EPR Characterization of Heterogeneously Functionalized Dendrimers

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ABSTRACT: EPR spectroscopy was used to evaluate the dynamic motions of dendrimer end groups for a G(4)-PAMAM dendrimer. Clusters of end groups were covalently tethered together and then selectively labeled with PROXYL groups upon removal of the tether. Analysis of the EPR spectra of the spin-labeled dendrimers suggests that initial end group proximity does not appreciably alter end group presentation on heterogeneously functionalized PAMAM dendrimers after tethers are released.

Introduction

Dendrimers have attracted a great deal of scientific attention.[1] Dendrimers find applications in diverse areas of research including drug delivery, gene therapy, bioimaging, photonics, and electronics.[2] With the burgeoning interest in dendrimers for practical applications has come the critical need for full characterization of dendrimers, and dendrimer characterization remains very challenging.[3]

One key research thrust for dendrimers involves targeted drug delivery. In many instances, dendrimers functionalized with a targeting functionality, with a prodrug, with a solubilizing agent, and with an imaging group will be highly desirable.[4] For dendrimers appended with more than one surface functional group, determining the relative locations of the termini emerges as a critical component of characterization. Techniques such as nuclear magnetic resonance (NMR) and mass spectroscopy (MS) are readily used to determine the relative amounts of different functionalities on the dendrimer, but NMR and MS generally do not provide information regarding the relative distributions of functional groups. Although several researchers have studied the likelihood of dendrimer end groups to backfold into the interior of the dendrimer,[5] dynamics and distributions of multiple end groups in relation to each other are less understood. Only a few simulations have been reported for dendrimers with more than one type of end group.[6]

Because they are readily available in large quantities, polyamidoamine (PAMAM) dendrimers continue to be particularly useful for many applications, and the in-depth characterization of PAMAM dendrimers is an area of active research. Electrophoretic mobility,[7] Fourier transform infrared spectroscopy and 2D IR correlation spectroscopy,[8] fluorescence and UV−visible spectroscopy,[9] and single photon counting techniques[10] are some examples of recently reported techniques for PAMAM dendrimer characterization. Computational investigations of the properties of PAMAM dendrimers are also actively pursued.[5d,11]

Electron paramagnetic resonance (EPR) is a promising technique with which to characterize the relative locations of PAMAM dendrimer end groups. Site-directed spin-labeling has been used to determine the relative distances between spin-labeled components of proteins, peptides, foldamers, and dendrimers.[12] Excellent precedent describing spin-labeled dendrimers and for EPR of dendrimers has been established.[13] Recently, we reported EPR linebroadening effects for G(4)-PAMAM dendrimers that were functionalized with 2,2,6,6-tetramethylpiperidine *N*-oxide (TEMPO) spin-labels and other (non-paramagnetic) functional groups.[14] At all of the loadings that we studied, a random distribution of spin-labels was observed. Comparison of experimental and simulated linebroadening effects indicated that neither significant clustering nor maximal separation of like surface functionalities occurred.

It has been suggested that the PAMAM dendrimer framework is flexible enough to allow extensive movements of the end groups.[1,3] Such dynamics could give rise to a random distribution of attached groups by scrambling any order imparted during synthesis. To determine the origin of the random presentation of dendrimer end groups, we selectively spin-labeled PAMAM dendrimers in a nonrandom fashion with 2,2,5,5-tetramethyl-1-pyrrolidinyloxy (PROXYL) radicals and then monitored their structural rearrangements by following temporal trends in dipolar linebroadening. Here, we describe the synthesis and EPR analysis of ethyl carbonate/PROXYL-spin-label functionalized PAMAM dendrimers.

