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HPLC Separation of Different Generations of Poly(amidoamine) Dendrimers Modified with Various Terminal Groups

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Polyamidoamine (PAMAM) dendrimers of different generations with various terminal groups were analyzed, for the first time, using a combination of high-performance liquid chromatography (HPLC), size exclusion chromatography (SEC), and matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) techniques. Separation of amine-terminated dendrimers from generation 1 through generation 9 ($G1NH_2$−$G9NH_2$) was achieved using reversed-phase HPLC with elution time increasing gradually as the density of terminal amine groups increases as a function of generation. Furthermore, separation of dendrimers with terminal amino, acetamide, hydroxyl, and carboxylate groups was obtained. It has also been shown that HPLC can be used to separate dendrimers based on some structural defects inherent during the syntheses of PAMAM dendrimers. MALDI-TOF mass spectra of $G1NH_2$ identify the major imperfections present during typical synthesis processes. The absolute molar masses (M_n) and molar mass distributions of the dendrimers were measured using the SEC system equipped with multiangle laser light scattering and refractive-index detectors. Findings from this study suggest HPLC can be a vital tool for characterization and preparative separation of PAMAM dendrimers.

Dendrimers are spherical, well-defined, highly branched macromolecules with two major chemical environments: a surface chemistry due to dense functional groups and a spherical interior, which is shielded from exterior environments. The unique architecture and relatively monodisperse structure of dendrimers result in unusual physical and chemical properties, which make them attractive for many novel applications including as a catalyst, as a host molecule, in analytical chemistry, in gene and drug delivery, and as a diagonistic reagent.[1−3]

Based on their size and molar mass, dendrimers fall between small molecules and polymers.[1] A wide variety of analytical techniques have been considered for characterizing dendrimers: size exclusion chromatography (SEC),[1,4] low angle laser light scattering,[1,5] infrared (IR) spectroscopy,[6] capillary electrophoresis,[7,8] MS (chemical ionization and fast-atom bombardment),[9] laser desorption, electrospray ionization,[10] matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF),[11] and ^1H, ^2H, ^{13}C NMR.[4,6,9,12] Unfortunately, the performance of all these techniques declines with increasing size of the dendrimers. A survey of the existing literature suggests the need for the further refinement of analytical methodologies for these macromolecules.

High-performance liquid chromatography (HPLC) is a widely accepted methodology in chemical laboratories for separation and purification of small molecules and protein and DNA biomacromolecules. Hence, HPLC can be exploited for the characterization of dendritic molecules.[13] To the best of our knowledge, this is the first reported work on the use of HPLC as a characterization technique for the identification and separation of polyamidoamine (PAMAM) dendrimers of various generations and dendrimer derivatives.

The PAMAM dendrimers are methodically constructed through repetitive alkylation and amidation steps in which each iteration yields the next higher generation of the dendrimer.[1] The controlled synthesis results in well-defined molecular masses, which increase as a function of the dendrimer generation. However,

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structural deviations are inevitable during the synthesis of the ethylenediamine (EDA)-core PAMAM dendrimers as reported by other researchers.[14] Structural imperfections can be divided into three main groups: (i) generational imperfections due to the presence of trailing generations and dimers; (ii) skeletal defects such as missing arms, intramolecular loops, etc., and (iii) substitutional deviations resulting from heterogeneity between molecules due to surface functionalization.[15]

Since separation is primarily based on adsorption between surface groups of the analytes and the stationary phase (column packing), HPLC is more suitable for separating components with second and third structural deviations, i.e., dendrimers with terminal defects and different surface functionalities. However, the identification of all or most of the defective compounds is even more challenging. The advent of mass spectroscopic methods such as electrospray denionization and matrix-assisted laser desorption/ionization can be utilized for the structural characterization of various fractions isolated with the help of HPLC. We have selected generation 1 amine surface terminated dendrimer ($G1NH_2$) and have obtained MALDI-TOF MS spectra of major fractions isolated using HPLC.

The surface amine groups of various generations of PAMAM dendrimers can be partially or completely substituted to other groups such as hydroxyl, acetyl, or carboxyl. The substitutions result in much different physicochemical properties and also modify the dendrimer platform for further conjugations with drugs, peptides, and proteins.[3] The potential of HPLC was investigated in this regard to determine the efficacy of separations of dendrimers with different terminal functional groups.

SEC or gel permeation chromatography (GPC) is the most preferred method for determining the absolute molar masses and molar mass distributions of polymers.[16] Since separation in SEC is based on the sizes of molecules, this is ideal for characterization of different generations. Also SEC can be used for detecting generational impurity (i.e., trailing generations, dimers, oligomers and supramolecular aggregates formed through intermolecular coupling). In addition, the purities of the dendrimers can be qualitatively determined from the polydispersity indices (M_w/M_n).

The specific objectives of the present article are as follows:

First: to ascertain the effectiveness of HPLC in terms of detecting and separating amine-terminated PAMAM dendrimers of generations 1 through 9 ($G1NH_2$–$G9NH_2$).

