements of protein−carbohydrate interactions are not well understood. Here, we report a systematic study of the effect on lectin binding of varying the loading of mannose surface residues on generations three through six PAMAM dendrimers. The degree of mannose functionalization was controlled by stoichiometric addition, and dendrimers were characterized using NMR and MALDI-TOF MS. Hemagglutination assays and quantitative precipitation assays were performed to determine the relative activity of the dendrimers. Using the mannose/hydroxyl-functionalized dendrimers reported here, we could systematically control both the degree of lectin clustering and the overall activity of the lectin with the dendrimer.

Introduction

The interaction between proteins and carbohydrates is critical in a wide variety of intercellular interactions. For example, viral and bacterial infections, antibody−antigen interactions, fertilization, and other cell−cell interactions all rely on protein−carbohydrate interactions.[1] The interaction of one carbohydrate with one protein, however, is quite weak. In the case of binding between hemagglutinin and monomeric α-sialosides, the association constant is only about 10^3 M^{-1}.[2] It is generally accepted that biological systems engage in multipoint attachment to augment the activity of the individual protein−carbohydrate interactions. Remarkably, the strength of multivalent protein−carbohydrate interactions is typically more than additive.[3] The advantage gained by a ligand when it presents multiple copies of a binding epitope to a receptor was first described by Lee and co-workers and is called the glycoside cluster effect.[1d,4] In most biological systems, this advantage is greater than would be expected simply due to the additive increase in the number of binding epitopes. While the glycoside cluster effect has been recognized for some time, the underlying cause for the enhancement in binding is still unclear.[3,5]

A number of synthetic multivalent ligands have been reported that take advantage of the glycoside cluster effect to act either as inhibitors or as effectors.[3,6−10] However, an empirical approach (i.e., trial and error) to the development of multivalent ligands has been necessary because the requisite underlying details of multivalent protein−carbohydrate interactions are not

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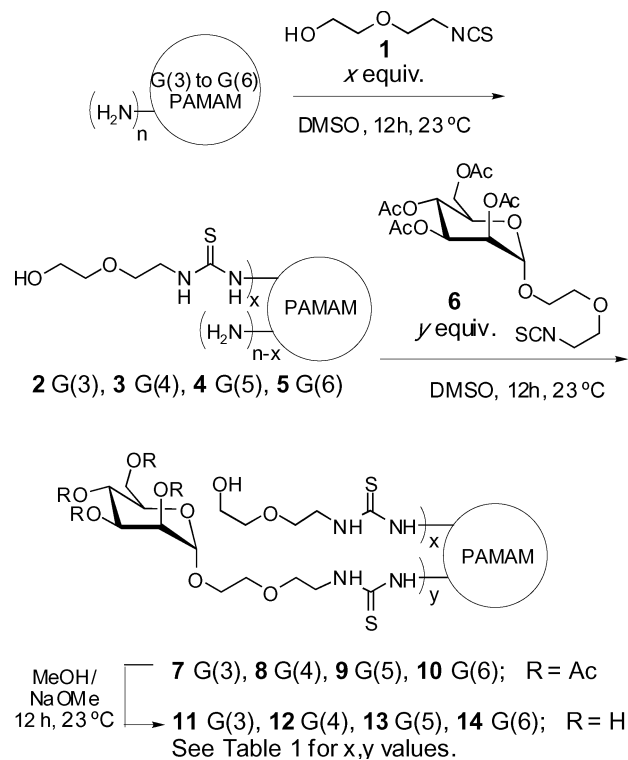
well understood. Presentation of multivalent carbohydrate ligands to various receptors has been achieved using a number of scaffold systems including glycoproteins[7] and linear polymers.[8] Dendrimers have been used extensively as scaffolds in recent years,[9] and many examples of saccharide-functionalized dendrimers have been reported.[10] However, these dendrimers are typically not large enough to simultaneously bind to multiple binding sites.

In a recent example, Roy et al. reported the lactose functionalization of a generation five poly(amidoamine) (PAMAM) dendrimer to study how the topology of binding site presentation and ligand display affects binding selectivity.[10g] Okada and co-workers reported the galactose and *N*-acetylglucose functionalization of PAMAM generations six, seven, and eight to study the effect that sugar density had on the ability of the dendrimer to recognize and encapsulate a naphthalenesulfonate salt, as well as the ability of these dendrimers to inhibit wheat germ agglutinin.[10h] Recently, we described the complete functionalization of generations one (G(1)) through six (G(6)) PAMAM dendrimers with mannose.[10i] Relative to methyl mannose, significant enhancements in binding toward Concanavalin A (Con A) were observed with the larger dendrimers because the larger dendrimers are capable of spanning multiple binding sites on Con A while the smaller dendrimers cannot.

While the study of fully loaded, saccharide-functionalized dendrimers provides valuable information regarding multivalent binding, varying the number of sugars on the surface should provide even more information. A number of systems have been reported that vary the loading of binding epitopes.[6a,7c,11] In general, the most highly functionalized scaffolds are not the ones with the highest activity. Often, a less functionalized ligand exhibits the greatest activity.

