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EPR and affinity studies of mannose–TEMPO functionalized PAMAM dendrimers†

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Mannose–TEMPO functionalized G(4)-PAMAM dendrimers with increasing mannose loadings have been synthesized and characterized by MALDI-TOF MS and EPR spectroscopy. Analysis of linebroadening effects in the EPR spectra of these dendrimers allowed us to determine the relative presentation of mannose and TEMPO on the dendrimer surface. Hemagglutination assays and affinity chromatography/EPR experiments to assess the activity of the mannose–TEMPO dendrimers with Concanavalin A are presented.

Introduction

Although multivalent protein–carbohydrate interactions play a critical role in many biological recognition events, multivalent therapeutic agents that utilize protein–carbohydrate interactions have been difficult to design. This is primarily because the fundamental requirements of protein–carbohydrate interactions are not well understood.[1] Carbohydrates are often covalently linked to macromolecules as a means of studying protein–carbohydrate interactions.[2] We[3] and others[4] have used dendrimers as scaffolds for carbohydrate display. Functionalization of the terminal amines on the surface of PAMAM dendrimers is readily accomplished to form homogeneous mannose functionalized[3b,c] and heterogeneous mannose–hydroxyl functionalized dendrimers.[3a]

Heterogeneous functionalization of dendrimers that incorporate varying loadings of saccharide residues is attractive because it allows for the fine-tuning of protein affinities and protein clustering properties of the macromolecules. Not only can the size of the (dendrimer) framework be changed easily by changing the generation of dendrimer used, but the display of sugars on the dendrimer surface can also be readily manipulated through heterogeneous functionalization.[5]

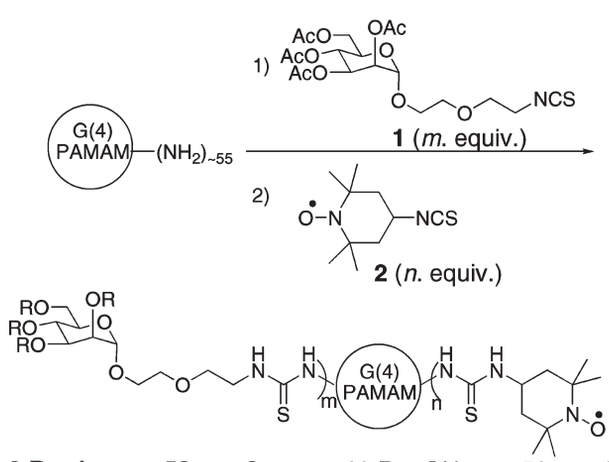
Recently, we studied EPR linebroadening effects for G(4)-PAMAM dendrimers functionalized with a TEMPO (2,2,6,6-tetramethylpiperidine *N*-oxide) spin label and another functional group. The relative locations of the spin labels were determined. At all loadings studied (5–95% TEMPO), the spin label was found to be randomly distributed on the surface of the dendrimer and therefore the other group (2,2,6,6-tetramethylpiperidine, propanol, *tert*-butane and phenol) must also present a random surface display.[6]

For our work using dendrimers to study protein–carbohydrate interactions, we use a synthesis strategy where peracetylated sugars are tethered to the dendrimer surface and then deprotected. Although the peracetylated sugars are almost certainly randomly distributed on the dendrimer surface, we were concerned that unmasking of the hydroxyl groups might create hydrogen-bonding networks. Clustering of sugars on the dendrimer surface comparable to the creation of rafts in lipid membranes could occur.[7] In order to effectively design heterogeneous multivalent recognition systems using the thiourea functionalization routes described here, it is imperative that we characterize the functional group distribution on the dendrimer. Moreover, although the work reported here is focused on PAMAM dendrimer functionalization, these studies are widely applicable to characterization of macromolecules and nanoparticles.

To evaluate the loading distribution of sugars on heterogeneously functionalized dendrimers, we synthesized mannose–TEMPO functionalized dendrimers. The synthesis, characterization, and EPR analysis of these compounds is reported here. In addition, the use of mannose–TEMPO functionalized dendrimers in hemagglutination assays and affinity chromatography experiments with Concanavalin A (Con A, a mannose binding lectin)[8] is described.

Results and discussion

In order to study the relative locations of the mannose residues on heterogeneously functionalized dendrimers, acetyl protected mannose–TEMPO functionalized dendrimers **3**–**9** were synthesized as shown in Scheme 1. Mannose derivative **1**[3a] and 4-isothiocyanatoTEMPO (**2**) were added sequentially at room temperature to G(4)-PAMAM in controlled ratios to obtain heterogeneously functionalized dendrimers **3**–**9**. The acetyl groups were removed with NaOMe–MeOH to form mannose–TEMPO functionalized dendrimers **10**–**16**.



