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Generational, skeletal and substitutional diversities in generation one poly(amidoamine) dendrimers

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Abstract

Structural deviations of ethylenediamine core polyamidoamine (PAMAM) dendrimers and derivatives can be defined as skeletal and/or substitutional diversities. Detailed analysis of dendrimer starting materials and derivatives is necessary to understand the intrinsic characteristics of commercial dendrimer materials and their variations related to subsequent surface modifications. In this paper, structural deviations of ethylenediamine core generation 1 PAMAM dendrimers (PAMAM_E1 or E1) are studied and determined in a frame of a systematic investigation using combined characterization techniques. A primary amine-terminated PAMAM dendrimer of generation 1 (E1.NH_2) was used as a starting material to synthesize glycidol (E1.N(Gly)OH) and acetamide-terminated (E1.NHAc) dendrimers. The purity and homogeneity of these dendrimers were extensively characterized by polyacrylamide gel electrophoresis (PAGE), capillary electrophoresis (CE), gel permeation chromatography (GPC), acid–base titration, nuclear magnetic resonance (NMR), matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) and electrospray ionization (ESI) mass spectrometry. PAGE and CE studies showed that electrophoretic mobilities at pH 2.5 are in the order of E1.NH_2 > E1.N(Gly)OH > E1.NHAc. Mass spectrometry and NMR investigations (^{1}H, ^{13}C DEPT-135, and ^{13}C NMR, COSY, HETCOR, NOESY) suggested that (a) the studied E1 dendrimers were *generationally* pure, (b) E1.NHAc and E1.N(Gly)OH dendrimers, and essentially had the same defects and *skeletal diversity* as E1.NH_2 did. The broad distribution of the main peak in the CE electropherogram of E1.N(Gly)OH revealed the incomplete hydroxylation of E1.NH_2 resulting in additional *substitutional diversity* between the dendrimer molecules. Potentiometric titration studies proved that overall numbers of terminal and tertiary amine groups also deviated from the theoretical values. NMR spectroscopy was applied for both qualitative and quantitative analysis of the structural defects of dendrimers and derivatives. E1.NH_2 and E1.NHAc exhibited only minor deviations from ideal structures and, respectively, displayed a narrow distribution; while E1.N(Gly)OH had a much broader distribution centered around 14 ± 3 glycidol substituents. The study of structural variations in generation 1 PAMAMs provides new insights for the characterization of higher generation PAMAM dendrimers and derivatives both in terms of the skeletal deviations as well as other resulting diversities related to dendrimer surface functionalization.

Keywords: Dendrimer; Distribution; Characterization

1. Introduction

The goal of this paper is to identify, discuss and explain typical structural diversities in generation 1 Starburst™ dendrimers, and to explore how dendrimer functionalization influences the various distributions of dendrimer derivatives.

The term ‘dendrimer’ may refer to a geometric structure, or to individual (macro)molecules with a tree-like architecture consisting of connectors and branching units that are symmetrically built around a core unit, as well as to a

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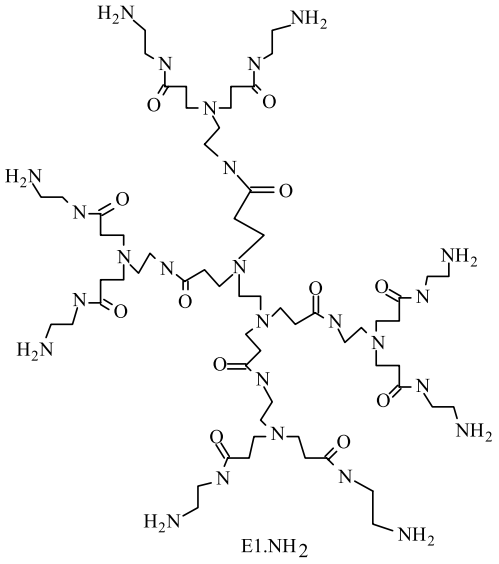
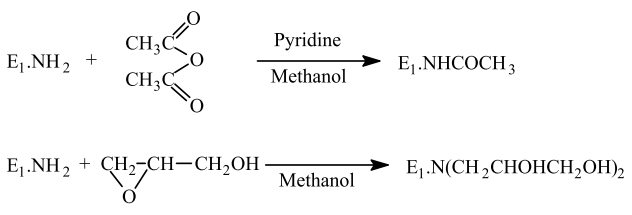
material composed of dendritic (macro)molecules. Dendrimers are often considered to be completely homodispersed at the molecular level, but practical dendrimer materials (Starburst™, Astramol™, etc.) synthesized in industrial quantities are always mixtures of dendrimer molecules that usually belong to a certain generation of a dendrimer family. Molecules in dendrimer materials may be very similar to each other, and their chemical analysis is not a trivial task. These materials—even at the lower generation levels should be considered as polymers with a very narrow molecular weight distribution [1,2] that are mixtures of various generations (*generational diversity*) and always contain various structural defects (*structural diversity*). However, theoretical simulations (e.g. [3,4]) use perfect geometric structures and therefore are unable to provide a complete understanding of dendrimer behavior. Many efforts have been made to investigate structural imperfections in dendrimers using different analytical methods, but no single analytical technique can extensively characterize dendrimer structural deviations. For instance, the application of phase partition methods like chromatography [5,6] and electrophoresis [7–9] are useful, but these methods cannot give information about the nature of the defects. Various mass spectrometry methods, with careful analysis, may provide precise molecular weights, but they cannot supply quantitative information regarding the distribution of the materials





Scheme 1. Schematic representation of the structure of E1.NH_2 and the synthesis of E1.NHAc and E1.N(Gly)OH PAMAM derivatives.

