

# An EPR and Fluorescence Depolarization Study of Intermolecular Interactions of Dendrimers at Medium and Highly Concentrated Aqueous Solutions

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The intermolecular dendrimer interactions between poly (amidoamine) dendrimers (SBDs) of different generations ( $G = 2, 4, \text{ and } 6$ ) were studied in aqueous solution by EPR and fluorescence depolarization. For the EPR studies a spin probe (TEMPO) was covalently linked to the SBDs, and for the fluorescence depolarization studies a fluorophor (fluorescein) was covalently linked to the SBDs. The mobility of the TEMPO labeled SBDs was determined by EPR over a range of the concentration of added SBDs (unlabeled SBD of the same generation) from 0.01 to 75% (w/w) in water solutions. The mobility of the TEMPO label was not influenced by added SBD below a concentration of approximately 5%, suggesting that no significant interdendrimer interactions, such as aggregation, occurred. Above a concentration of 30% SBD in water (w/w), the mobility of the TEMPO label decreased dramatically; the effect is mostly attributed to the viscosity increase at such high concentrations. Fluorescence depolarization studies, employing fluorescein-labeled dendrimers are in agreement with the EPR studies. © 2002 Elsevier Science (USA)

**Key Words:** dendrimer; EPR; nitroxide; fluorescence polarization.

## INTRODUCTION

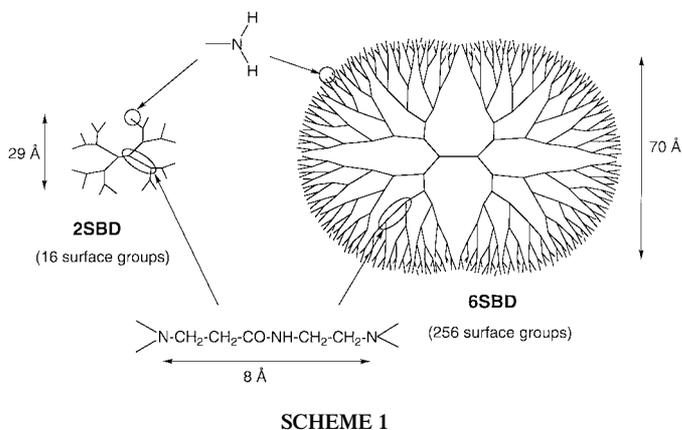
Starburst dendrimers (SBD) are a novel class of macromolecules possessing a well-defined molecular composition and constitution (1–3). The molecular architecture of dendrimers can be created by stepwise attachment of repeat unit layers or branches, termed generations, on a central core. The SBDs used in our studies are generated from amidoamine units emanating from an ethylenediamine core, terminated with amino groups at the surface (Scheme 1) (1). These water-soluble macromolecules are uniform in size for a given generation, with a high density of primary amino groups mostly in close proximity to the surface (4). The sizes of the SBDs systematically increase from ca. 2 to 15 nm as the generations ( $G$ ) increase from  $G = 1$  to 10. In the past a series of studies were performed to investigate the intramolecular morphology of dendrimers using photochemical and spectroscopic probe methods (5–8). However, little attention has been given to investigations of interdendrimer

interactions (9–12). Uppuluri *et al.* employed rheological methods to study the flow behavior of dendrimers in ethylenediamine solutions (9). It was found that over the entire range of shear rates and shear stress applied, the dendrimer solutions showed Newtonian flow behavior with constant viscosities with respect to both shear rates and shear stress. It was also found that with increasing generation of dendrimers the viscosity increases and, especially for the later generations, no evidence for entanglements of chains between different dendrimer molecules was observed (9, 10). Most traditional polymers of similar molecular weight would be highly entangled at the higher concentrations investigated. The low degree of entanglement between separate dendrimers was explained by the surface congestion and noninterpenetrability of the individual dendrimer molecules especially for the later generations (9, 10).

