

Carboxylated Starburst Dendrimers as Calibration Standards for Aqueous Size Exclusion Chromatography

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Standards commonly employed to calibrate size exclusion chromatography columns usually do not themselves have well-defined sizes. Carefully characterized spherical solutes in the appropriate size range are therefore of considerable interest. The chromatographic behavior of carboxylated starburst dendrimers—characterized by quasielastic light scattering and viscometry—on a Superose size exclusion column has been explored. Carboxylated starburst dendrimers appear to behave as noninteracting spheres during chromatography in the presence of an appropriate aqueous mobile phase. The dependence of the retention time on solute size seems to coincide with data collected on the same column for Ficoll, an uncharged, quasi-spherical polysaccharide. Chromatography of the dendrimers yields a remarkable correlation of the chromatographic partition coefficient with the generation number; this result is in part a consequence of the exponential relationship between the generation number and the molecular volume of the dendrimers.

INTRODUCTION

Size exclusion chromatography (SEC) is often referred to as a relative molecular weight (MW) method, in the sense that a chromatographic system must be calibrated with standards of known molecular weight. There are in fact three aspects to the problem of obtaining correct MW values from SEC. The first is the determination of the relationship between the solutes' molecular sizes and the retention time for the column in use. This can be carried out in principle with almost any well-characterized set of solutes, preferably of narrow molecular weight distribution. However, we must recognize at the outset that the molecular size relevant to SEC may not be well-defined (strong convictions exist in the biochemical community that this size is the Stokes radius¹ and in the polymer community that it is the viscosity radius,^{2,3a} but this matter is unresolved⁴ at the present time). The second requirement is that of knowing the relationship between the

molecular dimension and the molecular weight for the solute of interest. Such relationships are often available, e.g. via the Mark-Houwink equation for flexible chain macromolecules.⁵ The third consideration is the question of solute-packing interactions; if the analyte or the reference compounds are adsorbed onto or partitioned into the stationary phase, the separation is not based on size alone, and attempts to determine either molecular dimensions or mass are probably useless. In practice, the first two questions are usually answered simultaneously: if the calibration is done with compounds similar in structure to the analyte, it is assumed that the empirical relationship between MW and retention time so established is valid for the unknown. The third step is usually not studied explicitly, but the absence of significant interactions is commonly assumed on the basis of prior experience with a given class of solutes on a particular column.

It should be evident from the preceding comments that the ideal calibration standards would fulfill the following requirements: (a) their MW distributions should be negligibly small; (b) it should be possible to determine their molecular dimensions in an unambiguous manner; (c) they should not adsorb to the packing in question. Few solutes satisfy these criteria, and the situation is particularly problematic in the area of aqueous SEC. Proteins, which are commonly used for calibration standards, fulfill item (a) but are less satisfactory with regard to (b) and (c). Typically, one finds deviations around the best common curve for any calibration curve constructed with proteins, and it is difficult to know whether these deviations arise from variations of geometry for the different solutes (some of which are more spherical than others) or from differences in solute-packing interactions, due to differences in protein charge character or hydrophobicities. Nonionic polysaccharides, such as dextrans or pullulans, are representative of the class of flexible-chain water-soluble polymers. These are structurally uniform and do not interact with any stationary phase, but the interpretation of measured dimensions—such as radius of gyration or viscosity radius—in terms of the molecular volume controlling SEC is problematic. We have previously utilized in this regard fractions of Ficoll, a densely branched nonionic polysaccharide which may more closely approach a noninteracting sphere. However, its molecular weight polydispersity and its deviation from full compactness both impose difficulties in interpretation of SEC partition coefficients measured at chromatographic peaks vis-a-vis dimensional measurements, which themselves correspond to different averages over the distributions of molecular weights and conformations.

Carboxylated starburst dendrimers⁶ would appear to embody several of the features sought for aqueous SEC standards. While these materials are not yet commercialized,

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(1) Cantor, C. R.; Schimmel, P. R. *Biophysical Chemistry. Part II. Techniques for the Study of Biological Structure and Function*; W. H. Freeman: New York, 1980; p 675.

(2) See, for example: Rosen, S. L. *Fundamental Principles of Polymeric Materials*; John Wiley & Sons: New York, NY, 1982; p 70.