[10–12]. NMR is a powerful tool to analyze the quality and purity of dendrimers [13], but it cannot determine the molecular distribution of PAMAM species. Even though ^{1}H and ^{13}C NMR spectra of organic compounds can usually be assigned easily by means of 2D techniques, NMR is limited to determine only the average defects of the individual dendrimer molecules. In this scenario, a combination of different analytical techniques is necessary to understand the structural properties of dendrimers.

Polyamidoamine (PAMAM) dendrimers are synthesized via the divergent approach, which includes alternating reiterations of exhaustive Michael addition followed by amidation [14–16]. Each iteration of the reaction sequence (Michael addition and amidation) leads to the formation of the next generation. The number of terminal groups and molecular weight approximately doubles between generations. PAMAM dendrimers are often considered as monodispersed, highly branched, and globular assemblies. However, commercial PAMAM materials always contain 'trailing generation(s)' as well as dimers, and real PAMAM molecules are always missing arms and have molecular loops [7,9,17]. These defects result from the four main types of side reactions during the synthesis: (1) a retro-Michael reaction gives rise to asymmetrical structures due to missing arms; (2) dimers may form in the amidation step; (3) intramolecular cyclization (molecular loops) may also occur during the same step as ethylenediamine is a bifunctional reactant and (4) the residual ethylenediamine (EDA) acts as a new core to initiate a trailing (g-1) generation. Commercial high generation PAMAMs are relatively more pure than their corresponding low generations, because of the ultrafiltration step, which is usually employed to purify the as-formed dendrimers above generation 3. Smaller generation PAMAMs are very difficult to purify because of their relatively small molecular weight. Therefore, understanding the structural deviations of low generations is crucial for further characterization of their descendents, the higher generation PAMAMs.

In this present study, we illustrate the necessity of using multiple methods for PAMAMs by comparing the analysis results of acetamide (E1.NHAc) and hydroxyl-terminated derivatives (E1.N(Gly)OH) synthesized from the same starting material, a primary amine terminated PAMAM (E1.NH_2). Synthesis schemes of these derivatives are shown in Scheme 1 and the expected theoretical differences in the number of structural amines are shown in Table 1. An ideal E1.NH_2 would contain six tertiary (–N=) and eight primary amines (–NH_2). Upon exhaustive acetylation, all the terminal amine groups are transformed to acetamide groups, but the six tertiary amines remain intact. Similarly, when E1.NH_2 is reacted with excess glycidol, all terminal primary amines are expected to be transformed into tertiary amines (through secondary amines), thus the total theoretical number of tertiary amines in this molecule is 14, which is the sum of the original number of –N= and –NH_2 groups. Comparison of the analytical results of these derivatives to

Table 1
Average numbers of amine functionalities of E1.NH_2, E1.NHAc, and E1.N(Gly)OH PAMAMs

Dendrimer	Theoretical values		Practical values[a]		Summary of real values		
	Primary amines	Tertiary amines	Primary amines	Tertiary amines	Primary amines	Secondary amines	Tertiary amines[b]
E1.NH_2	8	6	7.7±0.3	4.6±0.3	7.7[c]	0	5.6
E1.NHAc	0	6	N/A	4.6±0.3	0	0	5.6
E1.N(Gly)OH	0	14	N/A	12.3±0.4[d]	0	2.5[e]	10.8[e]

[a] As measured by potentiometric titration analysis.
[b] Numbers of tertiary amines are based on full protonation of dendrimers [20]. They are equal to those measured using potentiometric titration plus 1.
[c] Confirmed by both PAGE and potentiometric titration analysis.
[d] The secondary amines of E1.N(Gly)OH cannot be differentiated from tertiary amines (see Fig. 5(b)).
[e] Based on both NMR quantification and potentiometric titration analysis.

those of the parent dendrimer is necessary to determine the real composition because every method has its advantages and limitations.

We applied a variety of analytical techniques to investigate the structural characteristics of this set of E1.NH_2, E1NHAc and E1.N(Gly)OH, including polyacrylamide gel electrophoresis (PAGE), capillary electrophoresis (CE), gel permeation chromatography (GPC), acid–base potentiometric titration, mass spectrometry (MS) and various NMR techniques. The combined analytical results provide complementary information about the real composition of practical materials at the generation 1 (G1) level, as detailed below.

2. Experimental section

2.1. Materials

G1 PAMAM dendrimer (E1.NH_2) with ethylenediamine (EDA) core was purchased from Dendritech (Midland, MI, USA). Acetic anhydride, glycidol, pyridine, 2,3-diaminopyridine (2,3-DAP), phosphoric acid, methanol were obtained from Aldrich and used as received. Water was purified by a Millipore Milli-Q Plus 185 purification system providing H_2O with a resistivity of 18.2 MΩ/cm. All the other chemicals were reagent grade unless otherwise described.