Burke, Kiessling, and co-workers recently reported a small trimannoside macromolecule that induced formation of a soluble lectin cluster. The authors made the point that "complex carbohydrate side chains of glycoproteins may induce lectin clustering at the cell surface." This lectin clustering might facilitate processes such as cell signaling.[12] Multivalent carbohydrate-bearing scaffolds may ultimately be used to control lectin clustering and to increase the affinity of the lectin for the carbohydrates.

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Scheme 1. The Synthesis of Mannose/Hydroxyl-Functionalized Dendrimers

We have synthesized a series of mannose- and hydroxyl-functionalized G(3)−G(6)-PAMAM dendrimers to systematically study the effect of carbohydrate loading on the activity of the dendrimer for a lectin. For any generation of dendrimer, the number of sugars present can be changed without significantly changing the size of the dendrimer. On the other hand, by using different generations of PAMAM dendrimers, we can vary the size of the molecule. As is demonstrated below, changing the size of the dendrimer affects the number of lectins that can be bound by the dendrimer. Controlling the amount of mannose on the dendrimer surface controls the relative activity of the dendrimers for the lectin. Thus, using the mannose/hydroxyl-functionalized dendrimers that we report here, we could systematically change both the degree of lectin clustering and the overall activity of the lectin with the dendrimer.

Results

The general procedure for the heterogeneous functionalization of PAMAM dendrimers is shown in Scheme 1. A DMSO solution of commercially available PAMAM dendrimer (generations three through six) and an appropriate volume of a DMSO solution of **1**[10i] were combined and stirred overnight to form partially functionalized dendrimers **2**−**5**. An appropriate volume of a DMSO solution of **6**[10i] corresponding to *y* equivalents was added to the reaction and stirred overnight to form fully functionalized (acetyl protected) dendrimers **7**−**10**. Dendrimers **7**−**10** were purified by dialysis in DMSO (cellulose, M_w cutoff 1000 g/mol), lyophilized, and globally deprotected using Zemplén conditions in 1:1 H_2O:MeOH to afford dendrimers **11**−**14**. The products were purified by dialysis against water and lyophilized. Table 1 shows a summary of the MS data and of

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**Table 1.** Summary of Characterization Data for Dendrimers **7**−**14**

M_w (Da) **7**−**10**[a]	M_w (Da) **11**−**14**	number of sugars[b]
11 590 (**7a**)	10 900 (**11a**)	4
12 700 (**7b**)	11 800 (**11b**)	6
14 100 (**7c**)	12 050 (**11c**)	12
15 100 (**7d**)	12 600 (**11d**)	15
17 080 (**7e**)	13 600 (**11e**)	21
18 370 (**7f**)	14 300 (**11f**)	25
21 000 (**7g**)	15 930 (**11g**)	29
22 300 (**8a**)	21 220 (**12a**)	7
23 700 (**8b**)	22 340 (**12b**)	8
24 100 (**8c**)	22 500 (**12c**)	9
24 800 (**8d**)	22 500 (**12d**)	13
25 800 (**8e**)	23 300 (**12e**)	15
26 200 (**8f**)	23 100 (**12f**)	18
27 900 (**8g**)	24 500 (**12g**)	21
28 500 (**8h**)	23 800 (**12h**)	27
31 600 (**8i**)	26 600 (**12i**)	30
31 950 (**8j**)	26 600 (**12j**)	31
30 500 (**8k**)	25 000 (**12k**)	32
32 900 (**8l**)	27 100 (**12l**)	35
32 000 (**8m**)	25 200 (**12m**)	37
35 000 (**8n**)	28 100 (**12n**)	40
37 000 (**8o**)	29 100 (**12o**)	46
38 400 (**8p**)	30 000 (**12p**)	51
39 000 (**8q**)	30 600 (**12q**)	55
42 800 (**9a**)	42 750 (**13a**)	4
41 500 (**9b**)	40 100 (**13b**)	8
47 300 (**9c**)	45 000 (**13c**)	17
51 800 (**9d**)	47 400 (**13d**)	33
58 100 (**9e**)	50 600 (**13e**)	51
59 700 (**9f**)	48 100 (**13f**)	67
64 500 (**9g**)	54 200 (**13g**)	74
69 400 (**9h**)	55 600 (**13h**)	86
73 600 (**9i**)	58 500 (**13i**)	95
79 200 (**10a**)	77 400 (**14a**)	11
81 000 (**10b**)	78 000 (**14b**)	18
84 400 (**10c**)	80 100 (**14c**)	26
86 400 (**10d**)	80 100 (**14d**)	34
87 900 (**10e**)	81 000 (**14e**)	40
93 200 (**10f**)	83 100 (**14f**)	52
99 200 (**10g**)	87 100 (**14g**)	73
106 500 (**10h**)	91 000 (**14h**)	95
106 300 (**10i**)	89 500 (**14i**)	100
111 000 (**10j**)	93 900 (**14j**)	102
114 000 (**10k**)	96 000 (**14k**)	122
122 900 (**10l**)	97 700 (**14l**)	146
126 000 (**10m**)	99 900 (**14m**)	151
132 000 (**10n**)	101 200 (**14n**)	178

[a] M_w is the weight average molecular weight. [b] Number of sugars = [M_w (**7**−**10**) − M_w (**11**−**14**)]/172 (172 = mass of four acetyl groups), or number of sugars = [M_w (**7**−**10**) − M_w (**2**−**5**)]/477 (477 = mass of **6**; M_w (**2**−**5**) data are in the Supporting Information), or number of sugars = [M_w (**11**−**14**) − M_w (**2**−**5**)]/306 (306 = mass of deacetylated **6**). Wherever possible, results from these three calculations are averaged.

the average number of mannose surface residues for each generation of dendrimer. Reversing the order of the addition of **1** and **6** caused no change in the desired ratio of **1**:**6** in the product, implying that **1** and **6** have comparable reactivities.