Second: to obtain separation among various surface-functionalized PAMAM dendrimers of the same generation. In this context, amine-, acetamide-, hydroxyl-, and carboxyl-terminated G1 and G4 dendrimers have been investigated.

Third: to determine absolute molar mass and molar mass distribution (MWD) of dendrimers with various generations and terminal groups. The MWDs and polydispersity values can be used to determine purity of the materials at least semiquantitatively.

Fourth: to asses the efficacy of HPLC in separating species with minor structural deviations.

Fifth: to identify primary structural defects present during a typical synthesis of amine-terminated G1 dendrimer. This is accomplished by analyzing MALDI-TOF mass spectra of major fractions isolated using HPLC.

To the best of our knowledge, this is the first reported work on using HPLC as an analytical technique for detection and separation of different generations of EDA-core PAMAM dendrimers with various terminal functionalities.

EXPERIMENTAL SECTION

Materials. EDA-core PAMAM dendrimers of generations 1–9 (G1–G9) were purchased from Dendritech (Midland, MI) in methanol solution. The surfaces of amine-terminated PAMAM dendrimers of selected generations 1 and 4 ($G1NH_2$ and $G4NH_2$) were converted to acetamide, hydroxyl, and carboxyl groups by reacting with acetic anhydride, glycidol, and succinic anhydride, respectively. Details of the conversion procedure can be found elsewhere.[15,17]

High-Performance Liquid Chromatography. The reversed-phase (RP) HPLC system consisted of a System GOLD 126 solvent module, a model 507 autosampler equipped with a 100-μL loop, and a model 166 UV detector (Beckman Coulter, Fullerton, CA). A Jupiter C5 silica-based RP-HPLC column (250 × 4.6 mm, 300 Å) was purchased from Phenomenex (Torrance, CA). Two Phenomenex Widepore C5 guard columns (4 × 3 mm) were also installed ahead of the Jupiter column. The mobile phase for elution of different generations of PAMAM dendrimers was usually a linear gradient beginning with 100:0 (v/v) water/acetonitrile (ACN) at a flow rate of 1 mL/min. Trifluoroacetic acid (TFA) at 0.14 wt % concentration in water as well as in ACN was used as a counterion to make the dendrimer surfaces hydrophobic. To ascertain the effect of using excess TFA on the HPLC results, $G4NH_2$ samples were prepared at two different concentrations of TFA—0.14%, the usual concentration in the mobile phase, and 1%. Under the same gradient conditions, the eluograms show no marked differences except for the dead volume peaks. The results suggest there is no side reaction such as hydrolyzation of the aliphatic amide groups due to the presence of excess TFA in aqueous media. Elution profiles of selected samples ($G1NH_2$, $G5NH_2$) taken after 1 week do not show any appreciable change, which confirm the stability of the PAMAM dendrimers in 0.14% aqueous solutions of TFA. Unless otherwise specified, the injection volume in each case was 50 μL with a sample concentration of 1 mg/mL and the detection of eluted samples was performed at 210 nm. The analysis was performed using Beckman's System GOLD Nouveau software.

Size Exclusion Chromatography/Gel Permeation Chromatography. SEC experiments were performed using an Alliance Waters 2690 separation module (Waters Corp., Milford, MA) equipped with a Waters 2487 UV absorbance detector (Waters Corp.), a Wyatt Dawn DSP laser photometer (Wyatt Technology Corp., Santa Barbara, CA), an Optilab DSP interferometric refractometer (Wyatt Technology Corp.), and TosoHaas TSK-Gel Guard PHW 06762 (75 × 7.5 mm, 12 μm), G 2000 PW 05761 (300 × 7.5 mm, 10 μm), G 3000 PW 05762 (300 × 7.5 mm, 10 μm), and G 4000 PW (300 × 7.5 mm, 17 μm) columns. Column

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temperatures were maintained at 25 ± 0.2 °C with a Waters temperature control module. Citric acid buffer (0.1 M concentration) with 0.025% sodium azide in water was used as a mobile phase. The pH of the mobile phase was adjusted to 2.74 using NaOH and the flow rate was maintained at 1 mL/min. Sample concentration was ~2 mg/mL, and an injection volume of 100 μL was used for all samples. Molar mass moments of the PAMAM dendrimers were determined using Astra software (version 4.7) (Wyatt Technology Corp.).

MALDI-TOF Mass Spectrometry. MALDI-TOF mass spectra were acquired using a Waters TofSpec-2E spectrometer in a reflection mode. The matrix solution was 10 mg/mL α-cyano-4-hydroxycinnamic acid (CHCA) dissolved in ACN/ethanol (50:50). The TFA salt form of the separated samples was isolated and collected. The dissolved samples were then lyophilized to recover the materials. Each isolated sample was then added to a 50:50 mixture of methanol/water to obtain an approximate concentration of 0.25 mg/mL. Equal volumes (5 μL) of each sample solution and matrix solution were well mixed. Then, a 1-μL solution of the mixture was injected on the spots of the target plate and evaporated to dryness. The spectrometer was calibrated with a mixture of known peptides in the CHCA matrix.