3 R = Ac, m = 52, n = 3
4 R = Ac, m = 49, n = 6
5 R = Ac, m = 41, n = 14
6 R = Ac, m = 27, n = 28
7 R = Ac, m = 14, n = 41
8 R = Ac, m = 6, n = 49
9 R = Ac, m = 3, n = 52

NaOMe, MeOH

10 R = OH, m = 52, n = 3
11 R = OH, m = 49, n = 6
12 R = OH, m = 41, n = 14
13 R = OH, m = 27, n = 28
14 R = OH, m = 14, n = 41
15 R = OH, m = 6, n = 49
16 R = OH, m = 3, n = 52

Scheme 1 Synthesis of mannose–TEMPO functionalized G(4)-PAMAM dendrimers.

† Electronic supplementary information (ESI) available: Tables of MALDI-TOF MS and EPR characterization data for compounds **3**–**16** and **22**–**24**; tables of masses of materials that were isolated in affinity chromatography experiments; EPR absorbance spectra for **3**–**16** and second integral values (11 pages). See http://www.rsc.org/suppdata/ob/b4/b411643g/

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Table 1 MALDI-TOF MS characterization data for **3**–**9**

	M_W after **1** was added	Number of sugars	M_W after both **1** and **2** were added	Number of TEMPOs
3	37740	52	38390	3
4	36310	49	37570	6
5	32060	40	34940	14
6	26100	27	32490	30
7	19220	13	28130	42
8	16090	6	26470	49
9	15590	5	26590	52

Theoretical ratios of mannose to TEMPO are shown in Scheme 1 (m:n) and include 95%, 90%, 75%, 50%, 25%, 10%, and 5% mannose functionalization. Actual ratios of mannose to TEMPO were determined from MALDI-TOF MS. The M_W of G(4)-PAMAM starting material was found to be 13170 g mol^{-1}, which suggests that, on average, each dendrimer has fifty-five terminal amino groups.[9] Subtraction of 13170 from the M_W of each dendrimer after addition of **1** and division of this value by 477 (the molecular weight of **1**) gives the average number of sugars on each dendrimer. Subtraction of the intermediate M_W from the M_W obtained for **3**–**9** and division of this value by 213 (the molecular weight of **2**) gives the average number of TEMPO residues per dendrimer. The difference in M_W for **10**–**16** and **3**–**9** divided by 168 (the molecular weight of four acetyl groups) serves as a check of the number of sugars per molecule. Table 1 shows the experimentally determined number of sugars and TEMPO residues for dendrimers **3**–**9**. In all cases, our calculated number of functionalized endgroups is between 54 and 57, which agrees very well with our calculated number of amino groups on the G(4)-PAMAM (55 endgroups on average) and suggests that essentially complete reactivity of the primary amines is observed. Whether primary isothiocyanate **1** or secondary isothiocyanate **2** is added first has virtually no effect on the degree of dendrimer functionalization (Table S2 and S3 of the supporting information†). Since we are not using excess isothiocyanate, it seems reasonable to assume that the isothiocyanates are reacting with the primary amines on the dendrimer rather than the secondary amines that are produced when incomplete/retro Michael addition occurs. Addition of **1** to some of the secondary amines (rather than the primary amines on the dendrimer surface) is a possibility, but the calculated values of number of endgroups based on the MS data suggest that the dendrimer's amino termini react preferentially.

The EPR spectra obtained for **3**–**9** are shown as a stack plot in Fig. 1a (normalized to peak A). After removal of the acetyl groups, EPR spectra were obtained for **10**–**16** (Fig. 1b). Dendrimers with high loadings of TEMPO have broader spectra than dendrimers with lower TEMPO loadings (**9** and **16** *vs.* **3** and **10**). Qualitatively, the stack plots of **3**–**9** and **10**–**16** are nearly identical. The A/B peak height ratio has shown itself to be a reasonable measure of linebroadening within the confines of our system and also has strong precedent in other systems, so we rely on this value to evaluate linebroadening effects.[10,11] A graph of the A/B peak height ratio *versus* the percent loading of TEMPO (Fig. 1c) shows the nearly perfect overlay of the results for acetylated and deprotected dendrimers. The only differences in the linebroadening effects are at very low and very high TEMPO loadings, where small concentration measurement errors or remaining unreacted **2** would cause the most significant changes in the EPR spectra. Indeed, when placed in the context of A/B peak height ratio *vs.* TEMPO loading for a variety of heterogeneously functionalized dendrimers, differences in linebroadening effects between **3** and **10** and between **9** and **16** are negligible (Fig. S1 in the supporting information†).