Representative MALDI-TOF mass spectra are shown in Figure 1.[13] Partially functionalized dendrimers **3**−**5** and fully functionalized dendrimers **7**−**10** were evaluated by MALDI-TOF MS. Comparison of these values to the molecular mass data of **11**−**14** and to the unfunctionalized PAMAMs allowed us to determine the number of sugars on the surfaces of the dendrimers. The MALDI peaks are broadened and are at lower

(13) For an excellent example of the use of MALDI-TOF for dendrimer characterization, see: Krska, S. W.; Seyferth, D. *J. Am. Chem. Soc.* **1998**, *120*, 3604 and references therein.
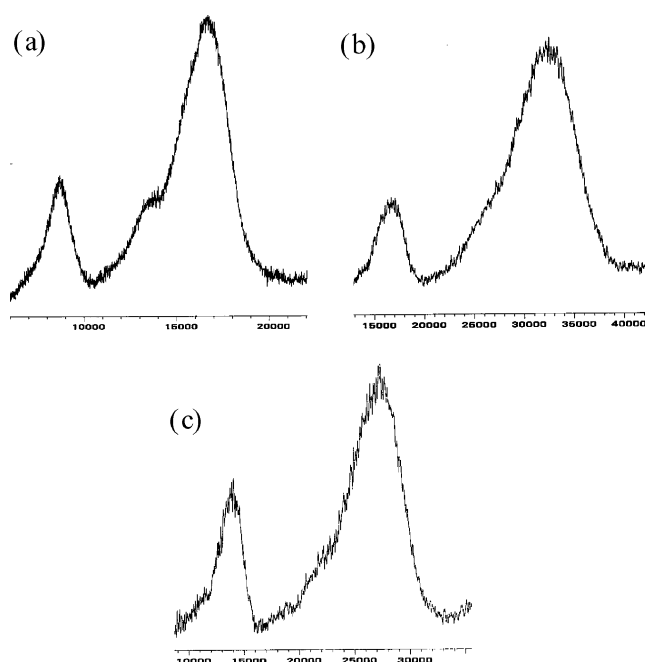
Figure 1. MALDI-TOF MS for (a) **3l**, (b) **8l**, and (c) **12l**.

mass values than the theoretical values because reactions toward the synthesis of the PAMAM starting material do not all give 100% yields. The loss of terminal $CH_2CH_2CONHCH_2CH_2NH_2$ units and intramolecular bis-amide formation (using 1 equiv of ethylenediamine) rather than intermolecular addition of 2 equiv of ethylenediamine are known to occur.[14] However, the polydispersities of the compounds do not increase to any appreciable amount upon reaction with either **1**, **6**, or both, strongly suggesting that the observed peak broadening is a function of the starting material and not of the reaction sequence. In all cases, the polydispersities of the dendrimers were less than 1.02.[15,16]

Although the acetylated products **7**−**10** were not appreciably soluble in water or methanol, the dendrimers **11**−**14** with deacetylated hydroxyl groups on mannose were water soluble unless the ratio of **1**:**6** was above 9:1. While protection of the mannose hydroxyl groups is not necessary during thiourea formation, it proved advantageous to leave the acetyl groups on the sugars because the acetyl groups provide sharper NMR signals than do any signals on the deprotected dendrimer. The ratio of **1**:**6** on the surface of the dendrimer was determined by integration of appropriate peaks in the ^{1}H NMR spectrum to compliment the MS data in Table 1. Figure 2a shows the NMR of peracetylated **8l**. The broad peak at 4.56 ppm is from the OH group from the hydroxyethanol unit. The peaks at ∂ 5.07, 4.88, 4.11, 4.00, and 3.95 ppm correspond to the protons on

(14) (a) Tomalia et al. have reported that the average molecular weights of PAMAM dendrimers are smaller than the theoretical weights; our MALDI results are consistent with Tomalia's electrospray results: Tolic, L. P.; Anderson, G. A.; Smith, R. D.; Brothers, H. M.; Spindler, R.; Tomalia, D. A. *Int. J. Mass. Spectrom. Ion Processes* **1997**, *165/166*, 405−418. (b) For a more in-depth discussion of MALDI-TOF MS characterization of mannose-functionalized dendrimers, see: Woller, E. K.; Cloninger, M. J. *Biomacromolecules* **2001**, *2*, 1052−1054.
(15) Polydispersity = M_n/M_w. Because of the finite nature of dendrimer size (compared to linear polymers), polydispersity values for dendrimers are usually much closer to 1 than they are for other polymers. The actual value is not so important; that the polydispersity does not increase upon dendrimer functionalization implies uniform dendrimer functionalization rather than high variation in the number of mannose groups per dendrimer.
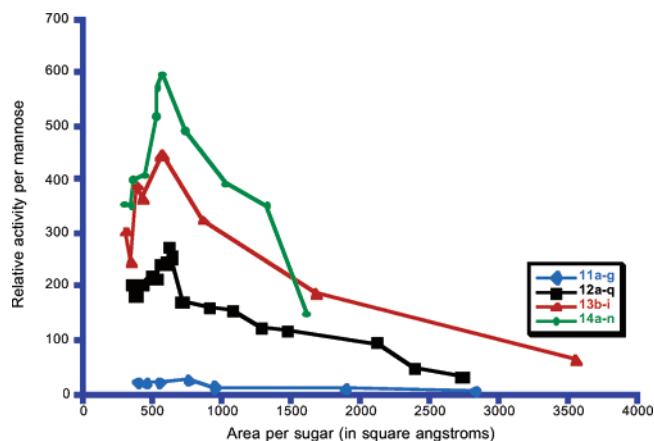(16) The small peak in all of our MALDI spectra at one-half the maximum mass is the $z = 2$ peak.