RESULTS AND DISCUSSION

HPLC Analysis. It is well known that amine-terminated PAMAM dendrimers adsorb tenaciously to a variety of solid media, especially if the medium is hydrophilic. This property presents difficulties for analysis by conventional chromatography. In ion-pair RP-HPLC, polycationic dendrimers form complexes with counterion TFA through ionic interactions that are adsorbed on C5 reversed-phase HPLC columns. The hydrophobicity of the PAMAM−TFA complex depends primarily on the surface density of the primary amine groups.

Figure 1 shows the chromatograms of generation 1 through generation 9 amine-terminated dendrimers ($G1NH_2$−$G9NH_2$). The gradient used was 0−40% ACN (balance water) over 30 min. The counterion TFA concentration was 0.14 wt %, which resulted in a pH of 2.25 in the aqueous phase. Figure 1 clearly demonstrates that the elution time of PAMAM dendrimers increases as a function of the number of generation.

The full generation dendrimers possess terminal primary amine as well as tertiary amine groups. Reported titration data of PAMAM dendrimers show the two types of protonation events for the amines.[7] The terminal primary amines have a p*K* in the range from 9 to 10, whereas the interior tertiary amines exhibit a p*K* between 4 and 5. At the acidic pH of 2.25, which is well below the two p*K* regimes, protonation of both primary and tertiary amines is favored.[18]

The increase of average radius of gyration (R_g) of PAMAM dendrimers as a function of generation is stronger than linear but weaker than quadratic (see Figure 3 of ref 19).[20,21] On the other hand, the number of terminal groups doubles (i.e., increases logarithmically) and the molecular mass approximately doubles

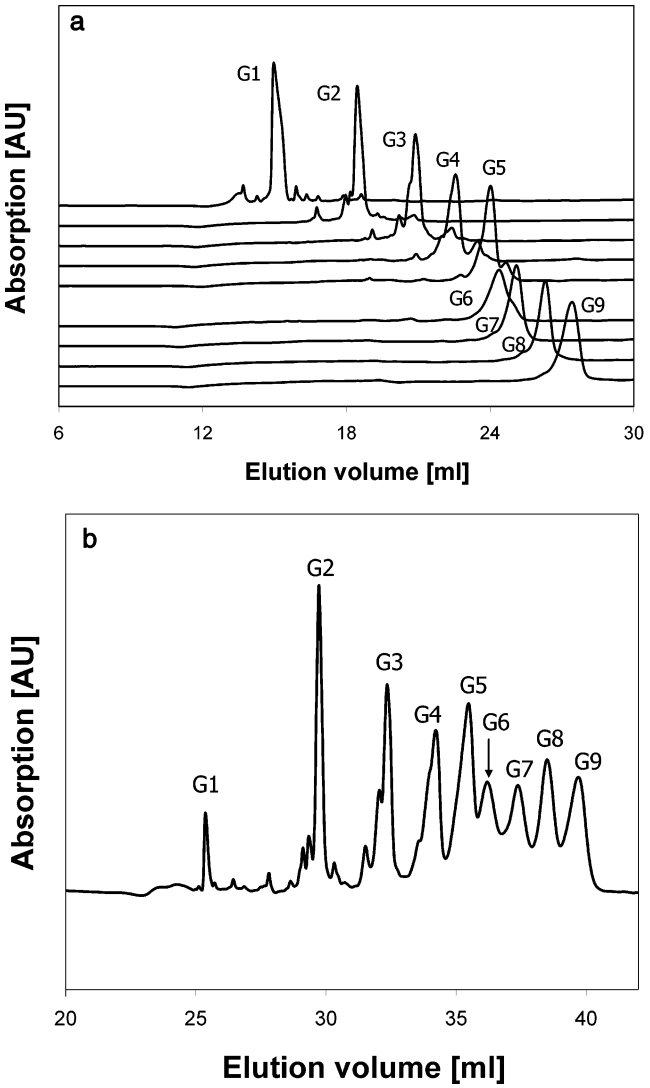

Figure 1. (a) Chromatograms of amine-terminated generation 1−generation 9 dendrimers ($G1NH_2$−$G9NH_2$) injected separately. The solvent flow rate was 1 mL/min, and the concentration of each analyte was ~1 mg/mL. The gradient used was 0−40% ACN (balance water). The detection wavelength of the UV was 210 nm. (b) Chromatogram of the mixture of $G1NH_2$−$G9NH_2$ dendrimers obtained after injecting 100 μg of the mixture (the concentration of each generation in the mixture was ~ 0.11 mg/mL). The gradient selected for the separation was 100% water for 10 min and a linear gradient of 0−50% ACN (balance water) in 40 min. The detection wavelength of UV was 210 nm.

with addition of each generation through successive iteration of the reaction sequence. As a result, the formal charge-to-mass ratio (assuming all terminal groups are carrying a charge) from generation to generation essentially remains constant. Therefore, the PAMAM dendrimers from G1 to G9 provide a series of molecules of nearly equivalent mass density of charges that span a molecular mass range from 1000 to nearly 0.5 million Da.