In this report we investigated possible self-aggregation of dendrimer molecules by two spectroscopic methods, EPR spectroscopy and fluorescence depolarization studies. For the EPR studies a spin probe (TEMPO) was covalently linked to the dendrimer, and for the fluorescence depolarization studies a fluorophor was covalently linked to the dendrimers. Both probes are sensitive to motion. The mobility of the probe labeled dendrimer should decrease, if aggregates of dendrimers are formed.

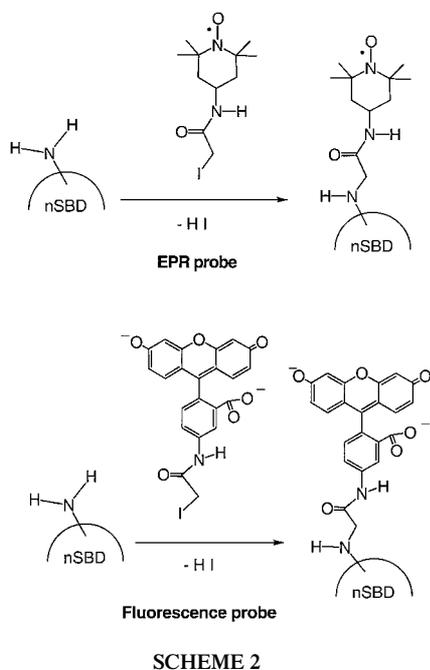
## EXPERIMENTAL SECTION

The synthesis and characterization of SBDs have been previously described in detail (1). Labeling of the dendrimers (nSBD-T;  $n = 2, 4, 6, 8$ ) with TEMPO was accomplished employing a modified method used by Pauly *et al.* for labeling DNA-nucleosides (13). To a solution of 2 mmol (in surface groups) of nSBD in 20 mL, 0.05 mmol 4-(2-iodoacetamide)-TEMPO (Molecular Probes) in 1 mL DMF was added and stirred for 6 h at room temperature. The mixture was then extracted six times with methylene chloride and hexane to remove the free TEMPO. For further purification, dialysis in water was used employing Spectra/Por cellulose ester membranes from Spectrum



Medical Industries, Inc. (MWCO:10000 for 6SBD-T and 8SBD-T; MWCO:2000 for 2SBD-T and 4SBD-T). The labeling grade was evaluated by EPR as one label for approximately 50 surface amino groups of SBD. Fluorescein labeled dendrimers (2SBD-FI) were synthesized analogously as nSBD-T using 5-iodoacetamidofluorescein (Molecular Probes) and purified by dialysis. The labeling grade was evaluated by UV-vis absorption spectroscopy as one label for approximately 80 surface amino groups of SBD. The reaction schemes for both syntheses are shown in Scheme 2.

The EPR spectra were recorded using a Bruker EMX spectrometer operating at X band (9.5 GHz). Fluorescence polarization experiments were performed on a Spex FluoroMax-2 Spectrometer using automated Glan Thompson polarizers. All measurements were performed at 20°C with air saturated aqueous solutions in millipore filtered water.



## RESULTS AND DISCUSSION

The EPR spectrum of 2SBD-T was measured in diluted aqueous solutions (0.1 mM) and is shown in Fig. 1 (Spectrum a). The spectrum displays a triplet with a  $^{14}\text{N}$  hyperfine splitting of 16.8 G, which is typical for TEMPO derivatives in aqueous solutions (14). The mobility parameter, the average correlation time for motion ( $\tau_c$ ), was determined employing Eqs. [1]–[3], where  $\Delta H_0$  is the line width of the central line,  $h_0$ ,  $h_1$ , and  $h_{-1}$  are the peak heights for the central line, low field line, and high field line, respectively:

$$\tau_B = B^* \Delta H_0 [(h_0/h_1)^{1/2} - (h_0/h_{-1})^{1/2}] \quad [1]$$

$$\tau_C = B^* \Delta H_0 [(h_0/h_1)^{1/2} + (h_0/h_{-1})^{1/2} - 2] \quad [2]$$

$$\tau_c = (\tau_B \tau_C)^{1/2}. \quad [3]$$

Considering anisotropic Brownian motion, two correlation times for motion were calculated,  $\tau_B$  and  $\tau_C$ , and averaged according to Eq. [3]. The constants  $B^*$  and  $C^*$  were determined earlier for aqueous solutions ( $B^* = C^* = 6.5 \times 10^{-10}$  s/gauss) (15). For further details of the calculation of  $\tau_c$  see Refs. (15–19).