2.2. Synthesis of E1.NHAc and E1.N(Gly)OH

2.2.1. Synthesis of E1.NHAc

To a 3.19 g methanol stock solution of E1.NH_2 dendrimer (dry weight 1.13 g) was added 2.56 ml of pyridine, and the mixture was diluted to 40 ml by methanol in a 250-ml round-bottom flask. Methanol solution containing 1.6 g acetic anhydride (molar ratio of Ac_2O/–NH_2 = 2.5:1) was added dropwise into the dendrimer/pyridine mixture solution while vigorously stirring, and the reaction was maintained for 24 h. The methanol was then removed from the reaction mixture on a rotary evaporator. The oily crude product was diluted with H_2O and dialyzed (MWCO 500) against water (six times 4 l) within three days to remove the excess of reactants and byproducts. The water was removed from the retentate by rotary evaporation. Then the product was redissolved in water and lyophilized. Yield: 1.16 g, 82.7%.

2.2.2. Synthesis of E1.N(Gly)OH

To an 8.59 g methanol stock solution of E1.NH_2 (dendrimer dry weight 3.04 g) in a 250-ml round bottom flask, a methanol solution of 3.15 g glycidol (molar ratio of Gly/NH_2 = 2.5:1) was added dropwise while stirred. After 24 h the methanol was removed on a rotary evaporator, the oily crude product was diluted with water and dialyzed (MWCO 500) against water (six times 4 l) in three days to remove byproducts of reactants. The water was removed from the retentate by rotary evaporation and the product was redissolved in water then lyophilized. Yield: 4.06 g, 73.0%.

2.3. PAGE analysis

Analysis of PAMAM dendrimers by PAGE was performed on a Micrograd vertical electrophoresis system (Gradipore, Sydney, Australia) with a commercial power supply (Model 500/200; BioRad, Hercules, CA, USA). Experiments were performed on 10×8 cm gels in a vertical electrophoresis unit (Model Protean I; BioRad). Precast 4–20% gradient express gels for PAGE were obtained from ISC BioExpress (Kaysville, UT). Tris–glycine (TG) native buffer (pH = 8.3) was purchased from Invitrogen (Carlsbad, CA). It was diluted with water by a factor of ten to prepare the running buffer. PAGE separations typically required 50 min at 200 V. Reverse polarity was used for the analysis of the polycationic E1.NH_2 and its derivatives. Into every sample well 2 μl of a sample solution composed of 1 μl 1 mg/ml PAMAM dendrimer and 1 μl methylene blue sucrose dye solutions (50% sucrose, 1% methylene blue) was injected. Developed gels were stained with 0.025% Coomassie Blue R-250 in 40% methanol and 7% acetic acid aqueous solution overnight. The gels were destained with 7% (v/v) acetic acid and 5% (v/v) methanol in water and analyzed with the NIH Image software (Developed at the

US National Institute of Health and available via the internet at http://rsb.info.nih.gov/nih-image/).

2.4. CE analysis

The CE instrument used in this work was purchased from Agilent Technologies (Waldbronn, Germany). Uncoated quartz capillaries (75 μm ID × 64.5 cm) were obtained from Agilent Technologies. These capillaries have an effective length of 56 cm. The capillary temperature was maintained at 25 °C, and separation voltage was kept at 20 kV for all the separations. An on-capillary UV diode-array detector was used, operating at wavelengths of 200, 210, 250 and 300 nm. Samples were introduced by hydrodynamic injection at a pressure of 50 mbar. Both silanized and uncoated capillaries were tested for the E1 PAMAM materials. Since there was no difference in terms of resolution and reproducibility the uncoated capillaries were used. Before initial use, the capillaries were rinsed with 1 M NaOH for at least 15 min, washed with deionized water for 15 min, and then conditioned with the running buffer for an additional 15 min. Before each injection, the capillary was rinsed with a sequence of 1 M NaOH (5 min) solution, followed by deionized water (5 min), then with the running buffer (5 min). Phosphate (50 mM) buffer (pH = 2.5, Agilent Technologies, Waldbronn, Germany) was used as running buffer. When PAMAM dendrimers are directly dissolved in pH 2.5 (50 mmol/l) phosphate buffer, an undesirable pH-change may occur depending on the sample concentration due to the high content of nitrogen ligands. In this study, the pH of the aqueous dendrimer solution was adjusted to pH 2.5 by 0.2 M phosphoric acid to form a salt. Dendrimer salt solutions were then freeze-dried for three days using a Labconco system. The dry PAMAM dendrimer phosphate salts were then dissolved in a calculated amount of phosphate buffer (pH = 2.5) to give a solution equivalent to c = 1 mg/ml PAMAM at the required pH. As a result of this procedure, every nitrogen atom in the dendrimer molecule is protonated, the buffer is not depleted, and no aggregation occurs between individual dendrimers. The electroosmotic flow (EOF) can be checked by injection of 10% acetone. It is sufficiently small and negligible at pH 2.5. Therefore, one cannot use neutral marker to detect the absolute mobilities of PAMAM dendrimers. In this study, 2,3-DAP (0.05 mg/ml in the dendrimer sample solution) was utilized as an internal standard. In the absence of electroosmotic flow at pH = 2.5, the electrophoretic mobility μ_0 for cations and polycationic dendrimers can be calculated from a simple equation [18] which is expressed as

$$\mu_0 = \frac{lL}{Vt_m} \quad (1)$$

where l is the effective length of the capillary (from inlet to detector window), L is the total length of the capillary (both in cm), V is the applied voltage, and t_m is the migration time. We assume that the internal standard experiences the same varied CE conditions as PAMAM dendrimer samples do thus the change in t_{IS} (IS denotes internal standard) reflects the changes in the run conditions in the column. The electrophoretic mobility of the internal standard in the absence of dendrimers was found to be always constant (e.g., for 2,3-DAP $t_{IS} = 9.2 \pm 0.4$ min., average of nine runs). Consequently, the relative PAMAM migration times ($t_{rel} = t_D/t_{IS}$, D denotes dendrimers) should be constant (alternatively, these data can be multiplied by the independently determined t_{IS} value and further the resulting data can be expressed as *normalized migration times*, which are directly comparable). Thus, the use of the relative, or normalized electrophoretic mobility values should permit direct comparison between various runs for PAMAM dendrimers of varying generations and functional groups.