Experimental Section

General Methods. G(4)-PAMAM dendrimer was purchased from Dendritech Inc. (Midland, MI), and 1-Oxyl-2,2,5,5-tetramethylpyrrolin-3-carboxylate *N*-hydroxy succinimide ester was purchased from Toronto Research Chemicals. Compound **1** was synthesized as reported previously.[14a,15]

MALDI. Matrix assisted laser desorption ionization (MALDI) mass spectra were acquired using a Bruker Biflex-III time-of-flight mass spectrometer. Spectra of all functionalized dendrimers were obtained using a *trans*-3-indoleacrylic acid matrix with a matrix to analyte ratio of 3000:1 or 1000:1. Cytochrome C (MW 12 361 g/mol), and Trypsinogen (MW 23 982 g/mol) 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 ns. Ions were accelerated at 19 000−20 000 V and were 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 (PDI = M_w/M_n) of the broad peaks were calculated by using the Polymer Module included in the software package. The peaks were analyzed using the continuous mode.

NMR. ^{11}B NMR spectra were recorded on a Bruker DRX 500 spectrometer (160.5 MHz), and chemical shifts are relative to $BF_3 \cdot Et_2O$ and are externally referenced. A baseline correction was applied to suppress the broad signal of the boron incorporated in the glass sample tube and in the inset. ^{1}H NMR spectra were

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recorded on Bruker DPX 300 (300 MHz) and Bruker DRX 500 (500 MHz) spectrometers. Chemical shifts are reported in ppm from tetramethylsilane with the residual protic solvent resonance as the internal standard (chloroform: ~ 7.25 ppm; dimethyl sulfoxide: ~ 2.50 ppm). Data are reported as follows: chemical shift, multiplicity (s = singlet, bs = broad singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet, app = apparent), integration, coupling constants (in Hz) and assignments. COSY spectra were recorded on a Bruker DRX 600 spectrometer (600 MHz). ^{13}C NMR spectra were recorded on a Bruker DRX500 (126.5 MHz) spectrometer with complete proton decoupling. Chemical shifts are reported in ppm from tetramethylsilane with the solvent as the internal standard ($CDCl_3$: ~77.0 ppm).

EPR. CW (continuous wave) EPR spectra were recorded at ~9.2 GHz for all spin-labeled samples, in a 3:1 DMSO:glycerol solvent at 76 K in a Varian E-109 spectrometer modified by the incorporation of an external field-sweep unit obtained from the University of Denver. The computer interface system provides for sweep control and data acquisition in a Labview environment. Recording conditions were established to avoid artificial broadening of the EPR spectra.

Estimation of Degree of Dendrimer Functionalization. Upon formation of borate ester **3**, the proton chemical shift of the methylene proton next to the hydroxyl group ($NHC(S)NHCH_2CH_2C\boldsymbol{H_2}OH$) moved from 3.3 ppm (overlapped with broad dendrimer methylene protons peak) to 3.6 ppm, which was not overlapped with methylene proton signals from the dendrimer. Integration of this peak plus the central methylene peak ($NHC(S)NHCH_2C\boldsymbol{H_2}CH_2OH$ and $NHC(S)NHCH_2C\boldsymbol{H_2}CH_2OB$) at 1.5 ppm) indicates the number of borate ester equivalents that have formed (see Figures S3 and S4 of the Supporting Information; the central methylene peak of the borate ester, $NHC(S)NHCH_2C\boldsymbol{H_2}CH_2OB$, shifted downfield ~0.1 ppm and was visible as a shoulder on the left side of the central methylene peak, $NHC(S)NHCH_2C\boldsymbol{H_2}CH_2OH$). The number of equivalents of ethyl carbonate and of PROXYL-spin-label were estimated from ^1H NMR and MALDI analysis of hydrolyzed **4**. The ^1H NMR spectrum of hydrolyzed **4** shows a central methylene peak of ethyl carbonate ($NHC(S)NHCH_2C\boldsymbol{H_2}CH_2OC(O)OCH_2CH_3$) at 1.7 ppm and a hydroxyl methylene peak ($NHC(S)NHCH_2C\boldsymbol{H_2}CH_2OH$) at 1.5 ppm. The ratio of peak integrations for these peaks and the MALDI−TOF MS analysis of hydrolyzed **4** and of **6** were used for estimation of degree of functionalization by PROXYL.