For 2SBD-T in aqueous solutions at 20°C a rotational correlation time of  $\tau_c = 0.23$  ns was determined. With increasing dendrimer generation the size and molecular weight increase, as does the surface congestion (1, 8). Therefore, rotational motion of a TEMPO labeled dendrimer should decrease with increasing dendrimer generation. Indeed,  $\tau_c$  increases for 4SBD-T, 6SBD-T, and 8SBD-T ( $\tau_c = 0.29, 0.43, 0.61$  ns, respectively).

To study interdendrimer interactions, the EPR spectra of solutions of 2SBD-T (0.1 mM) in the presence of different amounts of 2SBD (unlabeled dendrimer) were recorded. If the dendrimers interact with each other, e.g., forming aggregates, then the mobility of the TEMPO label should decrease and would be reflected by an increase in  $\tau_c$ . Up to a concentration of about 10 mM of 2SBD (3.26%, w/w), no spectral change in the EPR spectra could be observed, and accordingly,  $\tau_c$  remained constant (0.23 ns). At concentrations above 10 mM of 2SBD spectral changes were observed. Figures 1b and 1c show examples of typical EPR spectra at higher concentration of 2SBD. At very high concentrations of 2SBD (above 50%, w/w) the anisotropic components of the magnetic tensor parameters get resolved, which is common for radicals under slow motion condition. As a result the spectrum changes from a “three-line-spectrum” (Figs. 1a and 1b) into a “slow motion spectrum” (Fig. 1c). Employing Eqs. [1]–[3] the mobility parameter ( $\tau_c$ ) was calculated for three-line-spectra and plotted against the concentration of 2SBD (Fig. 2).

In case of the broad anisotropic spectra (e.g., spectrum c of Fig. 1) Eqs. [1]–[3] do not apply. Therefore, the mobility parameter was determined by computer simulation of the spectral line shape using the program by Schneider and Freed (20). The following assumptions were made, which were reasonable, since the chemical variations among the investigated systems are small: (i) The  $g_{ii}$  components of the  $\mathbf{g}$  tensor for the

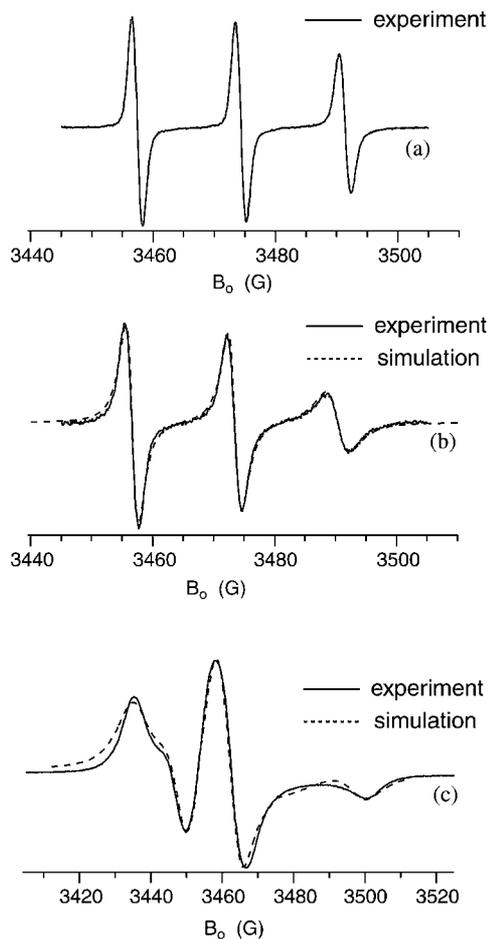


FIG. 1. EPR spectra of aqueous 2SBD-T solutions (0.1 mM) in the presence of different amounts of 2SBD (0% (a); 44% (b); 74% (c) w/w) at 20°C. Solid lines, denote experimental spectra; dashed lines, simulated spectra.