(a)

(b)
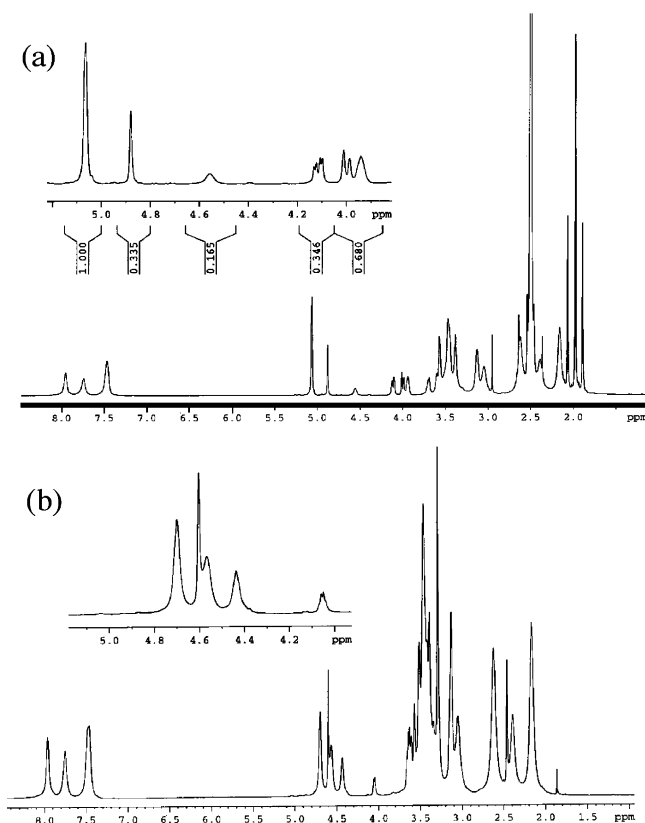
Figure 2. ^{1}H NMR spectra (500 MHz) of (a) **8l** and (b) **12l**.

the carbon chain of mannose. For the example shown in Figure 2a, the ratio of **1:6** is 1:2, which corresponds to 66% mannose. Figure 2b shows the same dendrimer after deacetylation, when the overlap of peaks makes quantitative analysis by NMR essentially impossible.

Concanavalin A (Con A) is a lectin that is commonly used to probe protein−carbohydrate interactions.[17] At a pH of 7 or greater, Con A exists as a tetramer. The binding clefts (one per monomer) are located 65 Å apart, and Con A shows specificity for α-D-mannose and, to a lesser extent, α-D-glucose. Con A and various derivatives have been used to assess the binding ability of a wide variety of mannose-containing molecules in a number of assays; hence, we chose to study the interaction of the dendrimers reported here with Con A.[5a,18]

As a means to determine the activity of dendrimers **11−14**, we performed a hemagglutination assay.[19] While this assay does not provide information regarding association constants of binding, it does provide data that are useful in making a general comparison of the effect variable mannose loading has on the activity of these glycodendrimers. In a recent review article discussing the various assays that have been used to study protein−carbohydrate interactions, Lundquist and Toone note that the hemagglutination assay is "a quick, simple assay that orders soluble ligands in a rough order of binding affinity."[5]

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(18) Mann, D. A.; Kiessling, L. L. In *Glycochemistry: Principles, Synthesis, and Applications*; Wang, P. G., Bertozzi, C. R., Eds.; Marcel Dekker: New York, New York, 2001; pp 221−275.

(19) Osawa, T.; Matsumoto, I. *Methods Enzymol.* **1972**, *28*, 323−327.

Figure 3. The relationship between activity toward Con A (relative to methyl mannose) on a per mannose basis and loading of sugars (expressed as area per sugar in Å^2) for dendrimers **11−14** (**13a** is off scale; lines through the points are shown only for clarity and do not represent curve fitting analysis).

The hemagglutination assay was performed by adding erythrocytes (rabbit) to preincubated solutions of Con A and varying concentrations of dendrimer. The concentration of Con A was kept around 1 μM for all assays. At this concentration, no deleterious precipitation of ligand and lectin was observed. The lowest amount of dendrimer that caused inhibition of agglutination was determined, and the results are shown in Figure 3. Each reported value represents at least three assays (see Supporting Information for data in tabular form).