2.5. Gel permeation chromatography (GPC) measurement

GPC was used to determine the average molecular weights of the as-prepared E1 PAMAM dendrimers. GPC experiments were performed using an Alliance Waters 2690 separation module (Waters Corporation, Milford, MA, USA) equipped with a Waters 2487 UV absorbance detector (Waters Corp.), a Wyatt Dawn DSP laser photometer (Wyatt Technology Corporation, Santa Barbara, CA), and an Optilab DSP interferometric refractometer (Wyatt Technology Corporation). Citric acid buffer (0.1 M) with 0.025% sodium azide in water was used as mobile phase. The pH of the mobile phase was adjusted to pH = 2.74 using NaOH and the flow rate was maintained at 1 ml/min. Sample concentration was kept at approximately 2 mg/ml and 100 μl was injected for all samples. Molar mass moments of the PAMAM dendrimers were determined using Astra software (version 4.7) (Wyatt Technology Corp.).

2.6. Potentiometric acid–base titration

Titration was carried out manually using a Thermo Orion 230A plus pH meter and burette at room temperature, 23 ± 1 °C. Approximately 50 mg of PAMAM dendrimers were dissolved in 30 ml solution of 0.1 M NaCl [19]. Titrations were performed using 0.0951 N HCl, and 0.1021 N NaOH was used for back-titration. The number of primary amines and tertiary amine groups was determined from the back-titration curves using the first derivatives.

2.7. Matrix-assisted laser desorption-time of flight (MALDI-TOF) and electrospray ionization (ESI) mass spectrometry

MALDI-TOF mass spectrometry was performed using Micromass TofSpec-2E mass spectrometer. Reflection mode was selected as the operation mode. 10 mg/ml beta-indoleacrylic acid in acetonitrile/H_2O (v/v = 70:30) was

used as the matrix. One milligram dendrimer samples were dissolved in 1 ml methanol, and then diluted 50 times by methanol to get a final concentration of 0.02 mg/ml. Equal volumes of 0.02 mg/ml dendrimer solution and matrix solution were well mixed. Then, 1 μl solution of the mixture was injected on the spots of target. The instrument was calibrated with four peptides of Angiotensin I, Substance P, Renin Substrate, and ACTH (clip 18–39)) (each peptide has an inject amount of 5 picomole) in an alpha-cyano-4-hydroxycinnamic acid matrix. The matrix was prepared at a concentration of 10 mg/ml in 50% acetonitrile and 50% ethanol. These peptides show $[M+H]^+$ molecular ions at the following mono-isotopic masses: 1296.6853, 1347.7359, 1758.9331 and 2465.1989. All of these standards were purchased from Sigma.

ESI mass spectrometry measurements were performed on a Micromass VG Platform instrument equipped with a single quadrupole mass analyzer using the ESI technique, with nitrogen as a nebulizing gas, and a flow of 350 l/h. Samples of 10 μl were injected into a solvent stream of 50% MeCN and 0.1% formic acid. A scan range of 50–2000 *m/z* was used.

2.8. NMR techniques

^{1}H NMR, ^{13}C NMR, ^{1}H COSY, ^{1}H–^{1}H NOESY and ^{13}C–^{1}H HETCOR NMR spectra were recorded by means of a Bruker AVANCE DRX 500 instrument. Dendrimer samples were dissolved in D_2O to give a $c=5$ mg/ml solution.

3. Results and discussion

3.1. PAGE analysis

PAGE analysis was used to evaluate the purity and homogeneity of dendrimers. In PAGE gel, dendrimers are visualized based on the amount of dye bound to the materials, thus the detection of dendrimers is not directly quantitative. In addition, application of gradient gels necessitates the use of a 'standard' calibration mixture because the retention factor (r_F) values depend on the changing gel density. Fig. 1(a) compares the PAGE electropherograms of E1.NH_2, E1.NHAc, and E1.N(Gly)OH and the semi-quantitative analysis results obtained using NIH Image program are shown in Fig. 1(b). It is clear that only E1.NH_2 and E1.N(Gly)OH migrated under the applied electric field at pH = 8.3. E1.NH_2 travels and can be stained because of its primary amines (13.6% of molecules with defective structure can also be observed as a 'tail'). E1.NH_2 had the highest mobility, which can be explained by the different protonation states, as the primary amine groups are protonated at pH 8.3. E1.NHAc can be stained, but it does not migrate under these conditions indicating that all of the terminal amine groups have been converted to acetamide groups making the molecule neutral at pH 8.3 [17]. Surprisingly, the E1.N(Gly)OH product moves and it is stained, suggesting that it carries some positive charges at this pH. The polycation nature of E1.N(Gly)OH may result from incomplete substitution of the terminal primary amines, but more cannot be concluded from PAGE results.