We conclude that the surface patterning of peracetylated mannose–TEMPO dendrimers and of unmasked mannose–TEMPO dendrimers is the same. Comparison of Fig. 1c to computer simulations of various dendrimer loading patterns

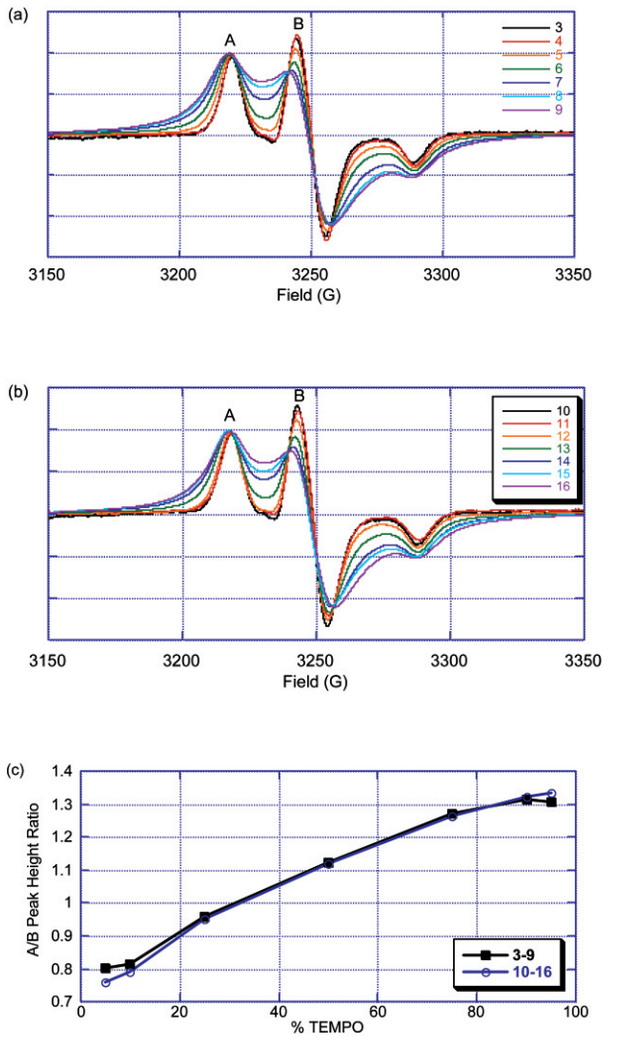


Fig. 1 (a) EPR spectra of **3**–**9**, normalized to peak A. (b) EPR spectra of **10**–**16**, normalized to peak A. (c) A plot comparing the A/B peak height ratio *versus* percent TEMPO loading for dendrimers with and without acetyl protecting groups.

strongly suggests that all dendrimers **3**–**16** have a random distribution of surface functional groups.

Hemagglutination assays[12] were performed on dendrimers **10**–**12**, and the results were compared with previous results using mannose-hydroxyl functionalized dendrimers to ensure that the spin label did not affect lectin binding. The first three entries in Table 2 summarize our results, which were comparable to previously reported values for dendrimers lacking the TEMPO groups. Unfortunately, dendrimers **13**–**16** were insoluble in aqueous solutions and could not be studied with lectins.

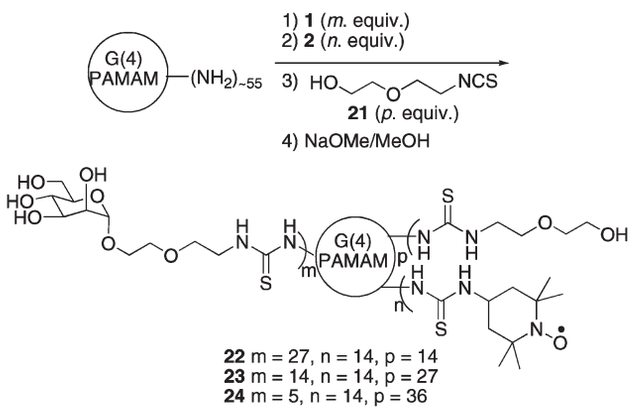
To increase the water solubility of the spin labeled dendrimers with low sugar loadings, dendrimers **22**–**24** were synthesized as shown in Scheme 2. Isothiocyanates **1**, **2**, and **21**[3a] were sequentially added to G(4)-PAMAM in controlled ratios at room temperature, and MALDI-TOF MS were obtained after addition of each component. Removal of the acetyl groups was accomplished using NaOMe–MeOH. MALDI-TOF characterization data is given in the supporting information.† As for **10**–**16**, theoretical numbers of functional groups are shown in Scheme 2 and values determined from MALDI-TOF MS characterization are reported in Table 2.