(3) The term "hydrodynamic radius" is inexact because either the intrinsic viscosity or the diffusion coefficient may be used to obtain an "equivalent sphere" radius for any solute, the former via the Einstein relation and the latter via Stokes' equation. Although both of these are hydrodynamic measurements, the radii so obtained will not be identical for a highly asymmetric solute, since the first arises from energy transfer among adjacent solvent layers, and the latter from the frictional solute-solvent interface. Therefore, we follow Potschka^{3b} in referring to R_v as the viscosity radius. (b) Potschka, M. *Anal. Biochem.* 1987, 162, 47.

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Table I. Hydrodynamic and Chromatographic Characterization of Carboxylated Starburst Dendrimers

generation	10 ⁻³ MW	[η], cm ³ /g	R_{η} , nm ^a	10 ⁶ D , cm ² /s	R_s , nm ^b	K_{SEC} ^c
0.5	0.92			2.66	0.92	0.74
1.5	2.17			1.91	1.28	0.68
2.5	4.67	4.15	1.45	1.67	1.47	0.62
3.5	9.67	4.58	1.92	1.00	2.45	0.56
4.5	19.7	4.77	2.46	0.79	3.10	0.51
5.5	39.7	4.88	3.13	0.67	3.65	0.45
6.5	79.6	5.16	4.03	0.54	4.50	0.39
7.5	160.	5.87	5.30	0.41	5.95	0.34

^a From data in columns 2 and 3, via eq 4. ^b From data in column 5, via eq 3. ^c From measured peak retention volumes, via eq 5.

their uses in many areas of research have been explored. With regard to SEC, their compact and symmetrical structures could make them rather close to rigid spheres so that the dimensions obtained by a variety of scattering or hydrodynamic techniques could be unambiguously identified with the dimension controlling SEC migration. The mode of synthesis guarantees that they are monodisperse. Lastly, the negative charge on the distal groups would prevent adsorption, since nearly all commonly used aqueous SEC packings have some negative surface charge.⁷ With regard to repulsion effects, one would expect some decrease in retention times at higher pH's and low ionic strength, under which conditions the negative charge on typical SEC stationary phases would enhance the exclusion of these solutes. These effects become essentially negligible at moderate ionic strength and pH on most columns.

In the current work we evaluate carboxylated starburst dendrimers (hereinafter referred to simply as dendrimers) as calibration standards on a Superose column. For this stationary phase, we know that electrostatic repulsive forces are negligible, even for, e.g., highly charged DNA fragments, in a 0.38 M pH 5.8 phosphate mobile phase⁸ and furthermore that proteins of various isoelectric points elute along a common curve in this same medium.⁹ In order to properly characterize the dendrimers, we obtained the Stokes radius by quasielastic light scattering (QELS) and the viscosity radius from the known MW and intrinsic viscosity (all measurements made in the SEC mobile phase solvent). The nature of the calibration curve obtained with these solutes is discussed vis-a-vis current understanding of the SEC separation mechanism.

EXPERIMENTAL SECTION

Materials. Carboxylated starburst dendrimers were prepared by methods described elsewhere.¹⁰ These molecules are synthesized by a series of stepwise condensation reactions on an initial trifunctional amine, and consequently molecular size grows in three dimensions with each subsequent step (called a "generation").¹⁰ The samples employed in these studies are described in Table I. All measurements were made in a 9:1 mixture of NaNO₃:NaH₂PO₄, pH = 5.5, 0.38 M, which had been previously shown to minimize electrostatic interactions between a variety of proteins and this stationary phase.⁹ This solvent is referred to below as the "standard mobile phase".

Methods. *Quasielastic Light Scattering (QELS).* Light-scattering measurements were made using one of two systems. With a Brookhaven (Holtville, NY) system equipped with a 72-channel digital correlator (BI-2030 AT) and using a Jodon 15-mW He-Ne laser (Ann Arbor, MI), QELS measurements were

made at scattering angles from 30 to 150°. Dendrimer samples at concentrations of ca. 2 mg/mL were filtered through 0.20- μ m Acrodisc filters (Gelman) prior to analysis. Counts were typically collected for 1 h. We obtained the homodyne intensity-intensity correlation function $G(q,t)$, with q , the amplitude of the scattering vector, given by $q = (4\pi n/\lambda) \sin(\theta/2)$, where n is the refractive index of the medium, λ is the wavelength of the excitation light in a vacuum, and θ is the scattering angle. $G(q,t)$ is related to the time correlation function of concentration fluctuations $g(q,t)$ by

$$G(q,t) = A(1 + bg(q,t)^2) \quad (1)$$

where A is the experimental baseline and b is the fraction of the scattered intensity arising from concentration fluctuations.