3.2. CE analysis

CE was used to analyze the purity of dendrimers based on their different electrophoretic mobility. Quantitative results regarding the purity and homogeneity can be achieved using CE based on the electrophoretic mobility of the components including charge-distributions between the molecules. We analyzed these dendrimers using a bare-fused silica capillary (i.d. 75 μm, total length 64.5 cm and effective length 56 cm) and internal standard 2,3-DAP. Fig.

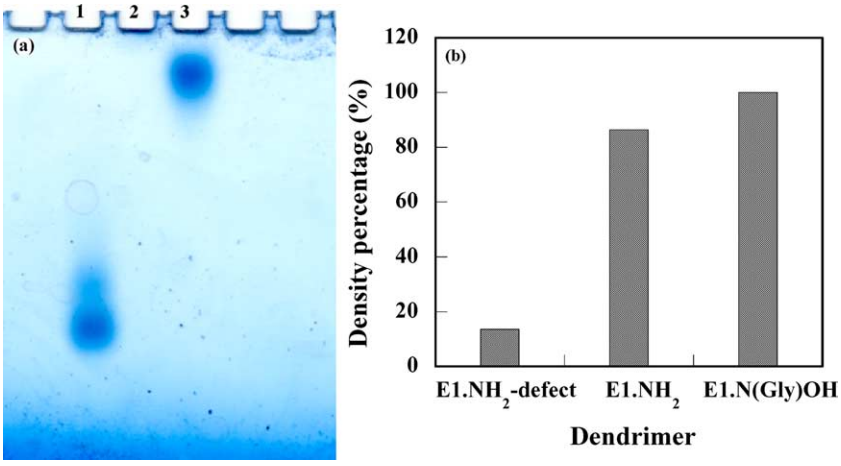


Fig. 1. (a) PAGE electropherograms of E1.NH_2, E1NHAc, and E1.N(Gly)OH. Separation was performed on a 4–20% gradient polyacrylamide gel using a standard Tris–Glycine native buffer (pH = 8.3). Lane 1: E1.NH_2; Lane 2: E1NHAc; and Lane 3: E1.N(Gly)OH. (b) Histogram of the dendrimer distribution in which the heights of columns are proportional to the density values of the bound dye determined by the NIH Image software.

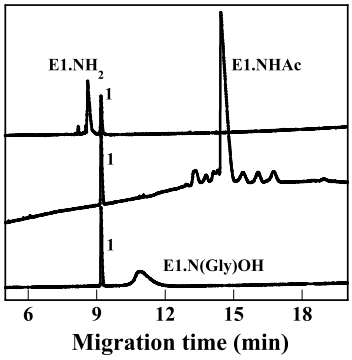


Fig. 2. Normalized CE electropherograms of E1.NH_2, E1.NHAc and E1.N(Gly)OH (normalized migration time of the internal standard, t_{IS} = 9.20 min, the average value of 7 runs, detection wavelength at 210 nm). Peak 1: 2,3-diaminopyridine. CE was performed using a bare-fused silica capillary (i.d.75 μm, total length 64.5 cm and effective length 56 cm). Injection time: t = 3 s.

2 shows normalized CE electropherograms of E1.NH_2, E1.NHAc and E1.N(Gly)OH.

Relative migration times (t_d/t_i, migration time ratio of dendrimers and 2,3-DAP) of all of these dendrimers are constant and their RSD values are all less than 2% (Table 2), indicating that good reproducibility has been achieved. Electrophoretic mobility of generation 1 PAMAM decreased after surface modifications, as expected. At pH 2.5, the observed electrophoretic mobility was in the order of E1.NH_2 > E1.NGlyOH > E1.NHAc, i.e., in the same order as their theoretical net charge/mass ratio (Table 2). Shape of the peaks are also compared in Fig. 2, where the main peaks of E1.NH_2 and E1.NHAc are much narrower than E1.N(Gly)OH. (The minor peaks have to be yet identified.) In the Section 3.5, we will further discuss the differences in the molecular weight distributions of the materials studied. It can be clearly seen that the intensity of the main peak of E1.NHAc is much higher than that of E1.NH_2. After acetylation, there are more small visible peaks appeared in the E1.NHAc electropherogram (Fig. 2), and the differences between various components of E1.NHAc seem to be magnified compared to the starting material E1.NH_2.

Deconvolution of the CE peaks using the PEAKFIT program (SYSTAT Software Inc., Point Richmond, CA 94804) provides more quantitative results. Fig. 3 shows the deconvolution of E1.NH_2 (a), E1.NHAc (b), and E1.N(Gly)OH (c) CE peaks in the presence of 2,3-DAP, and E1.NH_2 (d) in the absence of 2,3-DAP internal standards. Because the 2,3-DAP peak overlaps with E1.NH_2 peaks, deconvolution on E1.NH_2 electropherogram was performed both in the presence and absence of 2,3-DAP (Fig. 3(a) and (d)). Deconvolution lead to very similar profiles (see Fig. 3(a)). The major peak, corresponding to the ideal E1.NH_2, represents 79.3% of the total peak area in Fig. 3(d), while in E1.NHAc (Fig. 3(b)) the ideal component is 72.6% of the total peak area. E1.N(Gly)OH (Fig. 3(c)), displays only one broad peak, as all the individual peaks are hidden under an envelope. In this case, it is not feasible to differentiate the various species by CE. Then, mass spectrometry (vide infra) will be amenable for the further analysis of this material. CE results indicate that after acetylation, the differences between substituted PAMAM molecules are amplified, whereas the differences diminish after hydroxylation.