The results of the hemagglutination assays on **22** and **23** with Con A are shown in entries 4 and 5 of Table 2. Good agreement was observed between values obtained for mannose–TEMPO dendrimers and previously synthesized mannose–hydroxyl dendrimers with similar saccharide loadings. The water solubility of dendrimer **24** was too low to obtain good data in the hemagglutination assay. Because **10**–**12** and **22**–**23** show

Table 2 Hemagglutination assay results for spin labeled and non-spin labeled mannose functionalized dendrimers

	Number of sugars	Relative activity[a]	Relative activity without TEMPO[b]
10	52	410 ± 310	180 ± 10
11	49	290 ± 120	200 ± 10
12	41	170 ± 80	220 ± 10
22	26	200 ± 100	170 ± 40
23	12	100 ± 50	120 ± 40
24	7	NA[c]	30 ± 25

[a] All values are relative to methyl mannose, and concentrations are adjusted to reflect per sugar values. [b] On a per mannose basis and relative to methyl mannose, the activity of mannose/hydroxyl functionalized dendrimers with equivalent numbers of sugars. Values are taken from reference 3*a*. [c] Because of the low water solubility of **24**, this value could not be determined.



Scheme 2 Synthesis of tri-functionalized dendrimers.

activities in the hemagglutination assay comparable to those of mannose–hydroxyl functionalized dendrimers, we conclude that the presence of the TEMPO spin label does not significantly influence protein binding. Of course, dendrimers with TEMPO only (no mannose) show no affinity toward Con A in the assay. The spin label and the hydroxyl group both serve in the assay merely as inactive space-holding groups that separate the mannose residues.

In a mixture of randomly functionalized dendrimers, it is certainly possible that a small population of dendrimers will be responsible for the majority of the activity in the hemagglutination assay. We wanted to determine whether the activity that we have observed towards Con A is caused by the bulk dendrimer mixture (randomly mannose functionalized) or some minor component (where the sugars are clustered or are far apart on the dendrimer surface). To address this point, we performed affinity chromatography with Con A–sepharose using mannose–TEMPO dendrimers followed by EPR spectroscopy. In affinity chromatography, molecules with low affinity towards Con A should elute before those with high affinity. The linebroadening effects in the EPR spectra of the eluted dendrimers can then be compared to the linebroadening effects in the EPR spectrum of the original mannose–TEMPO dendrimer. Variations in linebroadening effects would be indicative of loading patterns deviating from random.

Solutions of mannose–TEMPO dendrimers **10**–**12** and **22**–**24** were run through columns packed with Con A-bound-sepharose. EPR spectra were obtained of the eluted samples and were compared with those of the starting solutions in order to study any differences in linebroadening effects. Results are summarized in Table 3. In all cases except **24**, the dendrimer remained on the column until it was eluted by addition of concentrated methyl mannose. Dendrimer **24** was eluted off the column in the first 1–2 fractions, which is consistent with the lower relative binding seen in the hemagglutination assay of comparably loaded mannose–hydroxyl dendrimers (entry 6, Table 2). In all cases,

Table 3 A/B peak height ratios for mannose–TEMPO dendrimers before and after affinity chromatography

	mL eluted[a]	Initial A/B ratio	A/B ratio after column[b]
10	22	0.77	0.76
11	22	0.79	0.74
12	22	0.92	0.86
22	22	0.94	0.79
23	22	0.99	0.93
24	1–2	0.78	0.85

[a] The fraction in which EPR active material was recovered. [b] The reported values are the average of three trials.

one-half to two-thirds of the original EPR-active material was recovered. Loss of mass probably occurs primarily because of weighing errors for the small quantities (≤3 mg per run) of dendrimer involved.

From the affinity chromatography/EPR experiments, we conclude that the results observed in the hemagglutination assay are due to the bulk dendrimer material binding strongly to Con A. In other words, a small amount of very active dendrimer (with clustered or disparate mannose residues) is not responsible for the activity that we observe in the hemagglutination assay.