The diffusion coefficients were calculated using

$$D = \Gamma\lambda^2/16\pi^2 \sin^2(\theta/2) \quad (2)$$

where Γ is the reciprocal of the diffusion time constant, which is obtained from the slope of $\ln g^2(q,t)$ vs t plots. The diffusion coefficient, D , is directly related to the Stokes radius, R_s , by Stokes' equation

$$D = kT/6\pi\eta R_s \quad (3)$$

where k is Boltzman's constant, T is the absolute temperature, and η is the viscosity of the solvent.

QELS was also carried out with an Oros (Biotage Co. Charlottesville, VA) Model 801 "Molecular Weight Detector" which employs a 30-mW solid-state 780-nm laser and an avalanche photodiode detector. Samples were introduced into the 7- μ L scattering cell (maintained at 26.5 \pm 0.4 °C) through 0.2- μ m Anotec filters. Scattering data (90°) were analyzed via cumulants. There were no significant differences between the results obtained using the two instruments. (General discussions of QELS data analysis may be found in ref 11.)

Viscometry. Measurements were made with a Schott AVSN automatic viscometer equipped with a 10-mL capacity glass Type 531 capillary viscometer, at 25.0 \pm 0.02 °C. Dendrimer samples were dissolved in our standard mobile phase at concentrations ranging from 6 to 17 mg/mL and filtered through 0.45- μ m Gelman filters. Efflux times were obtained with precisions of \pm 0.05 s. However, the very low viscosities of these materials made it difficult to obtain $[\eta]$ with better than 2% precision. The viscosity radius was obtained as¹²

$$R_{\eta} = \{3[\eta]M/10\pi N_A\}^{1/3} \quad (4)$$

where the units of R_{η} and $[\eta]$ are cm and cm³/g, respectively.

Chromatography. The chromatographic system was a Beckmann "system Gold" HPLC equipped with refractive index (ri) and UV detectors and a Superose 12 column (Pharmacia Fine Chemicals, Uppsala, Sweden). This last component consists of 10.5- μ m-mean-diameter cross-linked 12% agarose particles packed into a 30- \times 1-cm-i.d. column. The initial column efficiency was ca. 10 000 theoretical plates, but during the period of use described here declined to ca. 3500. The average pore size was 170 Å, as determined by the slope of a plot of $1 - K_{SEC}^{1/2}$ (for definition of K_{SEC} , see below) vs R_{η} for the nearly spherical solute Ficoll.¹³ The characteristics of Superose 12 packings and columns are described in detail in ref 14. Flow rates were determined by weighing of collections of timed eluant and were precise to better than \pm 0.2%. Peak retention volumes, V_e , were determined by triplicate injections with a repeatability of \pm 0.5%. The chromatographic partition coefficient then could be determined according to

$$K_{SEC} = (V_e - V_0)/(V_t - V_0) \quad (5)$$

where V_0 is the exclusion volume (interstitial packing volume), determined as 8.05 mL from the elution of DNA, and V_t is the total column liquid volume, determined as 21.82 mL from the