3.3. GPC measurements

GPC analysis provides the molecular weight and polydispersity information of the as-prepared E1 PAMAM materials. Fig. 4 shows the differential mass fraction profile of E1.NH_2, E1.NHAc and E1.N(Gly)OH dendrimer materials. The peaks of all three dendrimers appear to be symmetrical and have a close to Gaussian distribution. The polydispersity values similar to PDI = 1 (Table 2), suggest minimal differences for hydrodynamic volumes of E1 dendrimers. It is worthwhile to note that the range of molecular weights measured with GPC is close to the lower end of the detection limit. Compared with CE measurements, GPC appears to be less sensitive in terms of detecting

Table 2
Physicochemical parameters of generation 1 PAMAMs

Dendrimer	E1.NH_2	E1.NHAc	E1.N(Gly)OH
Theoretical M_w	1430	1766	2614
M_w measured by MALDI-TOF[a]	1430	1766	2468
M_n[b]	1398	1840	2385
M_w[b]	1431	1857	2411
Polydispersity[b]	1.024	1.019	1.011
Theoretical charge/mass ratio[c] (pH = 8.3)	5.59×10^{-3}	N/A	N/A
Theoretical charge/mass ratio[c] (pH = 2.5)	9.79×10^{-3}	3.40×10^{-3}	5.36×10^{-3}
Relative migration times (RSD)	0.94 (0.752%)	1.57 (1.01%)	1.19 (1.46%)
Electrophoretic mobility[c] (cm^2/Vs)	3.48×10^{-4}	2.08×10^{-4}	2.75×10^{-4}

[a] Obtained from the dominant peaks in MALDI-TOF spectra.
[b] Measured by GPC.
[c] Charge/mass ratios were calculated using the theoretical number of terminal and tertiary amine groups and theoretical molecular masses.

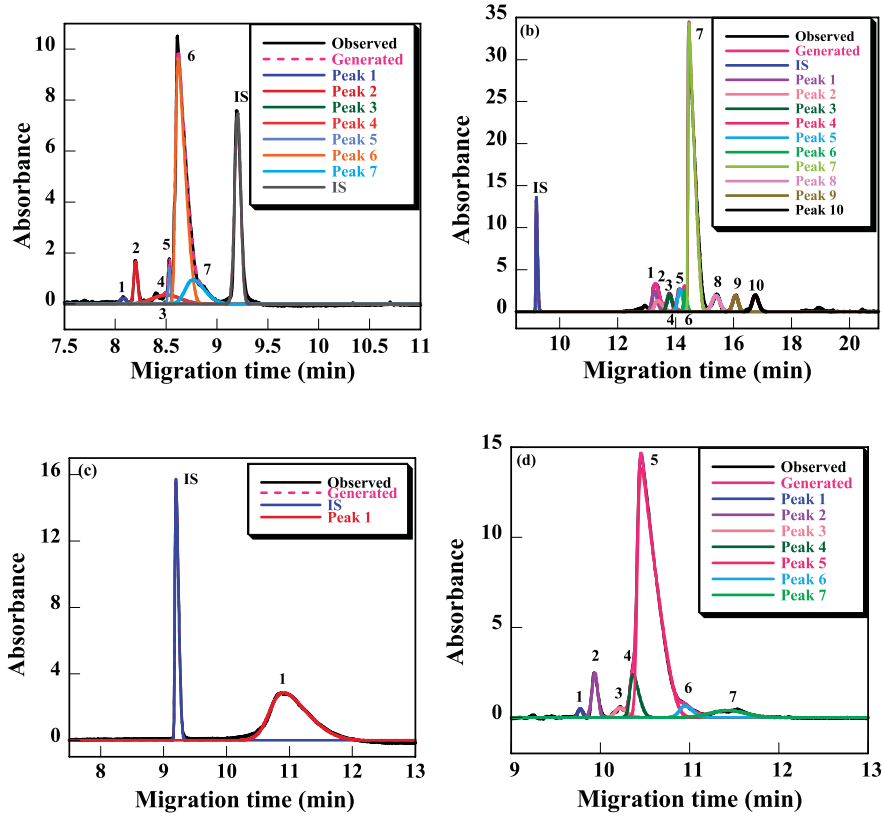


Fig. 3. CE migration peaks of E1.NH_2 (a), E1.NHAc (b) and E1.N(Gly)OH (c) deconvoluted by PEAKFIT. Deconvoluted electropherogram of E1.NH_2 without internal standard is also shown in (d). The major peak, corresponding to the pure E1.NH_2, possesses 79.3% of the total peak area in (d), while E1.NHAc constitutes 72.6% of the total peak area in (b). Regarding E1.N(Gly)OH (c), there is only one major broad peak, representing all the components.

structural differences, as all the measured species are all spherical at that pH. However, hydrodynamic volumes measured by GPC reflect the *average size distribution* of the entire dendrimer structure (including dimers, trailing generations, missing arms and molecular loops) and this is the best method to measure average molecular mass, even though CE is more sensitive in detecting charge distribution *differences* in various molecular species. The measured GPC molecular weight data may further be used to determine average numbers of terminal and tertiary amine groups of E1 PAMAMs after the acid-base titration data become available (as described below).