Conclusion

In summary, mannose–TEMPO functionalized PAMAM dendrimers have been synthesized and characterized by MALDI-TOF MS and EPR spectroscopy. The EPR spectra of dendrimers **3**–**16** indicate that both the acetylated mannose groups and the (deprotected) mannose residues are randomly distributed on the dendrimer surface. Comparison of the hemagglutination assay results for mannose–TEMPO and mannose–hydroxyl functionalized dendrimers suggests that the presence of a spin label has no affect on binding of the dendrimer to Con A. Finally, affinity chromatography/EPR experiments indicate that the results observed in the hemagglutination assay are due to the bulk dendrimer material binding strongly to Con A and are not caused by the presence of a few idealized dendrimers.

Experimental

General protocols

General reagents were purchased from Aldrich, Sigma, and Acros. Generation 4 PAMAM dendrimers were purchased from Dendritech as aqueous solutions. Methylene chloride was purified on basic alumina. $BF_3{\cdot}Et_2O$ was distilled from CaH_2. All other solvents and reagents were used as supplied. All MALDI-TOF reference standards were purchased from Sigma. Compound **21** was synthesized as previously reported.[3a]

General procedure for the synthesis of peracetylated mannose–TEMPO dendrimers 3–9

To a stock solution of G(4)-PAMAM in DMSO (500 μL, 25 mM in endgroups), aliquots of solutions of NCS-TEMPO (**2**, 25 mM) and of **1** (25 mM) in DMSO were added at room temperature so that the total added volume was 500 μL (see Table S1, supporting information†). The second reactant was added 48 h after the first, when analysis of the MALDI-TOF spectrum indicated complete reaction had occurred. The mixture was allowed to react for an additional 48 h after the second reactant was added. Completion was again determined through the analysis of the MALDI-TOF spectrum. A 100 μL aliquot was taken for EPR spectral analysis, and the remaining solution was used to synthesize dendrimers **10**–**16**. Tables S2 and S3 (supporting information†) provide MALDI-TOF MS and EPR characterization data for **3**–**9**.

General deacetylation procedure to form mannose–TEMPO dendrimers 10–16

Addition of 0.3 M NaOMe in methanol (1.0–1.3 equiv. per carbohydrate, see Table S4 in the supporting information†) to the DMSO solutions of **3**–**9** and then reaction at room temperature for 48 h afforded **10**–**16**. Reaction completion was determined by analysis of MALDI-TOF MS. A 100 μL aliquot was taken for EPR spectral analysis, and the remaining solution was purified on either Sephadex G25 gel in DMSO or in Amnicon centrifugation filters (M_WCO = 5 KDa.) and lyophilized. Table S5 (supporting information†) provides MALDI-TOF MS and EPR characterization data for **10**–**16**.

Synthesis of the tri-functionalized dendrimers 22–24

To a stock solution of G(4)-PAMAM in DMSO (1 mL, 25 mM in endgroups), aliquots of a solution of **2** (25 mM), **1** (25 mM) and **21** (25 mM) in DMSO were added sequentially at room temperature (total added volume = 1 mL, see Table S6, supporting information,† for amounts). The second reactant was added 48 h after the first, and the third reacted was added 48 h after the second. The functionalization of each reactant was confirmed by analysis of the MALDI-TOF spectrum. The mixture was allowed to react for an additional 48 h after the third reactant was added, upon which time MALDI-TOF MS indicated that the reaction had gone to completion. NaOMe in methanol was added so that there was 1.0 equivalent of NaOMe per mannose residue. After 48 h at room temperature, when MALDI-TOF MS indicated reaction completion, water was added to the mixture to quadruple the volume of the solution. The solutions were transferred to Amnicon centrifuge filters (M_WCO = 5 KDa.) and purified with water and lyophilized to dryness. Table S7 (supporting information†) provides MALDI-TOF MS and EPR characterization data for **22**–**24**.