coupling between the electron spin and the magnetic field ( $g_{xx} = 2.009$ ;  $g_{yy} = 2.006$ ;  $g_{zz} = 2.002$ ) are constant for all the spectra. (ii) The anisotropy of motion is constant ( $N = D_{//}/D_{\perp} = 10$ , where  $D_i$  are the components of the diffusional tensor). (iii) The Brownian model of diffusional rotational motion applies ( $D_i = 1/(6\tau_i)$ , where ( $\tau_{\perp}$ ) ( $\tau_{//}$ ) are the perpendicular and parallel components of the correlation time, respectively). To accomplish a good agreement between the experimental spectra and the simulation, a tilt of the main rotational axis ( $z$  towards  $x$ ) was included into the computation. This means that the TEMPO label is tilted from the perpendicular direction with respect to the dendrimer surface (20). This effect has already been found for the interaction of the TEMPO-labeled dendrimers with vesicles (21). For comparison, the program by Schneider and Freed (20) was also employed to simulate the EPR spectrum of 2SBD-T in the presence of 44% 2SBD in aqueous solution, which still displays a “three-line-spectrum” (Fig. 2b), but the rotational correlation time calculated according to Eqs. [1]–[3] is relatively high ( $\tau_c = 1.2$  ns). To compare the correlation times  $\tau_{\perp}$  and  $\tau_{//}$  from spectra simulation with  $\tau_c$ , calculated with Eqs. [1]–[3],

an averaged correlation time was obtained from the simulation ( $\tau_c = (\tau_{\perp} \tau_{//})^{1/2}$ ).

Similar experiments as described above were also performed for higher generation dendrimers, 4SBD and 6SBD. EPR spectra of 4SBD-T and 6SBD-T were recorded in the presence of different amounts of unlabeled 4SBD and 6SBD, respectively, and the correlation times ( $\tau_c$ ) were calculated. Figure 2 shows the dependence of  $\tau_c$  on the concentration of nSBD (Fig. 2 (top)) concentration in dendrimer molecules, Fig. 2 (bottom) concentration in wt%). Interestingly, the mobility of the TEMPO label was not influenced by added SBD below a concentration of approximately 5%, suggesting that no interdendrimer interactions such as aggregation occurred.

Above a concentration of 30% SBD in water (w/w) the mobility of the TEMPO label decreased dramatically; the effect is at least partly attributed to the viscosity increase at such high concentrations. Uppuluri *et al.* studied the flow behavior of highly concentrated dendrimers in ethylenediamine solutions (9). It was reported that the viscosity increases sharply with increasing dendrimer concentration. Therefore, we conclude that the main cause for mobility decrease of the TEMPO label at SBD concentrations above 30% is viscosity increase. But viscosity increase alone cannot explain all the results. The mobility of the TEMPO