Synthesis of Hydroxyl Functionalized G(4)-PAMAM Dendrimer 2. An aqueous solution of amine terminated G(4)-PAMAM dendrimer (418 μL of a 16.14% w/v solution in water, 67.5 mg, 5 μmol, M_w 13 500 g/mol, ~55 end groups) was lyophilized to afford a foamy residue. The residue was dissolved in 3 mL DMSO. To this solution 37.5 mg of 3-isothiocyanato-1-propanol (**1**) (320 mmol, 64 equiv) was added. The reaction mixture was stirred for 24 h at room temperature. After 24 h, an additional 10 mg of **1** (85 mmol, 17 equiv) was added and allowed to react for 24 h. The reaction mixture was dialyzed against DMSO (MW cutoff 1 kDa). The solution was lyophilized to give **2** as a colorless, oily solid, which was used without further purification. ^1H NMR (500 MHz, DMSO-d_6) δ 7.99 (bs, 1H, amide NHs), 7.81 (bs, 1H, amide NHs), 7.47 and 7.40 (bs, bs, 2H, $CH_2N\boldsymbol{H}C(S)N\boldsymbol{H}CH_2$), 4.47 (bs, 1H), 3.39 (bs, 6H), 3.13 and 3.07 (bs, bs, 5H), 2.66 (bs, 4H), 2.19 (bs, 4H), 1.59 (p, 2H, $C(S)NHCH_2C\boldsymbol{H_2}CH_2OH$) ppm. MALDI−TOF (pos) *m*/*z* 19 300.