The primary factor governing elution from a hydrophobic column surface is the density of dendrimer−TFA ion pairs. Since an excess of TFA was used in aqueous solutions to protonate the interior tertiary amine atoms and the peripheral amine groups, the density of the ion pairs is governed by the number of terminal amine groups. Generally speaking, the surface area of the dendrimers should go as the square of R_g. The actual surface areas

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Table 1. Ideal and Measured Molar Masses of Amine-terminated G1 through G8 PAMAM Dendrimers

different generation sample	theoretical molar mass, M_n (g/mol)	molar mass, M_n (g/mol)	polydispersity (M_w/M_n)	peak elution volume, mL	HPLC peak width at half-height
$G1NH_2$	1 430	1 398	1.024	14.97	1.00
$G2NH_2$	3 256	3 109	1.054	18.45	0.850
$G3NH_2$	6 909	6 648	1.143	20.88	1.085
$G4NH_2$	14 215	12 910	1.113	22.55	1.196
$G5NH_2$	28 826	26 010	1.104	24.02	1.206
$G6NH_2$	58 048	56 010	1.365	24.37	2.025
$G7NH_2$	116 493	113 700	1.277	25.10	1.370
$G8NH_2$	233 383	214 300	1.120	26.30	1.210
$G9NH_2$	467 162			27.42	1.717

Also listed are peak parameters obtained from HPLC eluograms.

and solvent-accessible surface areas of dendrimers as a function of generation are reported by Maiti et al. (see Table 4 in ref 19). Therefore, the surface charge density i.e., the density of the terminal amine groups, increases as a function of generation and so does the elution time.

In Figure 1a, the presence of well-defined peaks confirms relative purity of the dendrimer samples. The peak width at half-height ($W_{H/2}$) can be used as a qualitative measure of dispersity for different generation PAMAM dendrimers. The width values are normalized with the value for $G1NH_2$ and shown in Table 1. Since peak widths depend on the shape and steepness of the gradient, the numbers reported in Table 1 are only relative to the operating conditions used to generate chromatograms displayed in Figure 1a. In general, it is observed that the peaks get broader as a function of generation number. It is not surprising since the polydispersity increases with the increase of the number of generations due to successive iteration sequences involved during synthesis. The issue of polymolecular dispersity due to structural defects will be described later during the discussion of MALDI-TOF analysis of $G1NH_2$. On the other hand, the sensitivity of separation of structural defects decreases for higher generations. As a result, the increase in $W_{H/2}$ with increasing generation is moderate, from 1.0 to 1.7, as we move from G1 to G9.

Figure 1b shows separation of G1−G9 amine-terminated dendrimers from a single injection (total concentration 1 mg/mL, concentration of individual generations ~0.11 mg/mL). It is remarkable that, using a single column and gradient, separation of G1−G9 dendrimers spanning a molar mass range of 1430−467 162 Da and a R_g range of 7.63−46.47 Å[19] can be performed within 50 min.

The separations shown in Figure 1 could lead us to potential advantages of using HPLC for dendrimer analysis. The chromatographic technique can be used to assess purity of a given generation either qualitatively or semiquantitatively. Another benefit offered by HPLC is that it potentially can be scaled to preparative separations where relatively pure material (i.e., dendrimers without trailing generations and higher oligomer contamination) can be obtained for further studies or applications.

The amine termini PAMAM dendrimers can be modified with various reactive functional groups. Dendrimers are unique in this respect and this attribute has made dendrimers useful for numerous applications such as targeted drug delivery, gene therapy, magnetic resonance imaging, biosensing, and signal amplification.[3] Since separation is primarily based on surface properties, HPLC can be explored as a promising analytical method for detecting and separating different terminal group substitutions. In addition, the chromatograms can provide important information regarding the physicochemical properties of dendrimers.

Terminal groups of selected generations, G1 and G4 were modified to obtain cationic, anionic, and neutral surfaces. The various terminal groups considered are amine (cationic), hydroxyl, acetamide (neutral), and carboxyl (anionic). The modifications to hydroxyl (OH), acetamide (Ac), and carboxyl (COOH) surfaces were accomplished after reacting amine-terminated dendrimer of a specified generation with glycidol, acetic anhydride, and succinic anhydride, respectively. The details of the synthesis procedures are described by Shi et al.[15,17,22]

Figure 2a shows the eluograms of four different surface-functionalized G1 dendrimers obtained using 100% water for 5 min and a linear gradient of 0−100% ACN (balance water) in 25 min. The separation achieved among $G1NH_2$, G1Ac, G1OH, and G1COOH is remarkable. In this case, the peak elution time increases as the surface group is modified from amine to hydroxyl, acetamide, and carboxyl, respectively. At an acidic pH of 2.25, all the terminal groups are protonated and hydrophobicity increases as the surface changes from cationic (amine, NH_2) to neutral (hydroxyl, OH; acetamide, Ac), to anionic (carboxyl, COOH). Similar trends can be observed for G4 dendrimers, with the exception that in case of G4 hydroxyl surfaced dendrimer elutes faster than amine surfaced (Figure 2b). The separations achieved in the chromatograms of Figure 1 indicates that HPLC can be used to determine impurities bearing different surface functionalities, not to mention the fact that the chromatographic technique can be scaled to preparative separations where relatively pure surface-modified dendrimers are required.