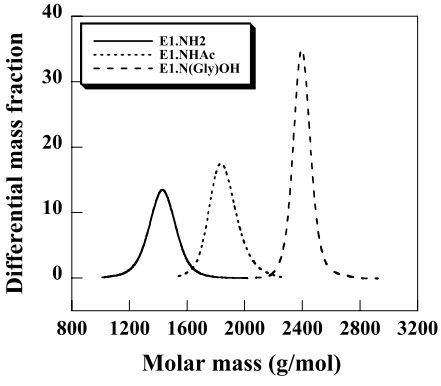


Fig. 4. GPC differential mass fraction profiles of E1.NH_2, E1.NHAc, and E1.N(Gly)OH.

3.4. Acid–base potentiometric titration of G1 polycationic PAMAM dendrimers

Potentiometric acid–base titration allows us to measure average numbers of primary and tertiary amine groups in dendrimer materials. Titration results for the studied materials are summarized in Table 1, and Fig. 5 shows representative titration curves of the E1.NH_2 starting material and E1.N(Gly)OH (titration curves for E1.NHAc are not shown). Forward titration starts from a 'native' protonation equilibrium, thus to measure the number of base functionalities we used the back-titration data that reflect the full deprotonation process of dendrimers. In Fig. 5(b), the first derivative peak (at 1.7 ml) in curve *C* corresponds to the full neutralization of excess HCl, the second derivative peak (at 4.45 ml) reflects the complete deprotonation of both tertiary and terminal secondary amines. The small amount of secondary amines cannot be measured by acid–base titration because their deprotonation capability is close to that of the tertiary amines. The measured tertiary amines for E1.N(Gly)OH using titration is close to the sum of the number of secondary amines and tertiary amines measured using NMR quantification (Table 1). In this case, both

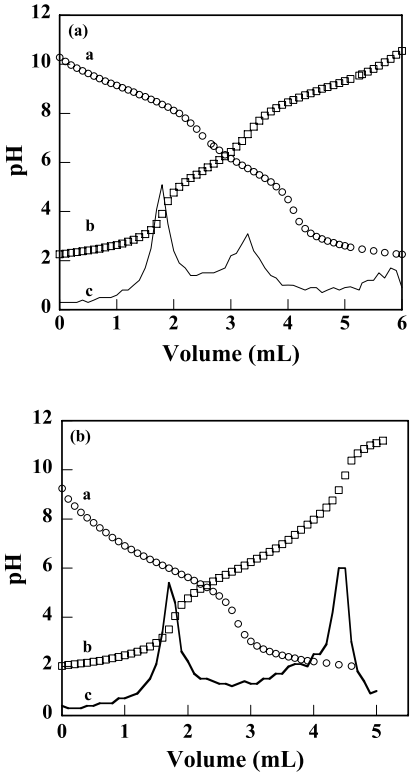


Fig. 5. Acid–base potentiometric titration curves of E1.NH_2 (a) and E1.N(Gly)OH (b). Curve a, b, and c correspond to the forward titration, to the back titration, and the first derivative of the back titration data, respectively.

techniques are supplementary to provide identical values. Average amounts of terminal and tertiary amines of E1.NH_2 can be easily measured by acid–base titration (Fig. 5(a)) because of the large difference between their pK_a values (for PAMAM terminal and tertiary amines are around 4–5 and 9–10, respectively, [15,16]). According to the literature [20], one of the core tertiary amines cannot be completely protonated even at pH 2. In our measurements, the minimum pH used was 2.1 in order to avoid any possible decomposition of dendrimers. Thus, the number of tertiary amine groups determined here by titration should be corrected to get the actual results. Due to the neutrality of terminal acetamide groups, only the tertiary amines participate in protonation and deprotonation process in E1.Ac, which allows us to measure the number of tertiary amines in the starting E1.NH_2 material separately. (The measured number of tertiary amines is identical to that of E1.NH_2, see Table 1.) These titration data can reliably reflect the *average* amine functionalities of these dendrimers.

3.5. Mass spectrometry

ESI and MALDI TOF mass spectrometry supply the mass-related information of particular species including the indication of specific structural defects [6,21]. (Hummelen et al. [21] used the terms of 'compositional and positional heterogeneity' for poly(propyleneimine) (PPI) dendrimers. These terms are similar to but not identical to our terminology, as composition may refer to atoms that constitute the molecules and 'positional' may refer to geometric localities for internal positions and terminal positions as well.) Interestingly, while ESI-MS and MALDI-TOF work equally well for E1.NH_2 and

Table 3
Structural defects and assignments of E1.NH_2, E1.NHAc and E1.N(Gly)OH based on MALDI-TOF mass spectrometry