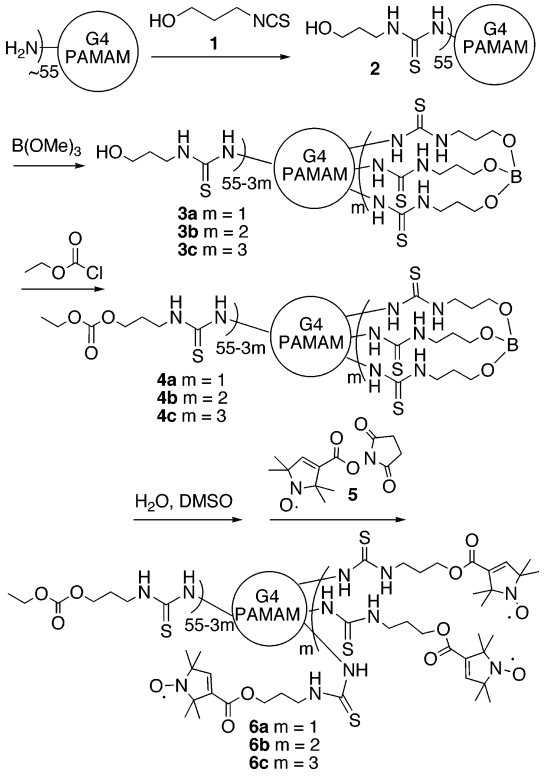
Formation of G(4)-PAMAM—Borate Ester Dendrimers 3a—c. To a 5 mL two-neck round-bottom flask equipped with an N_2 inlet and a rubber septum was added 50 mg of 4 Å MS. The flask was flame-dried under vacuum and flushed with N_2, and 50 mg of G(4)-PAMAM-OH **2** (2.6 μmol) dissolved in 1.5 mL DMSO-d_6 was added. Methyl borate (**3a**, 1.62 mg, 15.6 μmol; **3b**, 2.7 mg, 26 μmol; **3c**, 4.86 mg, 46.8 μmol) was added. The reaction mixture was stirred for 15 h at room temperature under N_2. The reaction mixture was concentrated in vacuo for 1 h at room temperature. To drive the reaction to completion, the reaction mixture was repeatedly freeze−pump−thawed under vacuum during a 5 h period. A 0.5 mL aliquot was removed for ^{11}B and ^1H NMR analysis. After confirming that triborate ester formation occurred without mono- or diborate formation, the product was used without purification. **3a**: ^1H NMR (500 MHz, DMSO-d_6) δ 8.02 (bs, 1H, amide NHs), 7.85 (bs, 0.8H, amide NHs), 7.49 and 7.42 (bs, bs, 1.7H, $CH_2N\boldsymbol{H}C(S)N\boldsymbol{H}CH_2$), 4.48 (bs, 0.7H), 3.65 (bt, 0.18H, $C(S)NHCH_2CH_2C\boldsymbol{H_2}OB$), 3.39 (bs, 6.3H), 3.13 and 3.07 (bs, bs, 5.3H), 2.67 (bs, 3.9H), 2.20 (bs, 3.9H), 1.59 (p, 1.7H, $C(S)NHCH_2C\boldsymbol{H_2}$-$CH_2OB$ and $C(S)NHCH_2C\boldsymbol{H_2}CH_2OH$) ppm. ^{11}B NMR (165 MHz, DMSO-d_6) 23 ppm. **3b**: ^1H NMR (500 MHz, DMSO-d_6) δ 8.0 (bs, 1H, amide NHs), 7.81 (bs, 0.8H, amide NHs), 7.47 and 7.40 (bs, bs, 1.8H, $CH_2N\boldsymbol{H}C(S)N\boldsymbol{H}CH_2$), 4.47 (bs, 0.7H), 3.66 (bt, 0.27H, $C(S)NHCH_2CH_2C\boldsymbol{H_2}OB$), 3.39 (bs, 6.5H), 3.13 and 3.07 (bs, bs, 4.9H), 2.67 (bs, 4.3H), 2.20 (bs, 4.1H), 1.59 (p, 1.9H, $C(S)$-$NHCH_2C\boldsymbol{H_2}CH_2OB$ and $C(S)NHCH_2C\boldsymbol{H_2}CH_2OH$) ppm. ^{11}B NMR (165 MHz, DMSO-d_6) 23 ppm. **3c**: ^1H NMR (500 MHz, DMSO-d_6) δ 8.0 (bs, 1H, amide NHs), 7.81 (bs, 0.8H, amide NHs), 7.47 and 7.40 (bs, bs, 1.6H, $CH_2N\boldsymbol{H}C(S)N\boldsymbol{H}CH_2$), 4.47 (bs, 0.7H), 3.66 (bt, 0.44H, $C(S)NHCH_2CH_2C\boldsymbol{H_2}OB$), 3.39 (bs, 5.9H), 3.13 and 3.07 (bs, bs, 4.2H), 2.67 (bs, 4.3H), 2.20 (bs, 3.1H), 1.59 (p, 1.57H, $C(S)NHCH_2C\boldsymbol{H_2}CH_2OB$ and $C(S)NHCH_2C\boldsymbol{H_2}CH_2OH$) ppm. ^{11}B NMR (165 MHz, DMSO-d_6) 23 ppm.

Formation of Ethyl Carbonate/Borate Ester G(4)-PAMAM dendrimers 4a—c. The DMSO solution of **3** was cooled to 0 °C, and 15 mg (130 μmol) of DMAP in 0.5 mL of dry pyridine and 12.4 μL (14.1 mg, 130 μmole) of ethyl chloroformate were added. The temperature was slowly raised to room temperature and the reaction mixture was stirred for 15 h under N_2. A 0.5 mL aliquot was removed for ^{11}B and ^1H NMR analysis. On the basis of spectral analysis of the aliquot, an additional 15 mg (130 μmole) of DMAP in 0.5 mL of dry pyridine and 12.4 μL (14.1 mg, 130 μmol) of ethyl chloroformate were added and allowed to react for an additional 24 h. A 0.5 mL aliquot was removed for ^{11}B and ^1H NMR analysis. After confirming that **4** had formed and that the triborate ester was intact and that partially hydrolyzed borate was not present, the reaction mixture was purified on Sephadex gel in DMSO. The borate ester of dendrimer **4** was hydrolyzed by dialysis against 1:1 DMSO−H_2O (MW cutoff 1 kDa), which was gradually changed to DMSO. The solution was lyophilized to afford a yellow oily solid, which was used without further purification.