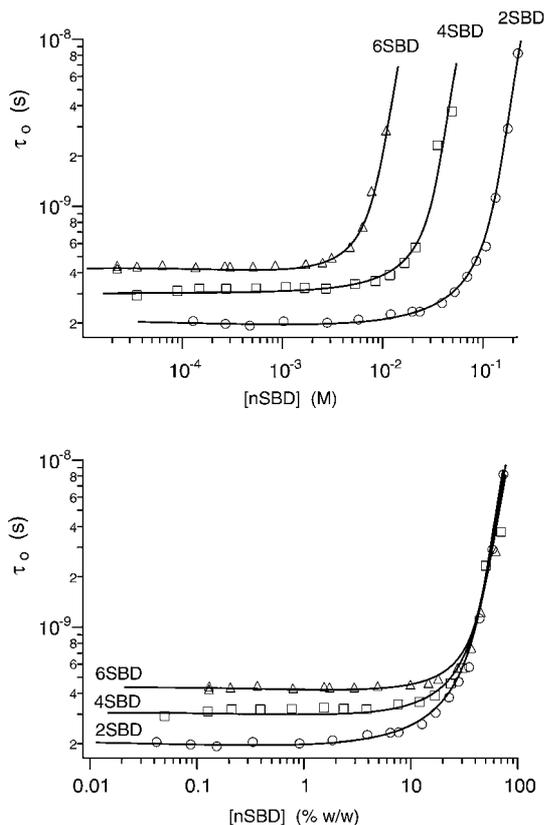


FIG. 2. Rotational correlation time of nSBD-T in aqueous solutions at 20°C in the presence of different amounts of nSBD (2SBD; 4SBD; 6SBD). (Top)  $\tau_c$  vs concentration of nSBD in molecules; (bottom)  $\tau_c$  vs nSBD concentration in % (w/w); [2SBD-T] = 0.1 mM; [4SBD-T] = 0.03 mM; [6SBD-T] = 0.01 mM.

TABLE 1  
Parameters Used for Simulation of EPR Spectra<sup>a</sup>

Generation	Concentration (%, w/w)	$\tau_{\perp}$ (ns) <sup>a</sup>	Tilt angle <sup>b</sup> (degree)
2SBD	44	2.0	60
2SBD	58	6.0	65
2SBD	74	26	70
4SBD	50	6.5	65
6SBD	45	2.0	60
6SBD	63	9.0	65

Note. The hyperfine A tensor components of nSBD-T were found to be  $A_{xx} = 6.0$  G,  $A_{yy} = 6.5$  G, and  $A_{zz} = 38$  G.

<sup>a</sup>  $\tau_{\perp}$ , perpendicular component of correlation time.

<sup>b</sup> Tilt angle of the main rotational axis of the nitroxide ( $z$  toward  $x$ ) with respect to the perpendicular direction from the dendrimer surface.

label above 30% SBD was similar for different generations at a fixed dendrimer concentration (w/w), which is not expected, since the viscosity of the solution for later generation dendrimers ( $G = 6$ ) is higher than for lower generations ( $G = 2$ ) (9). This suggests slightly stronger intermolecular dendrimer interactions for lower generations ( $G = 2$ ) than for later generations ( $G = 6$ ) at SBD concentrations above 30%. This is consistent with the notion, that the lower generations possess an open “starfish” structure, whereas the later generations possess a more closed and nearly spherical external surface (5, 8, 22). The open structure of the lower generations could allow for attractive interactions between the dendrimer molecules at high concentrations, which is prevented for the later generations due to the surface congestion.

At dendrimer concentrations higher than about 50% (w/w) the EPR spectra change from a “three-line-spectrum” (e.g., Fig. 1a,b) into a “slow motion spectrum” (e.g., Fig. 1c). Along with the increase in correlation time ( $\tau_{\perp}$ ) the tilt angle of the TEMPO label toward the dendrimer surface increases (Table 1). This can be explained by interactions of the TEMPO label of one dendrimer molecule with the surface groups of a neighboring dendrimer molecule. At high dendrimer concentrations, such as 50%, it is expected that the dendrimer molecules collide frequently with each other, which supports interactions of TEMPO labels of one dendrimer with the surface groups of another dendrimer. The increase in tilt angle with increasing dendrimer concentrations is in agreement with the increase in correlation time and viscosity.

Studying spin–spin interactions between TEMPO labels by EPR spectroscopy is another method for probing interactions between dendrimer molecules. If two TEMPO molecules interact with each other, either statically due to their vicinity or dynamically through collisions of mobile radicals, the EPR spectrum changes (1). In the experiments shown above, to a low concentration of TEMPO-labeled dendrimers (e.g., [2SBD-T] = 0.1 mM) unlabeled dendrimers were added. At these low SBD-T concentrations the TEMPO labels do not significantly interact with one other and spin–spin exchange was not observed. In the follow-

ing experiment the concentration of the TEMPO labeled dendrimers was increased systematically (e.g., 2SBD-T: from 0.1 to 40 mM). If the dendrimers interact with each other, e.g., forming aggregates, then line broadening of the EPR due to spin–spin interaction should be observed. Up to a concentration of about 10 mM of 2SBD-T (3.26%, w/w), which corresponds to a TEMPO concentration of approximately 0.4 mM, no modification in the EPR lineshape was observed, suggesting that no interdendrimer interactions such as aggregation occurred. At concentrations above 10 mM of 2SBD-T spectral changes were observed. The major cause for the spectral changes is probably attributed to the decrease in mobility, which was also observed in the experiments, in which unlabeled dendrimers were added to a low concentration of TEMPO labeled dendrimers ([2SBD-T] = 0.1 mM) (see above). In addition, dynamic and static interactions between 2SBD-T could cause additional spectral changes. Due to this complexity, the EPR spectra at [2SBD-T] > 10 mM were not further analyzed. Similar results were also observed with 6SBD-T.