Figure 3 shows the relationship between activity of the dendrimer relative to methyl mannose and area per sugar on the surface of the dendrimer. The relative activity was adjusted to a per mannose basis for direct comparison to α-methyl mannoside. The area available to each sugar was determined on the basis of the number of sugars per molecule (as identified by MALDI-TOF MS and NMR data) and the calculated surface area of the dendrimer (see the Discussion section for details). The relative activity (*y* axis) is reported versus the area per sugar rather than versus the number of sugars (*x* axis) because this provides the most valuable evaluation of results for different generations of dendrimers. The distance between the sugars, or the available area that each sugar can occupy on the dendrimer surface, has a significant effect on activity with Con A, but there is no correlation between generations when the number of sugars per dendrimer is considered.
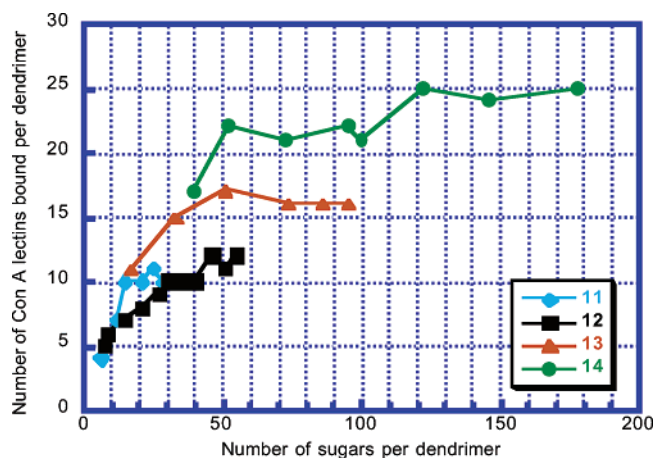
To gain insight into the stoichiometry between the dendrimers and Con A, a precipitation assay was performed. As described by Brewer and co-workers,[20] the ligand in varying concentration is incubated with a constant Con A concentration. If the Con A concentration is high enough (in this case, 33 μM), the mannose-functionalized dendrimer will precipitate the Con A. The supernatant is removed, the precipitate is washed with buffer and then redissolved by addition of monomeric methyl mannose, and the concentration of Con A is determined by UV absorbance at 280 nm. The ratio of dendrimer to Con A at the point that maximum precipitation of Con A is observed is considered to be the maximum stoichiometry of Con A to dendrimer. The results of the precipitation assay for **11−14** are summarized in Figure 4.

(20) Khan, M. I.; Mandal, D. K.; Brewer, C. F. *Carbohydr. Res.* **1991**, *213*, 69−77.

Figure 4. The maximum number of Con A lectins bound to each dendrimer versus the number of sugars per dendrimer for each generation.

Discussion

Newkome and co-workers have reported the reaction of mixtures of branched isocyanates with polypropylene(imine) dendrimers and with 12-cascade acid dendrimers and have shown that different ratios of functional groups can be added to the dendrimer surface.[21] In a similar fashion, we have used mixtures of isothiocyanates to functionalize PAMAM dendrimers with mannose and hydroxyl groups. We find that, when isothiocyanates of comparable reactivity are added to the dendrimer, the degree of functionalization can be easily controlled by stoichiometric control of reagents.[22] Indeed, sequential additions as described in Scheme 1 can be used to synthesize heterogeneously functionalized dendrimers even when one isothiocyanate is significantly more reactive than another (details to be published separately).

The relative amounts of mannose and hydroxyl groups on the dendrimer surface were determined both by MALDI-TOF MS and by NMR. While ^{1}H NMR integrations provide an excellent measure of the ratio of functional groups (see Figure 2a for example), we find that MALDI-TOF spectra give a much more accurate quantitative indication of how many mannose and hydroxyl residues are present on the dendrimers.[14] For example, the mass of **3l** is 16 300 Da, that of **8l** is 32 900 Da, and that of **12l** is 27 100 Da. The difference between **8l** and **3l** is the number of molecules of **6** added (M_w = 477); thus, (32 900 − 16 300)/477 = 34.8 sugars. Likewise, the difference between **12l** and **3l** is the number of deprotected carbohydrates (M_w = 306); thus, (27 100 − 16 300)/306 = 35.3 sugars. The difference between **8l** and **12l** is the result of the loss of four acetyl protecting groups per sugar (M_w = 172); thus, (32 900 − 27 100)/172 = 33.7 sugars. The average number of sugars is 34.6, which is rounded up to 35 sugars. This procedure was repeated for all dendrimers, and the number of sugars per dendrimer is shown in Table 1. There are several instances in Table 1, especially for M_w data for **11**−**14**, where the M_w values do not follow the expected increasing progression. This reflects the difficulty of obtaining MALDI MS on macromolecules and is why the number of sugars is determined by averaging values

obtained from multiple calculations with different MALDI spectra. For example, **14c** and **14d** have the same M_w, but averaging the three calculations described above indicates that **14c** has 26 sugars and **14d** has 34 sugars. The average number of sugars calculated from acetylated, deprotected, and partially loaded PAMAMs is more accurate than that which could be obtained from any one spectrum.