From the individual eluograms of G1 and G4 dendrimers (Figure 2c), we observe that the peak widths of G1OH and G4OH are considerably broader as opposed to the other surface functionalized dendrimers. The anomalous elution behavior of G1OH and G4OH and broad peaks for both of them can be the result of reaction stoichiometry between surface terminal amine groups and glycidol, the reagent used for surface modification. During the surface modification reactions, excess amounts of glycidol

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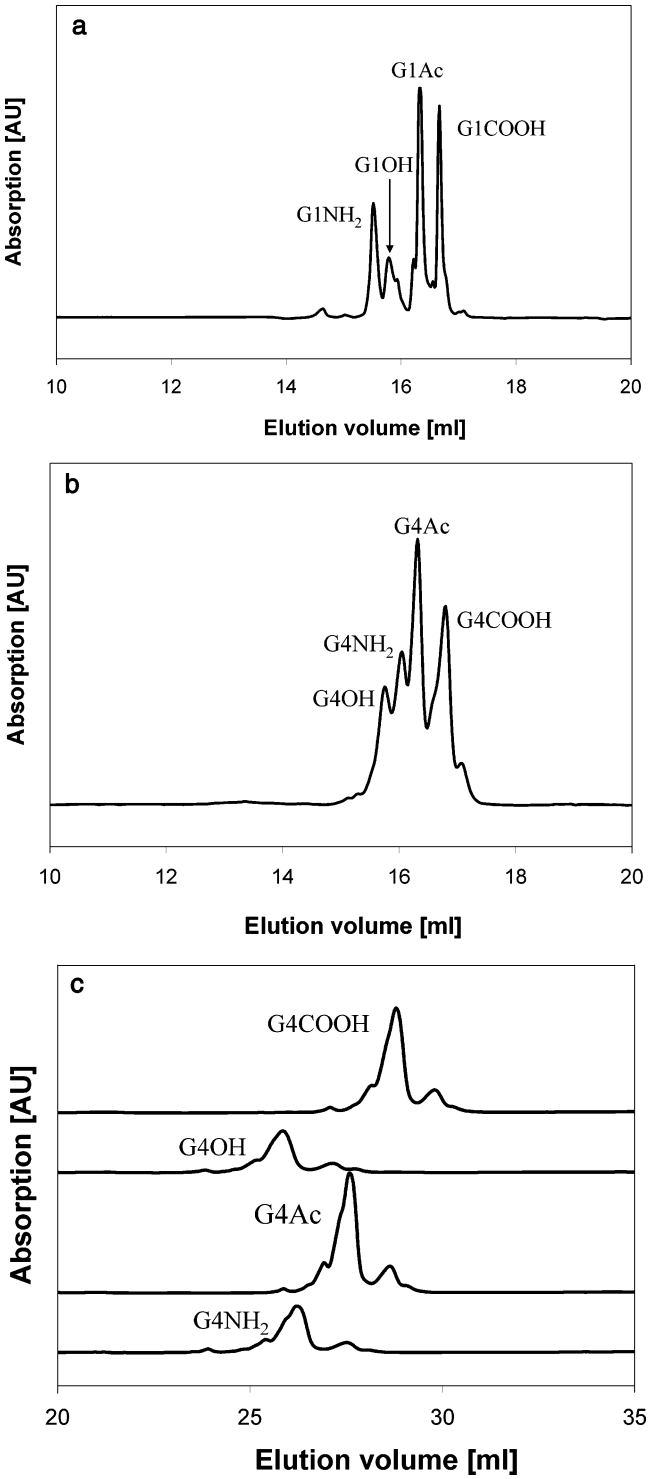

Figure 2. (a) Chromatogram obtained after injecting 100 μg of the mixture of equal amounts of $G1NH_2$, G1Ac, G1OH, and G1COOH. The gradient selected for the separation was 100% water for 5 min and a linear gradient of 0−100% ACN (balance water) in 25 min. The detection wavelength of UV was 210 nm. (b) Eluogram obtained after injecting 100 μg of the mixture of equal amounts of $G4NH_2$, G4Ac, G4OH, and G4COOH. A linear gradient starting with 0% ACN (balance water) and ending in 100% ACN over 40 min was used. (c) HPLC profiles of G4 dendrimers functionalized with various surface groups. A linear gradient starting with 0% ACN (balance water) and ending in 40% ACN in 40 min was used.