4-Isothiocyanato-2,2,6,6-tetramethylpiperidine *N*-oxide (2)

4-Amino-2,2,6,6-tetramethylpiperidine *N*-oxide (1.0 g, 5.8 mmol) was dissolved in 20 mL of aqueous 5% NaOH solution and cooled in an ice bath. Thiophosgene (600 mg, 400 μL, 0.9 equiv.) was added over 15 minutes (syringe pump) with rapid stirring. Immediate filtration and drying *in vacuo* over P_2O_5 gave 640 mg (57%) of pale orange product. Comparison of the EPR spectra of the product with the nitroxide reagent verified the preservation of the nitroxide radical. Characterization data was in agreement with previously published results. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.0 (br. s, 1 H), 2.2–1.0 (br. m, including singlet (4 CH_3 groups) at 1.2).[13]

MALDI

Matrix assisted laser desorption ionization (MALDI) mass spectra were acquired using a Bruker Biflex-III time-of-flight mass spectrometer. Spectra of dendrimers were obtained using a *trans*-3-indoleacrylic acid matrix with a matrix–analyte ratio of 3000 : 1 or 1000 : 1. Horse heart myoglobin (M_W 16952 g mol^{-1}), bovine serum albumin (M_W 66431 g mol^{-1}), Bradykinin (M_W 1061 g mol^{-1}), Cytochrome C (M_W 12361 g mol^{-1}), and Trypsinogen (M_W 23982 g mol^{-1}) were used as external standards. An aliquot corresponding to 12–15 pmol of the analyte was deposited on the laser target. Positive ion mass spectra were acquired in linear mode, and the ions were generated by using a nitrogen laser (337 nm) pulsed at 3 Hz with a pulse width of 3 nanoseconds. Ions were accelerated at 19–20 000 volts and amplified using a discrete dynode multiplier. Spectra (100 to 200) were summed into a LeCroy LSA1000 high speed signal digitizer. All data processing was performed using Bruker XMass/XTOF V 5.0.2. Molecular mass data and polydispersities of the broad peaks were calculated by using the Polymer Module included in the software package. The peaks were analyzed using the *continuous* mode. Delta values were set at minimum levels. As described in reference 6, the peak width at half height is evaluated to determine the overall sample homogeneity. Samples that deviate significantly from a normal distribution function give significantly broader peaks than those that are observed for the compounds described here.

Hemagglutination assays

Hemagglutination assays were performed at 22 °C in 0.1 M Tris–HCl buffer, pH 7.2, containing 0.15 M NaCl, 1 mM $CaCl_2$ and 1 mM $MnCl_2$. Serial two-fold dilutions of ligands (50 μL) were incubated with 1 μM Con A (50 μL) for two hours, after which 50 μL of 3% suspensions of rabbit erythrocytes was added. After one hour the inhibiting dose was determined by visual inspection. At the concentrations used, no precipitation of Con A was observed.

EPR

CW-EPR spectra were recorded at ~9 GHz for TEMPO-labeled dendrimers in 3 : 1 DMSO–glycerol solvent at 76 K in a Varian E-109 spectrometer modified by the incorporation of an external field-sweep control unit obtained from the University of Denver. The computer interface of the systems provides for sweep control and data acquisition in a LabView environment. For clarity of display, the spectra have been normalized to exhibit equal amplitudes at the point marked "A" in the spectra. Recording conditions were established to avoid artificial broadening of the EPR spectra. Serial dilutions were performed to establish conditions under which line-broadening from inter-dendrimer spin–spin interactions was eliminated.

Affinity chromatography

A 1 mL disposable syringe with a plug of glass wool was used as the column, and a second 1 mL syringe was placed on top of the first and connected with Parafilm™ to hold the buffer. The column was packed by running 1–2 mL of PBS-buffered saline through the column and then slowly pipetting the Con A sepharose gel into the column so that the gel was on the 1 mL mark of the syringe. The column was wrapped in aluminium foil because of the light sensitivity of the TEMPO-functionalized dendrimers and washed with 10 mL of PBS buffer for equilibration. A 100 μL solution of sample (approximately 30 mg mL^{-1} in PBS) was loaded onto the column, and PBS buffer (20 mL) was eluted through the column while 1 or 2 mL fractions were collected. For the affinity columns performed on **10**–**12**, 1 mL fractions were collected. For the affinity columns performed **22**–**24**, 2 mL fractions were collected. After 20 mL of PBS was eluted, 10 mL of 0.1 M methyl mannose in PBS was added to the column while 1 mL fractions were collected. All fractions were desalted (and mannose was removed) by either dialysis against water (M_WCO = 3.5 KDa.) or using Amnicon centrifugation tubes with water (M_WCO = 5 KDa.). Purified samples were lyophilized. The fractions that contained enough mass to obtain an EPR spectrum were dissolved in DMSO to make a 12.5 mM solution of endgroups and scanned using EPR. Up to 4 continuous fractions were combined to make an EPR sample if needed. Tables S8 and S9 in the supporting information indicate the masses of material eluted in each fraction.†

Acknowledgements

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