To confirm the reliability of the EPR label in reporting dendrimer interactions, a second, completely independent fluorescence label was examined. Fluorescence depolarization is a widely used method for investigating the mobility of molecules (23). To study interdendrimer interactions, a fluorophore (fluorescein) was covalently linked to dendrimers ( $G = 2$ ). Upon excitation of the fluorescence label (fluorescein) with linearly polarized light, the emission from fluorescein is also polarized. Due to rotational diffusion during its excited-state lifetime, some polarization is lost. The polarization loss is determined by the polarization value ( $p$ ), which is calculated by Eq. [4] from fluorescence intensities of the vertically and horizontally polarized emission,

$$p = (I_{//} - I_{\perp}) / (I_{//} + I_{\perp}), \quad [4]$$

where ( $I_{//}$ ) is the polarized fluorescence intensity parallel and

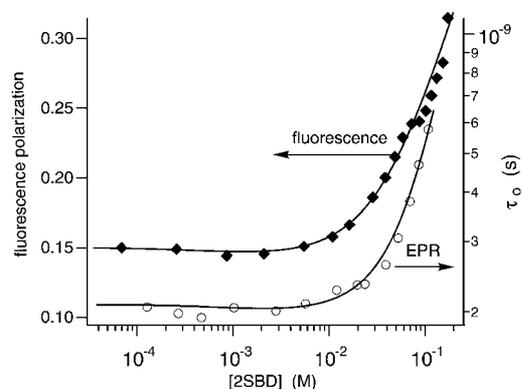


FIG. 3. Fluorescence polarization of 2SBD-FI (left axis, linear scale) ( $\lambda_{ex} = 485$  nm,  $\lambda_{em} = 525$  nm, [2SBD-FI] =  $1 \times 10^{-5}$  M) and rotational correlation time of 2SBD-T (right axis, logarithmic scale) ([2SBD-T] = 0.1 mM) in the presence of different amounts of 2SBD in aqueous solutions.

( $I_{\perp}$ ) perpendicular to the polarized excitation light (23). In aqueous solutions a polarization value of 0.15 was found for 2SBD-FI at a concentration of  $1 \times 10^{-5}$  M. Addition of unlabeled dendrimer (2SBD) up to a concentration of about 10 mM of 2SBD did not change the polarization value (Fig. 3). Above 10 mM of 2SBD the polarization increased due to hindered rotation. For comparison, the rotational correlation time, determined by EPR, vs dendrimer concentration is also shown in Fig. 3. It can be seen that fluorescence polarization studies and EPR results are in excellent agreement. With both methods no inter-dendrimer interactions were observed below a concentration of 10 mM of 2SBD. Above 10 mM of 2SBD the polarization value and the rotational correlation time from EPR measurements increase.

### SUMMARY AND CONCLUSION

The mobility of the TEMPO labeled SBDs ( $G = 2, 4,$  and  $6$ ) were determined by EPR over a range of the concentration of added SBDs (unlabeled SBD of the same generation) from 0.01 to 75% (w/w) in water solutions. Interestingly, the mobility of the TEMPO label was not influenced by added SBD below a concentration of approximately 5%, suggesting that no significant interdendrimer interactions such as aggregation occurred. Above a concentration of 30% SBD in water (w/w) the mobility of the TEMPO label decreased dramatically; the effect is mostly attributed to viscosity increase at such high concentrations. Fluorescence depolarization studies, employing fluorescein-labeled dendrimers, are in agreement with the EPR studies.

### ACKNOWLEDGMENTS

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