The hemagglutinations assays (summarized in Figure 3) reveal that there is a significant increase in activity (on a per mannose basis) for the fourth, fifth, and sixth generation dendrimers **12**−**14** as the number of mannose residues on the dendrimer is increased. Maximum activity occurs at just over 50% sugar loading for all generations (56% or 31 sugars on **12j**, 54% or 51 sugars on **13e**, and 53% or 95 sugars on **14h**; 100% loading is taken as the maximum number of sugars with which we were able to functionalize each generation, which is reported as **11g**, **12q**, **13i**, **14n**). The G(3)-functionalized dendrimers **11a**−**g** do not show a sharp increase in activity with increasing sugar functionalization, nor would such an increase be expected because these dendrimers are not large enough to span multiple binding sites of Con A. Figure 3 shows that, while the area available per sugar at maximum activity varies slightly for the different generations, the trend is similar in each case. What is most striking is that the maximum activity does not correlate to maximum sugar loading of the dendrimer. The decrease in activity as the number of sugars increases above about 50% loading is most likely a result of unfavorable steric interactions that are accumulating between Con A and the dendrimer. Similar loading effects have been observed in a number of systems in which the loading of the binding epitope was varied.[6a,11b,d,f,g]

That dendrimers can exhibit significant deformation has been previously reported.[23] In fact, dendrimers deposited on mica surfaces are dome-shaped; a G(5)-PAMAM dendrimer was reported to have a height of approximately 10 Å and a diameter of more than 150 Å in an AFM study.[22a] To determine the sizes of our mannose-functionalized dendrimers in solution (and thus the surface area available per sugar), we synthesized spin-labeled dendrimers and compared the line-broadening effects in the EPR spectra of the spin-labeled dendrimers to calculated line-broadening effects (see experimental procedure in the Supporting Information). Because our spin label (4-isothiocyanato-2,2,6,6-tetramethylpiperidine *N*-oxide or 4-NCS TEMPO) is six atoms shorter than our mannose tether **6**, we added 5 Å per surface group or 10 Å total to the measured diameters. Thus, the approximate diameters of **11**−**14** are 60, 78, 95, and 130 Å, respectively.[24]

As illustrated in Figure 3, the maximum activity occurs at slightly closer packing of the sugars as the generation increases. Dendrimer **11** reaches a maximum activity at approximately 750 Å^2 per sugar, **12** reaches it at 620 Å^2 per sugar, and **13** and **14** reach it at 550 Å^2 per sugar. A reasonable explanation is that the larger dendrimers are more flexible and can avoid unfavorable steric interactions better than smaller dendrimers.

Figure 5 shows the relationship between the concentration of sugars on the surface of the dendrimer and activity on a per

(21) (a) Newkome, G. R.; Childs, B. J.; Rourk, M. J.; Baker, G. R.; Moorefield, C. N. *Biotechnol. Bioeng.* **1999**, *61*, 243−253. (b) Newkome, G. R.; Weis, C. D.; Moorefield, C. N.; Baker, G. R.; Childs, B. J.; Epperson, J. *Angew. Chem., Int. Ed.* **1998**, *37*, 307−310.
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(23) (a) Li, J.; Piehler, L. T.; Qin, D.; Baker, J. R.; Tomalia, D. A. *Langmuir* **2000**, *16*, 5613−5616. (b) Uppuluri, S.; Keinath, S. E.; Tomalia, D. A.; Dvornic, P. R. *Macromolecules* **1998**, *31*, 4498−4510.
(24) Walter, E. D.; Sebby, K. B.; Singel, D. J.; Cloninger, M. J.: A short procedure is included in the Supporting Information; details regarding dendrimer size determination by spin label will be published separately.
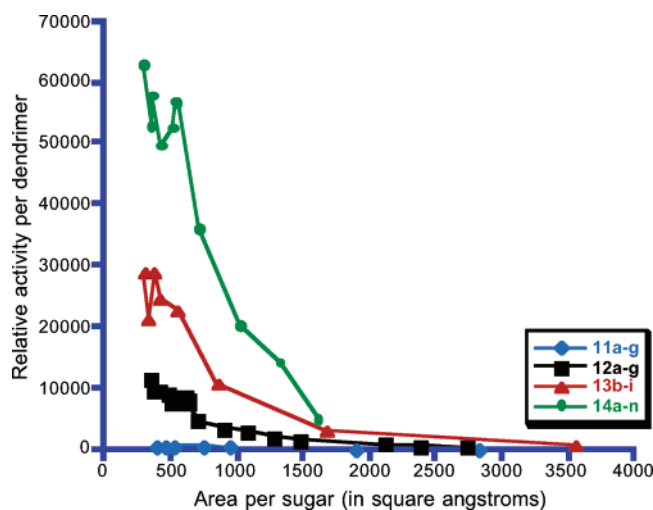
Figure 5. The relationship between activity toward Con A (relative to methyl mannose) on a per dendrimer basis and loading of sugars (expressed as area per sugar in Å^2) for dendrimers **11**−**14** (**13a** is off scale; lines through the points are shown only for clarity and do not represent curve fitting analysis).
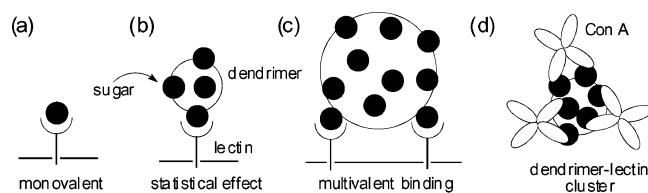

Figure 6. Expected binding motifs for Con A with mannose-functionalized dendrimers.

dendrimer basis (compared to methyl mannose). As expected, as the number of sugars increases, the activity for each generation of dendrimer continues to increase beyond the maximum observed activity on a per sugar basis (compare Figure 5 to Figure 3).