The ^1H NMR and MALDI−TOF MS data are for the hydrolysis product formed from **4**. **Hydrolyzed 4a**: ^1H NMR (500 MHz, DMSO-d_6) δ 7.98 (bs, 1H, amide NHs), 7.76 (bs, 1H, amide NHs), 7.56 and 7.41 (bs, bs, 1.7H, $CH_2N\boldsymbol{H}C(S)N\boldsymbol{H}CH_2$), 4.05 (m, 3.9H, $CH_2C\boldsymbol{H_2}C(O)O\boldsymbol{CH_2}CH_3$), 3.38 (bs, 3.2H), 3.14 and 3.05 (bs, bs, 5.99H), 2.36 (bs, 2.5H), 2.16 (bs, 4.8H), 1.77 (p, 1.89H, $C(S)$-$NHCH_2C\boldsymbol{H_2}CH_2OC(O)O$), 1.58 (p, 0.2H, $C(S)NHCH_2C\boldsymbol{H_2}CH_2OH$), 1.18 (t, 3.1H, $C(O)OCH_2C\boldsymbol{H_3}$) ppm. MALDI−TOF (pos) *m*/*z* 21 300. **Hydrolyzed 4b**: ^1H NMR (500 MHz, DMSO-d_6) δ 7.99 (bs, 1H, amide NHs), 7.77 (bs, 1H, amide NHs), 7.59 and 7.44 (bs, bs, 1.2H, $CH_2N\boldsymbol{H}C(S)N\boldsymbol{H}CH_2$), 4.05 (m, 3.4H, $CH_2C\boldsymbol{H_2}C(O)$-$O\boldsymbol{CH_2}CH_3$), 3.39 (bs, 5.9H), 3.13 and 3.04 (bs, bs, 5.58H), 2.20 (bs, 4.48H), 1.77 (p, 1.84H, $C(S)NHCH_2C\boldsymbol{H_2}CH_2OC(O)O$), 1.58 (p, 0.27H, $C(S)NHCH_2C\boldsymbol{H_2}CH_2OH$), 1.2 (t, 2.82H, $C(O)OCH_2C\boldsymbol{H_3}$) ppm. MALDI−TOF (pos) *m*/*z* 21 200. **Hydrolyzed 4c**: ^1H NMR (500 MHz, DMSO-d_6) δ 7.99 (bs, 1H, amide NHs), 7.78 (bs, 0.9H, amide NHs), 7.59 and 7.44 (bs, bs, 1.06H, $CH_2N\boldsymbol{H}C(S)N\boldsymbol{H}CH_2$), 4.05 (m, 2.75H, $CH_2C\boldsymbol{H_2}C(O)O\boldsymbol{CH_2}CH_3$), 3.39 (bs, 5.8H), 3.13 and 3.05 (bs, bs, 5.95H), 2.63 (bs, 3.58H), 2.4 (bs, 1H), 2.16 (bs, 2.7H), 1.77 (p, 1.13H, $C(S)NHCH_2C\boldsymbol{H_2}CH_2OC(O)O$), 1.58 (p, 0.25H, $C(S)NHCH_2C\boldsymbol{H_2}CH_2OH$), 1.16 (t, 1.9H, $C(O)OCH_2C\boldsymbol{H_3}$) ppm. MALDI−TOF (pos) *m*/*z* 20 700.