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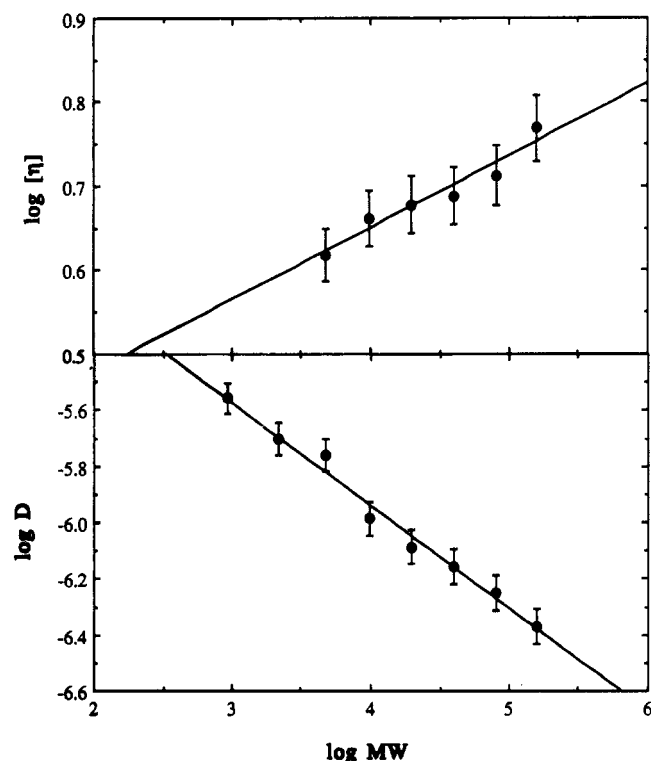


Figure 1. Double-logarithmic plots of the MW-dependence of intrinsic viscosity (upper) and diffusion coefficient (lower) of dendrimers, in standard mobile phase solution.

elution of acetone. K_{SEC} could thus be measured with a relative precision of about 2%.

Dendrimer samples typically exhibited a number of peaks in both ri and UV detection. That these were not chromatographic artifacts was established by collection and reinjection of the individual peaks. However, only the earliest eluting (and largest) peak changed with generation number, G . Furthermore, all other peaks had retention times too large to be consistent with the expected MWs or either R_η or R_S . We therefore conclude that these peaks are low-MW impurities, likely to be synthetic precursors of the final products. While the nature of these impurities is clearly of concern, there are a number of findings that support the conclusion that these species may be neglected in the evaluation of $[\eta]$, R_S , or K_{SEC} . First, the plot of $\log [\eta]$ vs $\log MW$ (Mark-Houwink plot) is quite linear (correlation coefficient of 0.98), whereas an abundance of low-MW impurities would be expected to produce more erratic error in the intrinsic viscosities. Furthermore, when the values of R_η obtained from $[\eta]$ were plotted against R_S , a regression coefficient of 0.99 was obtained. (It is noteworthy that because the values of $[\eta]$ are small and do not vary much (as one would expect for spherical solutes), the effect of errors in $[\eta]$ tend to be overwhelmed by the much larger and more variable MW term). The parameter R_S , obtained by light scattering, may be assumed to be relatively insensitive to the less massive, hence weakly scattering, impurities. We therefore suggest that the presence of the chromatographically detectable low-MW subspecies does not contribute significantly to either measurements of dimension. Consequently, their elution was neglected in the determination of K_{SEC} .

RESULTS AND DISCUSSION

Table I contains the results of viscometry and QELS for the dendrimers in the standard mobile phase, along with the chromatographic partition coefficients, measured in the same solvent. The dependences of the intrinsic viscosity and the diffusion coefficients on the MW are shown in the double-logarithmic plots in Figure 1. From the exponents of the equations $[\eta] = KM^a$ and $D = K'M^{a'}$, namely $a = 0.086$ and $a' = -0.36$, it is evident that the dendrimers approach the compactness of an impenetrable sphere, for which $a = 0$ and

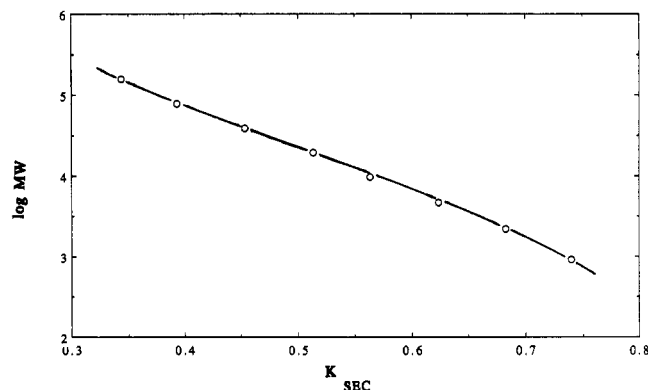


Figure 2. Dendrimer calibration curve, as log molecular weight vs chromatographic partition coefficient, on Superose 12.