To understand why activity continues to increase on a per molecular basis as the area per sugar decreases, the different possible motifs for dendrimer−Con A interaction must be considered. The most pertinent motifs are summarized in Figure 6. For small dendrimers and methyl mannose, only the monovalent interaction shown in Figure 6a is expected. For larger dendrimers that cannot span the distance between carbohydrate binding sites on the lectin but still have clusters of sugars in relatively close proximity, the statistical effect shown in Figure 6b is expected.[4] Large dendrimers can bind simultaneously to multiple binding sites on the lectin (they can bind multivalently) as depicted in Figure 6c.[3] Many dendrimer−lectin clusters and aggregates can also be envisioned. One example of a dendrimer−lectin cluster for large dendrimers is shown in Figure 6d.

Because precipitation assays suggest that Con A lectins are bound to the dendrimer in a bidentate fashion for **12**−**14** (see below), the effect of multivalent binding (as described in Figure 6c) should be the same for **12**−**14**. The continued increase in activity on a per dendrimer basis (Figure 5) can be attributed at least partly to some more subtle effect on binding than the ability of the dendrimer to span multiple binding sites on Con A. Because the graph in Figure 5 compares the number of molecules of dendrimer relative to molecules of methyl mannose, the simplest explanation for the increase in activity is a
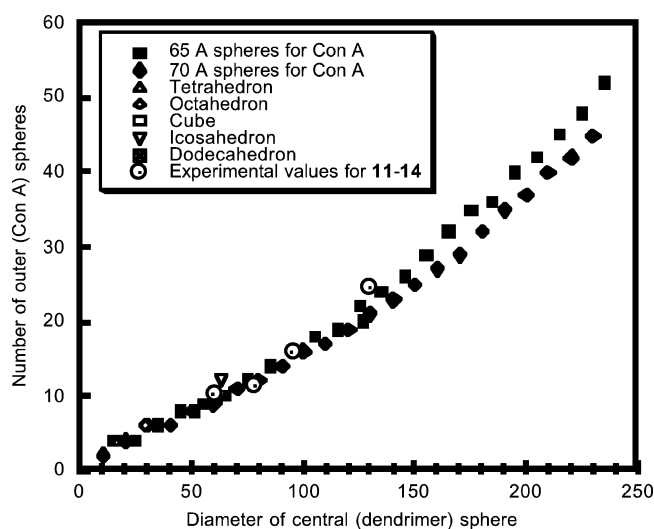

Figure 7. The calculated and the experimentally determined maximum number of Con A lectins that can be complexed by the dendrimers.

statistical effect (as in Figure 6b); that is, as the number of sugars available to a given binding site increases, the activity of binding increases. This modest increase in activity is not as dramatic as the increase gained for multivalent binding, but its effect is evident.

The results of the precipitation assay are summarized in Figure 4. The most obvious result of the precipitation assay is that, for each generation **11**−**14**, the number of Con A molecules binding to a dendrimer reaches a maximum and levels off. This maximum is 10−11 Con A lectins for **11**, 11−12 Con A lectins for **12**, 16 Con A lectins for **13**, and 24−25 Con A lectins for **14**. The theoretical maximum number of Con A molecules that would fit around **11**−**14** was determined computationally. The dendrimer was modeled as a central sphere with a diameter from 10 to 240 Å, with spherical Con A molecules (65 or 70 Å spheres to mimic Con A) placed on the dendrimer surface. As a check of our simulation, the results were compared to those obtained by placing the outer (Con A) spheres at the vertices of polyhedra with the inner (dendrimer) sphere at the center. Details are given in the Experimental Section, and the computational results are shown in Figure 7. Using the dendrimer diameters of 60, 78, 95, and 130 Å for **11**, **12**, **13**, and **14**, respectively (see above), we determined that the calculated number of Con A lectins that should fit around each dendrimer is in excellent agreement with the maximum number of Con A lectins obtained in the precipitation assay. The simulation predicts 9−10 Con A lectins for **11**, 12−13 Con A lectins for **12**, 15−16 Con A lectins for **13**, and 22−23 Con A lectins for **14**. The agreement, in all cases, is remarkably excellent in light of the simplicity of the model. Interestingly, the agreement tends to fall off with increasing dendrimer size, where the experimental values are systematically larger than the calculated values. In the sixth generation, the calculated value is 22−23, while the observed value is 24−25. We attribute this trend to an increasing flexibility of dendrimer shape with increasing generation number. This flexibility of the larger dendrimers enables a variability in surface area, for a presumably fixed molecular density and thus volume. Thus, calculation for a model dendrimer of spherical shape, in which the ratio of surface area to volume is minimized, gives a lower limit on the actual number of Con A lectins that can bind to the dendrimer;
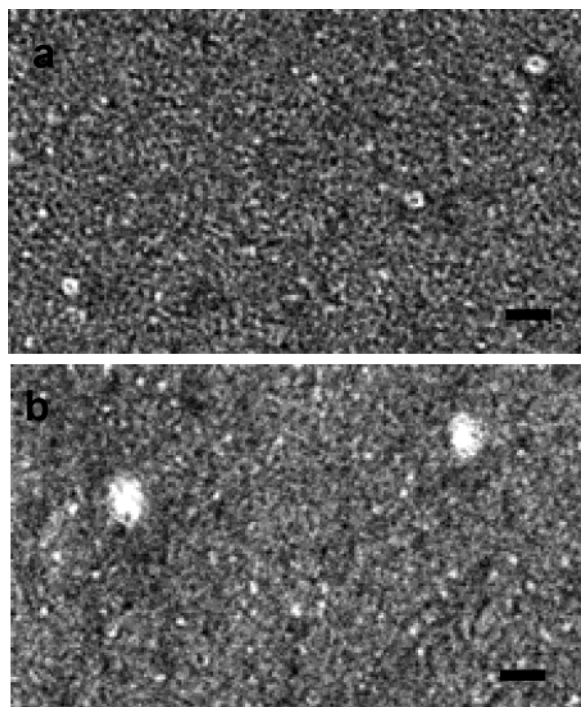
Figure 8. TEM images of aqueous solutions of (a) **14n** at a concentration of 0.023 mg/mL[25] and (b) **14n** (0.023 mg/mL) and Con A (0.18 mg/mL). Scale bar = 20 nm.