Formation of Ethyl Carbonate/PROXYL-Spin-Label Functionalized Dendrimers 6a—c. To a 5 mL two-necked round-bottom flask equipped with N_2 inlet and rubber septum was added 50 mg of 4 Å MS, and the flask was flame-dried under vacuum and flushed with N_2. Hydrolyzed **4** (30 mg) was dissolved in 0.7 mL DMSO-d_6. PROXYL-spin-label **5** (1 mg, 3.5 mmol) dissolved in 0.5 mL dry pyridine and 0.35 mg of triethylamine (3.5 mmol, 0.25 μL) was added. The reaction mixture was protected from light and stirred at room temperature for 24 h under N_2. After 24 h, an

Scheme 1. Synthesis of Ethyl Carbonate/PROXYL Functionalized Dendrimers



additional 1 mg (3.5 mmol) of **5** in 0.5 mL of dry pyridine and 0.25 μL (0.35 mg, 3.5 mol) of triethylamine were added and allowed to react for an additional 24 h. The reaction mixture was purified on Sephadex gel in DMSO and further purified by dialysis against DMSO (MW cutoff 1 kDa) in the dark. The solution was lyophilized to give a yellow oily solid, which was used for EPR analysis. **6a**: MALDI−TOF (pos) *m*/*z* 21 400. **6b**: MALDI−TOF (pos) *m*/*z* 21 500. **6c:** MALDI−TOF (pos) *m*/*z* 22 000.

Results and Discussion

Functionalization of G(4)-PAMAM Dendrimer. In order to introduce order to the dendrimer end groups, we selectively spin-labeled three neighboring dendrimer end groups to form **6** as shown in Scheme 1. Although 64 endgoups are theoretically present on a G(4)-PAMAM, we have shown 55 end groups in Scheme 1 because the weight-average molecular weight (M_W) for unfunctionalized G(4)-PAMAM corresponds to a macromolecule with 55 end groups.[16] First, hydroxyl functionalization of the G(4)-PAMAM dendrimer with 3-isothiocyanato-1-propanol (**1**) was performed to afford **2**. Borate esters **3** were synthesized by transesterification of methyl borate with alcohol functionalized dendrimer **2**. Azeotropic coevaporation was necessary, as the low boiling point of the methanol-methyl borate azeotrope (bp 54.5 °C, nearly equimolar in the two components) provided a convenient means of driving the reaction to completion.[17]

The formation of borate ester **3** was monitored in situ by ^{11}B NMR. The boron resonance (in DMSO-d_6) shifted from 18 ppm (methyl borate) to 23 ppm (**3**) upon exchange of all three ligands (Figure 1a). ^{11}B NMR characterization indicates that the boron is triply tethered to the dendrimer, since monoborate and diborate ester resonances are upfield of the observed signal (Figure 1b; see also Figure S6 in the Supporting Information). The chemical shift of the borate ester depends on the atoms directly coordinated to the boron center and on the coordination geometry of the boron atom.[18] The peak at 4 ppm is most likely

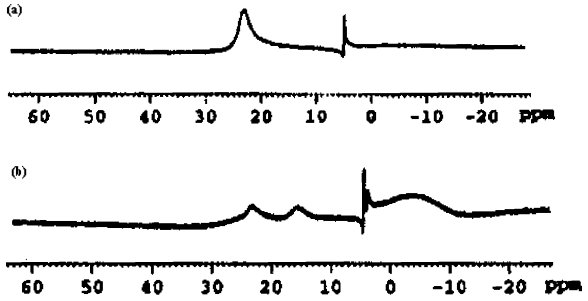


Figure 1. ^{11}B NMR of (a) triborate ester peak of **3c** at 23 ppm and (b) partially hydrolyzed borate ester **3c** with a mono and diborate peak at 17 ppm. Chemical shifts are relative to $BF_3 \cdot Et_2O$ and are externally referenced.

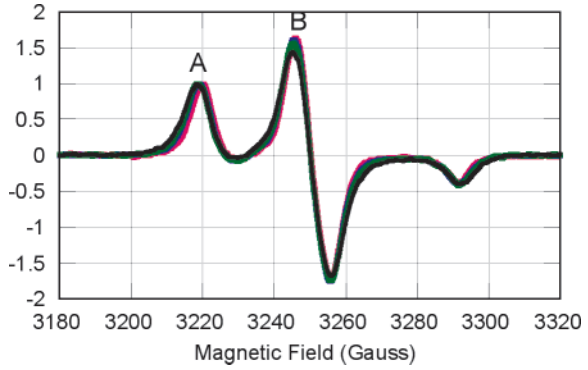


Figure 2. EPR spectra of **6a**−**c**, normalized to peak A. Pink = 20% random PROXYL label, blue = **6a**, green = **6b**, and black = **6c**.

a tetracoordinate borate that is not tethered to a dendrimer, since the location of the peak is consistent with this assignment[19] and since the narrow line width suggests a small molecule. The broad upfield rise is from the boron in the glass sample tube and in the inset.