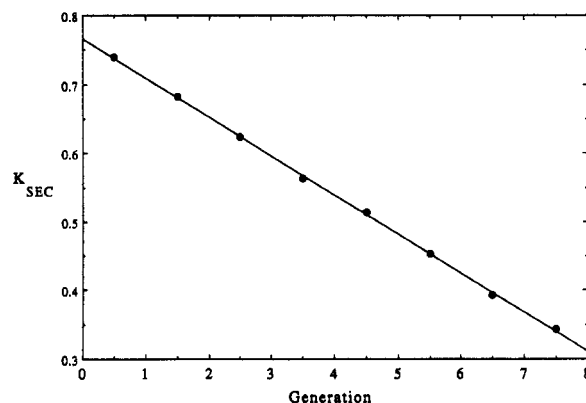


Figure 3. Dependence of chromatographic partition coefficient on generation number.

$a' = -0.33$. The relationship between the Stokes radius and the viscosity radius is given by $R_S = 1.15R_\eta$. For hard spheres, we would expect $R_S = R_\eta$; for flexible chains, $R_S/R_\eta = 0.5$ – 0.8 .¹⁵ The somewhat greater value for R_S in the present case may arise from the highly charged nature of the dendrimer, so that it diffuses along with a number of counterions. In an analogous system, we have found that the effective charge of anionic micelles measured by electrophoretic light scattering is far smaller than the formal charge.¹⁶ This means that the diffusing species encompasses a large number of counterions, i.e. that the electrophoretic shear plane is some distance from the average micellar ionic headgroup. The relevance to the present case is that the mutual diffusion coefficient is reduced by the counterion cloud, leading to somewhat elevated values for R_S .

Figures 2–4 show calibration curves for the dendrimers, presented in several ways: as K_{SEC} vs $\log MW$ in Figure 2, as K_{SEC} vs generation number in Figure 3, and as K_{SEC} vs solute dimensions— R_S or R_η —in Figure 4. Figure 4 also includes data for another compact, spherically symmetric solute, Ficoll, eluted under identical conditions, which were reported elsewhere.¹⁷

The coincidence of the Ficoll and dendrimer curves in Figure 4 is compelling evidence that both behave as spheres of radius R , i.e. that Ficoll and dendrimer samples of the same R_η or R_S are chromatographically indistinguishable. Since the former solutes are uncharged, these results also suggest that solute–substrate interactions are negligible for the latter. It is certainly possible that early elution of the dendrimers might be observed at higher pH and lower ionic

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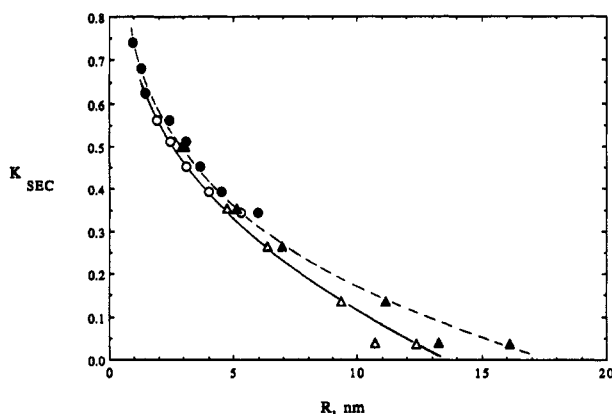


Figure 4. Dependence of chromatographic partition coefficient on solute radius, for dendrimers (circles) and for Ficolls (triangles). Open symbols are for viscosity radii; filled symbols are for the Stokes radii. Ficoll data are taken from ref 17.

strength. The somewhat greater divergence for the R_S plots of Figure 4 may arise from the effect of the electrical double layer of the dendrimers on their diffusion coefficients, without a parallel effect on their permeation behavior.