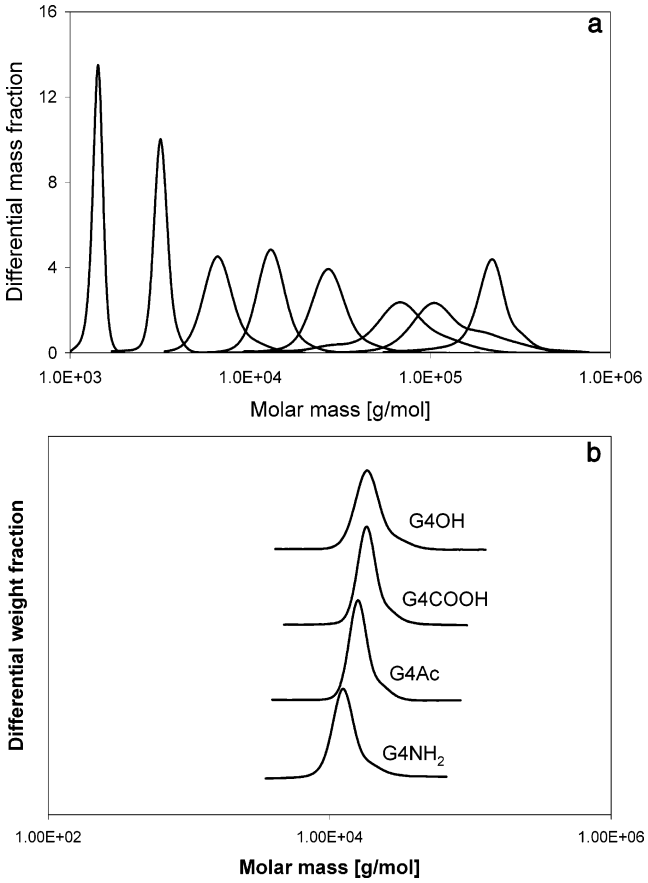

Figure 3. (a) Differential molar mass distributions of G1−G8 amine-terminated PAMAM dendrimers. The distributions were obtained after analyzing the SEC chromatograms detected with MALLS and refractive index (RI) detectors. The concentration of each analyte in the injected samples was ~2 mg/mL. The number average molar mass and polydispersity indices are listed in Table 1. (b) Molar mass distributions of G4 dendrimers functionalized with various surface groups. The number average molar mass (M_n) and polydispersity indices are listed in Table 2.

were used to convert the amines to hydroxyl groups. The reactivity of primary amine is higher compared to secondary amines. Accordingly, when excess glycidol is reacted with $G1NH_2$ or $G4NH_2$, at first all primary amine groups (at least theoretically) react with glycidol and become secondary amines. However, due to the presence of excess glycidol, some of the secondary amines also react with glycidol and convert to tertiary amines. Due to the two-step addition, the polydispersity of hydroxyl-modified dendrimers is high with respect to the modification of the secondary amine groups, which resulted in broader peaks. This is also evident from SEC results where polydispersity (M_w/M_n) of hydroxyl-terminated dendrimers are considerably higher than the other surface group dendrimers. Depending on the fraction of primary amines that convert to tertiary amines, the surface characteristics of the PAMAMs also change. Therefore, G1OH has higher elution time than $G1NH_2$ whereas G4OH has lower elution time compared to $G4NH_2$.

Size Exclusion Chromatography Measurements. SEC measurements are performed to determine the polydispersity, especially size-dependent dispersity of the different generations of dendrimers. Furthermore, SEC is the most reliable method to determine average molar mass of polymers. The SEC apparatus was equipped with three detectors—a multiangle laser light scattering (MALLS), a differential refractive index, and a

Table 2. Ideal and Measured Absolute Molar Masses of Various Surface Functionalized G1 and G4 Dendrimers

sample	terminal group	theoretical molar mass, M_n (g/mol)	measured molar mass, M_n (g/mol)	polydispersity (M_w/M_n)	peak elution volume, (ml)
$G1NH_2$	amine	1 430	1 398	1.024	21.6
G1Ac	acetamide	1 766	1 744	1.019	30.3
G1COOH	carboxyl	2 230	2 063	1.070	32.8
G1OH	hydroxyl	2 614	2 385	1.011	24.8
$G4NH_2$	amine	14 215	12 821	1.081	26.7
G4Ac	acetamide	16 903	15 373	1.161	29.0
G4COOH	carboxyl	20 615	18 205	1.106	31.5
G4OH	hydroxyl	23 687	18 916	1.113	25.5

Also listed are peak parameters obtained from HPLC chromatograms.

UV−visible. The use of a MALLS detector allows determination of absolute molar mass without the help of any calibration standards. This is of utmost importance in the case of dendrimers since no calibration standards are available.

Figure 3a shows the molar mass distributions (MWDs) of G1−G8 dendrimers. The evaluated number average molar masses (M_n) and polydispersity indices (M_w/M_n) are shown in Table 1 along with the theoretical molar masses. As expected, the molar masses of various generations are closer but less than the theoretical value, confirming the presence of structural defects. Like HPLC eluograms and for the same reason, polydispersity values are generally higher for higher generations. Considering the fact that separations in the columns are attained due to differences in size, the sharp peaks obtained in the MWDs indicate relative purity of the materials. The structural defects such as missing arms and intramolecular cyclization do not result in significant change in size and therefore do not increase the polydispersity values to a large extent. However, the presence of any trailing generations and dimer or oligomer formation through intermolecular cyclization are the major contributing factors for increasing polydispersity. Since molar mass approximately doubles with successive iteration of reaction sequence for synthesis of next higher generation, the dimers of any generation will have approximately similar molar mass as the next higher generation. The high molar mass tails in MWD of various generations, which overlap with the MWD of the next higher generation, suggest the presence of dimers after synthesis and purification. From the chromatograms, it can be inferred that not more than a few percentage of oligomers and trailing generations are visible for all the generations investigated.