Reaction of **3** with ethyl chloroformate was performed to afford **4**, where the remaining hydroxyl groups were masked. ^{11}B NMR spectra were obtained during the reaction to ensure that the borate ester was not prematurely hydrolyzed. After hydrolysis of borate ester **4**, dendrimers were functionalized by reaction with PROXYL NHS ester **5** to form **6**. The degree of functionalization was determined by ^{1}H NMR and MALDI−TOF MS.

EPR Studies. EPR spectroscopy was used to evaluate the dynamics of end groups. The ratio of the EPR spectral amplitudes at the positions marked "A" and "B" in Figure 2 provides a useful measure, analogous to that introduced by Korkorin,[20] of the interspin broadening. The A/B peak height ratio has shown itself to be a reasonable measure of linebroadening for our system[14a] and also has strong precedent in other systems.[21] Dendrimers bearing spin-labels that are in close proximity to one another, either because of high loading with spins or because lower spin loadings are arranged such that the spins are close together, have spectra with significant linebroadening (A:B > 1). Dendrimers with randomly or maximally separated spin-labels induce lower linebroadening effects (A:B < 1). The calculated A:B ratios for dendrimers with clustered, randomly distributed, and maximally separated spin-labels, as compared to the A:B ratios for **6a**−**c**, are shown in Table 1.

As shown in Table 1, the A:B ratios obtained from spectra of **6a**−**c** strongly suggest that the PROXYL groups are not clustered together. The difference between a random distribution and a maximally separated distribution of spin-labels cannot be readily determined at the loadings obtained for **6**, although a random distribution is most likely.[22]

Table 1. Comparison of A:B Ratios for Different Dendrimer Functionalization Patterns on a G(4)-PAMAM and for Experimentally Obtained Results with 6a−c[a]

	A:B ratio		
spin-label distribution	for 3 spins	for 6 spins	for 9 spins
clustered	0.95	1.05[b]	1.1[b]
random	0.72	0.75	0.76
maximally separated	0.72	0.72	0.72
6	0.61 (**6a**)	0.63 (**6b**)	0.69 (**6c**)

[a] Calculated values are taken from ref 14a. [b] These computed values are maximal values, as 2 (**6b**) and 3 (**6c**) clusters of 3 spins each may occur, reducing the A:B ratio.

The EPR spectra obtained for **6a−c** are shown in Figure 2 (spectra are normalized to peak A). EPR spectra of PROXYL-spin-labeled G(4)-PAMAM dendrimers **6a−c** showed no significant linebroadening effects, indicating that the spin-labels are not in close proximity to one another. Analysis of EPR spectra of **6a−c** suggests that, when end groups are not covalently tethered together, the dynamic and highly flexible nature of the PAMAM dendrimer induces end group distribution. Even for end groups that are initially clustered by triboronate ester formation, distribution results after release of the tether.

Conclusions

Ethyl carbonate/PROXYL-spin-label functionalized PAMAM dendrimers **6a−c** have been synthesized and characterized. The EPR spectra of dendrimers **6a−c** indicate that when end groups are not covalently tethered together, the dynamic and highly flexible nature of the PAMAM dendrimer induces end group distribution. Initial end group proximity is not enough to appreciably alter end group presentation on heterogeneously functionalized PAMAM dendrimers. To introduce order on a PAMAM dendrimer surface, we conclude that the end groups should be permanently covalently tethered together.

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Supporting Information Available: Figures showing spectra for **2**, **3**, **4**, and **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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