The values of R_S are consistently larger than R_η for Ficolls as well as for dendrimers. As suggested above, the surface charge and concomitant electrical double layer of the dendrimers may contribute to this effect. The Ficoll fractions are uncharged but differ from the dendrimers also in being polydisperse with respect to MW. Since the Stokes radius from QELS corresponds to the z-average component of the distribution, it must exceed the viscosity radius, which corresponds to the (smaller) viscosity average of the distribution. This is not a consideration for the monodisperse dendrimers and is unlikely to be very important for the viscosity radius plots for Ficolls, because for polymers with low α values, such as the value of ca. 0.35 found for Ficolls,¹⁸ the MW weight of the component corresponding to the measured viscosity radius is unlikely to diverge much from the MW of the component eluting at the chromatographic peak. However, these small uncertainties preclude any assertions about whether the effective dimension controlling SEC is the viscosity radius or the Stokes radius, based on the current data alone.

The dependence of K_{SEC} on generation number, G , is seen in Figure 3 to be strikingly linear; the correlation coefficient for this plot is found to be $r > 0.995$. This remarkable correlation deserves further analysis. We observed that the dependence of R_η on K_{SEC} and on G , respectively, is fit very well by the two empirical equations

$$R_\eta = 24.77 \times 10^{-1.97K_{SEC}} \quad (6a)$$

$$R_\eta = 0.7737 \times 10^{0.111G} \quad (6b)$$

Since the value of R_η is strongly influenced by the MW value for each dendrimer, which in turn is not a measured quantity but is rather a calculated one based on the synthetic procedure and some indirect evidence,⁶ we chose to verify the relationships of eqs 6a and 6b by examining the logarithmic forms of these two equations using measured values for R_S as well

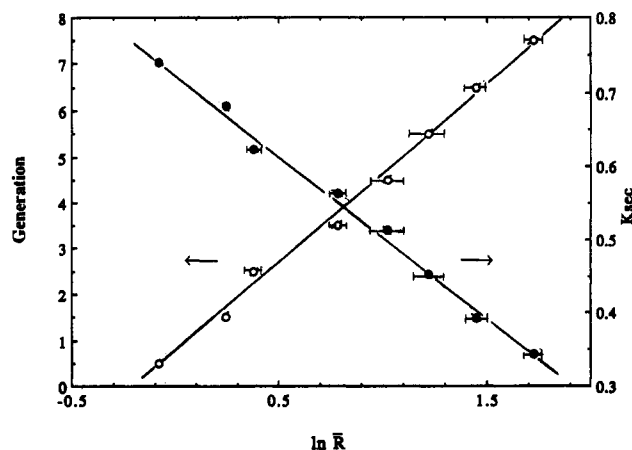


Figure 5. Dependence on solute radius of generation number (left axis) and chromatographic partition coefficient (right axis). \bar{R} is the mean of the Stokes and viscosity radii. The horizontal bars indicate the difference between these two dimensions: the left end of each bar corresponds to R_η , and the right end to R_S .

as R_η . This is done in Figure 5 in which we plot the dependence of both K_{SEC} and G on $\ln \bar{R}$, where \bar{R} is the mean of the radii measured by viscometry and QELS. The horizontal bars in this figure indicate the discrepancy between R_η and R_S . We observe that both K_{SEC} and G are linear functions of the logarithm of the solute radius.

The structure of the dendrimers ensures⁶ that the solute volume increases exponentially with G ; and since solute volume varies as R^3 , the linear relationship between $\ln R$ and G is not surprising. The observed dependence of K_{SEC} on $\ln R$ was not expected and does not conform to any of the commonly cited theoretical expressions for size exclusion,¹⁹⁻²² although experimental calibration curves often display a roughly linear dependence of, e.g., $\ln [\eta]M \sim \ln R_\eta$ on K_{SEC} in the region of intermediate K_{SEC} prior to the sigmoidal inflections at small and large permeation. In the present case, it is the exact linear relationship between K_{SEC} and $\ln R$ that leads to the remarkable correlation of K_{SEC} with G shown in Figure 3. The theoretical significance of this behavior merits further attention.

CONCLUSION

Carboxylated starburst dendrimers appear to behave as noninteracting spheres during chromatography on Superose size exclusion columns, in the presence of an appropriate aqueous mobile phase. The dependence of the retention time on solute size coincides with data collected on the same column for Ficoll, an uncharged, quasi-spherical polysaccharide. Chromatography of the dendrimers yields a remarkable correlation of the chromatographic partition coefficient with the generation number; this result is in part a consequence of the exponential relationship between the generation number and the molecular volume of the dendrimers.

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