The evaluated MWDs of different surface-modified G4 dendrimers are plotted in Figure 3b. The number average molar mass (M_n) and polydispersity values are listed in Table 2. As expected, the polydispersity values increase with surface modification. However, even after surface modifications, all G4 samples essentially exhibit relatively narrow polydispersity thereby confirming the success of the surface modifications as expected from chemistry.

HPLC along with MALDI-TOF MS Experiments. The mass spectra of $G1NH_2$ are analyzed primarily to determine the major structural defects during synthesis. The individual chromatograms of Figure 1a show each generation exhibits a few smaller peaks with less and higher elution times apart from the broad main peak. The breadth of the main peak as well as the presence of smaller peaks suggests the presence of multiple components as a result of structural defects during the syntheses of full generation dendrimers. Some of the synthesis products with minor structural defects can manifest themselves as shoulders or even can be embedded in the main peaks if changes in surface properties do not cause large differences in retention times. The essential result is the presence of polymolecular polydispersity.

Figure 4a shows a chromatogram obtained using a flatter gradient of 0−30% ACN (balance water) over 40 min. The gradient was chosen to separate various molecules with minimal structural defects while at the same time retaining the measurable sensitivities. Apart from a major peak, Figure 4a exhibits nine other significant peaks. MALDI-TOF mass spectra of each individual fraction marked A−J are obtained to identify major structural deviations.

The major components of the various peaks and the concomitant structural impurities are described in the next few paragraphs. It has to be noted that the objective of the present MS analysis is not to decipher all the structural defects that can be present in different full generation PAMAM dendrimers. A detailed description of various structural defects and accompanying reaction schemes can be found in the work of Peterson et al.[14] Here our two major objectives are (i) to find the typical structural defects corresponding to the major peaks and (ii) to ascertain the effectiveness of HPLC in separating relatively minor structural defects.

Spectra b and c in Figure 4 show the MALDI-TOF mass spectra of the major fractions identified as C and J, respectively, in Figure 4a. In Figure 4b, the sharp peak corresponding to 1430 Da confirms the presence of ideal $G1NH_2$ structure with eight terminal groups. Peaks at mass numbers 1087.8 and 1315.9 suggest that even the major fraction marked C has impurity due to structural defects that could not be separated by the column−gradient combination used here. The peak at m/z 1315.9 is due to one missing arm bearing molar mass 114 Da.

The spectra also show a component (714.4 Da) that has exactly half the mass of the full generation $G1NH_2$. In MALDI-MS, the appearance of multiple charged ions is usually suppressed. Also, the ion at a mass of (M + 2H)/2 is not visible. Hence, the component at 714.4 Da can be assumed as a dendrimer fragmentation product, where the central C−C bond in the EDA core is broken. The neutralization of the negatively charged part of the fragmented dendrimer by a proton results in a positive ion with half the dendrimer mass. Mass numbers exceeding the mass of