increased surface areas in the more flexible larger generations allow for the binding of some additional Con A lectins over this number. That the theoretical maximum number of complexed Con A lectins is obtained (or exceeded) in all cases qualitatively suggests a reasonably high affinity of Con A for **11**−**14**.

Although assay conditions such as Con A concentration are quite different for the hemagglutination assay and the precipitation assay, it is illuminating to evaluate the results of the two assays together. For dendrimers with per mannose activities of >100 (relative to methyl mannose) in the hemagglutination assay, the number of Con A molecules attracted to each dendrimer in the precipitation assay was less than or equal to one-half the number of sugars on the dendrimer surface (in 21 of 22 such dendrimers for which we have data). This indicates that each dendrimer with a hemagglutination activity of >100 probably binds Con A in a bidentate fashion (see "sugars per Con A" in Table S6 in the Supporting Information for all values). For the dendrimer with the highest activity in the hemagglutination assay in the fourth and fifth generational series (**12j** and **13e**), three sugars per Con A are stoichiometrically available in the precipitation assay. The value is higher (4.3 sugars per Con A) for **14h** in the G(6) series, but 3.4 sugars per Con A are stoichiometrically available for **14g**, the previous dendrimer in the series. On the dendrimer of each generation that shows the highest inhibition of hemagglutination, three (not two) sugars per Con A are present: either the extra sugar is an important component of the statistical effect (Figure 6b) or not all sugars are spaced properly for bidentate binding (even considering the dynamic nature of the dendrimers). Also, after the maximum possible number of Con A molecules have been

bound to the dendrimer, adding more sugars reduces the dendrimer's activity in the hemagglutination assay on a per sugar basis but does not lower the activity so much that fewer Con A molecules are bound in the precipitation assay.

The precipitation assay suggests that the surface of the dendrimer is saturated with Con A. However, the precipitation assay was performed at a significantly higher concentration of Con A and of dendrimer than was the hemagglutination assay. To evaluate the Con A/dendrimer binding motif at the concentration of the hemagglutination assay, transmission electron micrographs were obtained (Figure 8). In Figure 8a, individual dendrimers of **14n** can be observed. To obtain Figure 8b, **14n** and Con A were combined in a 1:7 ratio, and the particles roughly double in diameter. This is exactly what one would expect from **14n** surrounded by Con A (6.5 nm diameter). The TEMs strongly suggest that closely packed dendrimer−lectin clusters such as depicted in Figure 6d are formed in solution.

Taken together, the results from the hemagglutination assay and the precipitation assay suggest that we can independently attenuate lectin binding affinity and lectin clustering. Considering the huge number of lectins present on cell surfaces,[26] it is likely that recruitment of far more than 25 lectins occurs in many cellular processes. However, one could imagine using the results reported here to construct dendrimer/polymer superstructures with a high degree of control over both the lectin binding affinity and the number of lectins clustered into a specific area of the cell surface.

Conclusion

The formation of mannose/hydroxyl-functionalized dendrimers was achieved by the stoichiometric control of reactants. MALDI-TOF MS and ^{1}H NMR are useful techniques for the characterization of surface functionalization of heterogeneously functionalized denedrimers. The degree of mannose functionalization has a significant effect on the activity of the dendrimer toward Con A. By controlling the number of sugars present on the surface of the dendrimer, we could controld both the lectin binding activity (as measured in the hemagglutination assay) and the number of lectins clustered around the dendrimer (as measured in the precipitation assay).

Supporting Information Available: Experimental procedures and characterization data for **7**−**14** and experimental procedures for MALDI-TOF MS, hemagglutination assay, quantitative precipitation assay, computer modeling, TEM, and dendrimer size determination by EPR. Precipitation assay graphs and a table showing the relative activity in the hemagglutination assay of **11**−**14** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(26) Kilpatrick, D. C. *Handbook of Animal Lectins Properties and Biomedical Applications*; John Wiley & Sons: New York, 2000.