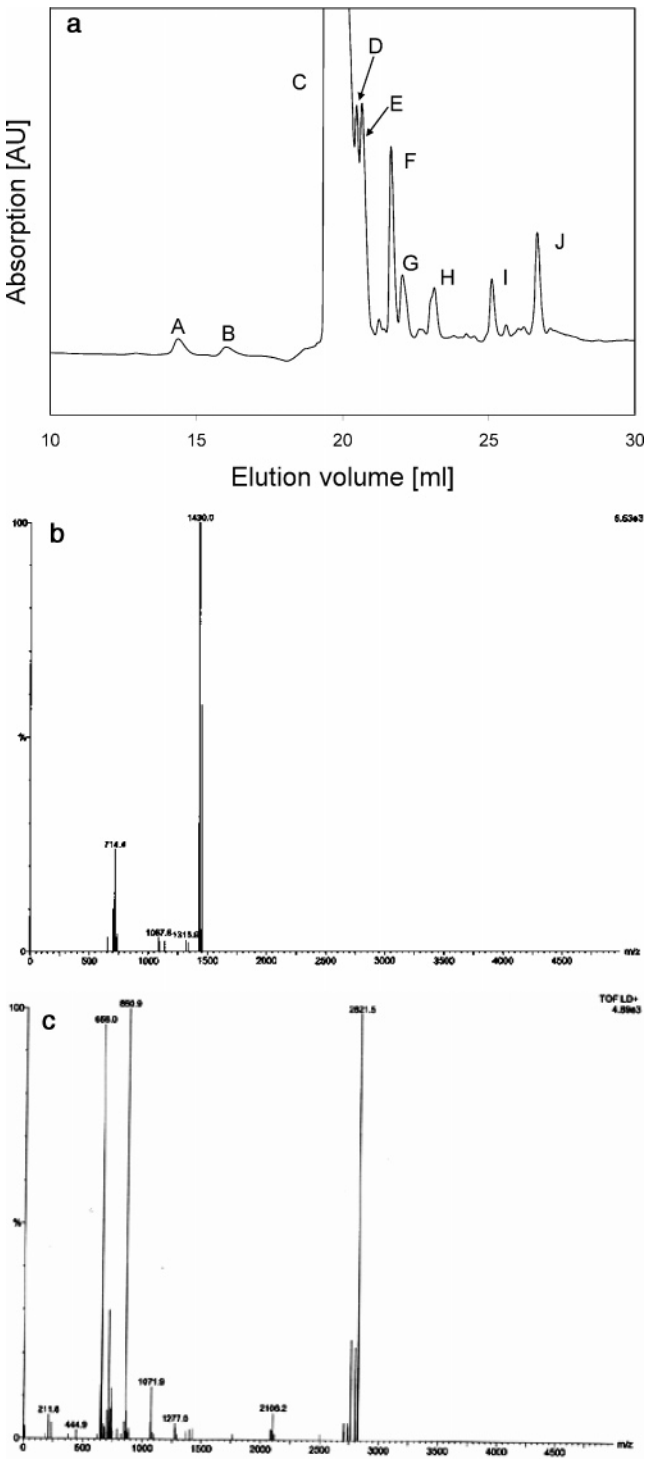

Figure 4. (a) Chromatogram of $G1NH_2$ obtained after eluting through a HPLC column using a linear gradient of 0−30% ACN (balance water) in 40 min. For clarity, the ordinate of the plot is amplified. (b) MALDI-TOF mass spectra of the main peak fraction (identified as C in (a)) of $G1NH_2$. (c) MALDI-TOF mass spectra of fraction J in (a).

by +22 observed in most cases are due to Na salts. The $[M + Na]^+$ ions are due to the random Na impurity in the samples.

The primary structural deviations during synthesis of a full generation dendrimer are due to intramolecular defects, missing repeating units, intramolecular loops, intermolecular aggregates (dimers), and half-generation and lower generation dendrimers (trailing generations). The major species identified from the MS spectra of A−J are listed below. For brevity, we have only shown the spectra of fractions C and J in Figure 4b and c.It is observed

fraction	experimentally determined molecular mass (Da)
A	656.1, 913.6, 1141.8, 1430.0
B	656.0, 853.5, 1078.1, 1201.9, 1290.1
C	714.4, 1087.8, 1315.9, 1430.0
D	656.0, 866.9, 1079.0, 1369.8
E	656.0, 866.9, 1078.0, 1369.9
F	656.0, 825.0, 1078.0, 1369.9, 1680.1
G	656.0, 860.9, 1072.0, 1680.0
H	656.0, 861.0, 1078.0, 1289.0, 1548.0, 1794.2
I	656.0, 861.0, 1072.0, 1649.0, 2343.4
J	656.0, 860.9, 1071.9, 1277.0, 2106.2, 2799.5

from the list above that some of the components with lower mass (e.g., 656.0, 861.0 Da) are present in all fractions, suggesting they are formed due to fragmentation. Also some components are observed in spectra of two adjacent fractions (e.g., m/z 1680.0 in both F and G), indicating incomplete separation. The structural deviations can be divided into three groups—missing repeating units, intramolecular cyclization, and intermolecular cyclization. Peterson et al.[14] listed seven different compounds. Our analysis shows even greater number of species.

Some of the products having lower molar masses than the ideal $G1NH_2$ are formed due to incomplete Michael addition, resulting in unsymmetrical dendrimer structures. These products are clearly seen in the MALDI MS spectra of the fractions eluting faster than the ideal $G1NH_2$. In general, the compounds with masses 1141.8, 1078.1, 1201.9, 1290.1, 1087.8, and 1315.9 Da fall into this category. For example, one missing arm results in a mass of 1316 Dal (1430−114), two missing arms results in 1202 Da (1430 − 2 × 114), and three missing arms results in 1088 (1430 − 3 × 114) Da.

Additionally, separated fractions contain compounds with structural errors form via cyclization during the amidation step of $G1NH_2$ synthesis. Since there are many (ideally 8) identical ester groups in the outer shell, different cyclization possibilities exist that can be either intramolecular cyclization or intermolecular dimer formation (see Figure 7 in ref 14). The intramolecular cyclized compounds have comparable molar mass and elution times. For example, products with masses 913, 1370, and 1548 Da fall in this category.[14] The dimeric products are the same as reported by Peterson et al.[14] and have molar masses of 2343 and 2800 Da.

Another source of structural error could be the retro-Michael reaction. Some of the products with missing repeating units can form either via incomplete Michael reaction or the retro-Michael reaction.[23] Also some products formed can be as a result of combination of two defects.

The salient finding is that HPLC can be used to even separate compounds with structural defects formed via different routes. The analyses suggest that combination of HPLC and MS can at least semiquantitatively determine amounts of impurities present.

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CONCLUSIONS

Findings from this study indicate that HPLC can be used successfully in the analytical scale and potentially in the preparative or semipreparative scale to separate PAMAM dendrimers of various generations, to isolate the same generation having different surface functionalities, and even to separate major structural defects inherently present during typical synthesis of full generation dendrimers.

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