Dendrimer	Molecular fragments	Number of structures in Fig. 7	Molecular fragments assignment
E1.NH_2	1430	1	Perfect E1.NH_2
	1370	2	Intramolecular cyclization (loop)
	1316	3	One missing arm
	1201	4	Two missing arms
	914	5	Two missing arms and one loop
	715		Double ion of 1430
E1.NHAc	1766	6	Perfect E1.NHAc
	1622	7	Intramolecular cyclization (loop)
	1496	8	Two missing arms
	1082	9	Two missing arms and one loop
	883		Double ions
E1.N(Gly)OH	2615	10	16 Glycidol substituents
	2541		15 Glycidol substituents
	2468		14 Glycidol substituents
	2393		13 Glycidol substituents
	2318		12 Glycidol substituents
	2245		11 Glycidol substituents
	2184	11	One loop +11 glycidol substituents
	2037	12	One loop+9 glycidol substituents
	1307		Double ions of 2615
	1233		Double ions of 2468
	1159		Double ions of 2318

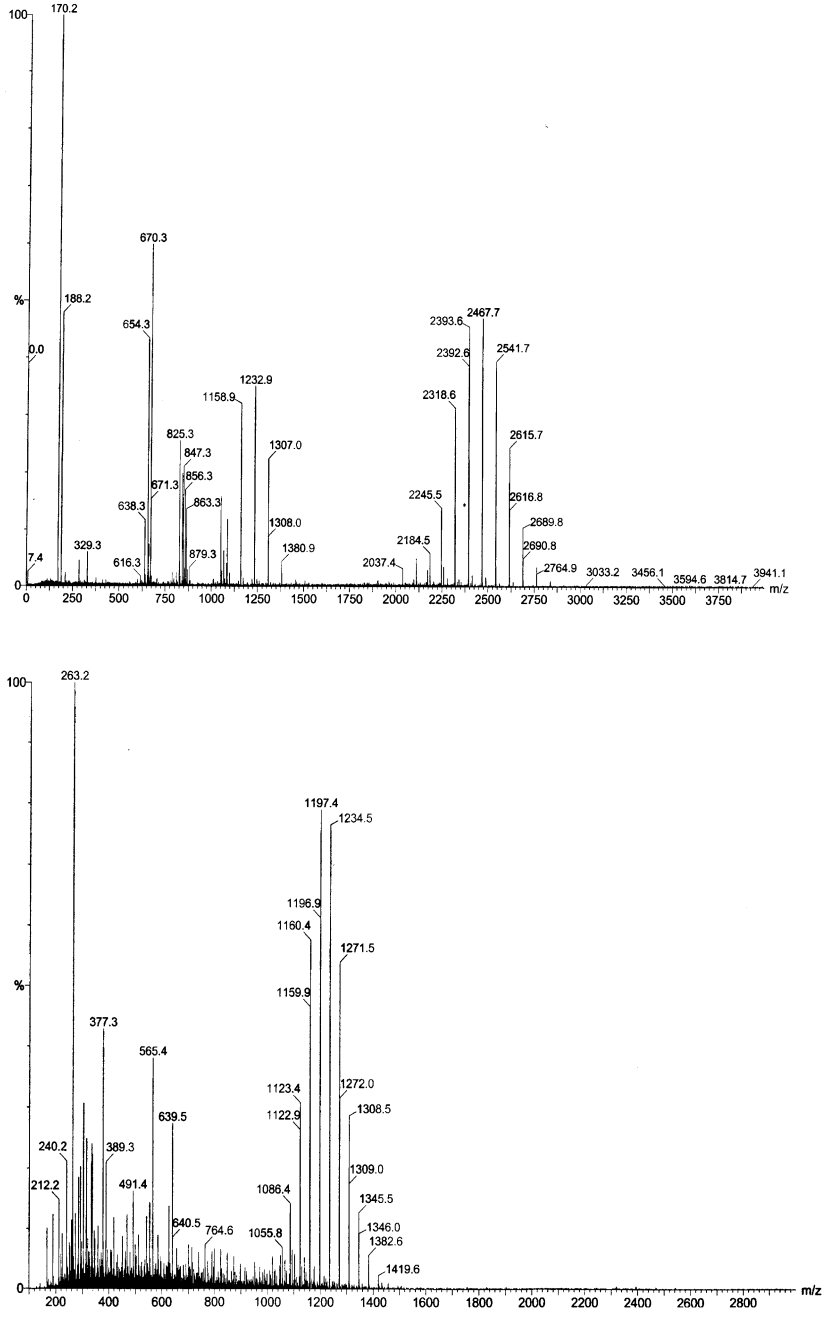


Fig. 6. Representative MALDI-TOF (top panel) and ESI (bottom panel) mass spectrum of E1.N(Gly)OH.

E1.NHAc (see Supporting Information), ESI-MS creates double-charged species from the E1.N(Gly)OH molecules (see Fig. 6 for representative MALDI-TOF and ESI mass spectra of E1.N(Gly)OH.).

The assignments of molecular fragments in the MALDI-TOF spectra [8] are listed in Table 3. Fig. 7 shows the possible PAMAM dendrimer molecular structures suggested by MALDI-TOF.

One has to keep in mind that although MS provides exact molecular masses of the detected species (which helps identify possible structural differences and molecular weight distribution patterns), it is not applicable for the straightforward quantification of the dendrimers because existence molecular fragments are generated by the method itself. PAMAM dendrimers are especially sensitive to the applied experimental conditions. Analysis of data shows that both E1.NH_2 and E1.NHAc have a relatively simple composition (see Supporting Information) compared with E1.N(Gly)OH. It seems that this observation may be inconsistent with the CE results in which the opposite observation was made, and only one broad migration peak was present in the E1.N(Gly)OH electropherogram. However, CE cannot differentiate between individual species according to the number of their terminal glycidol moieties,

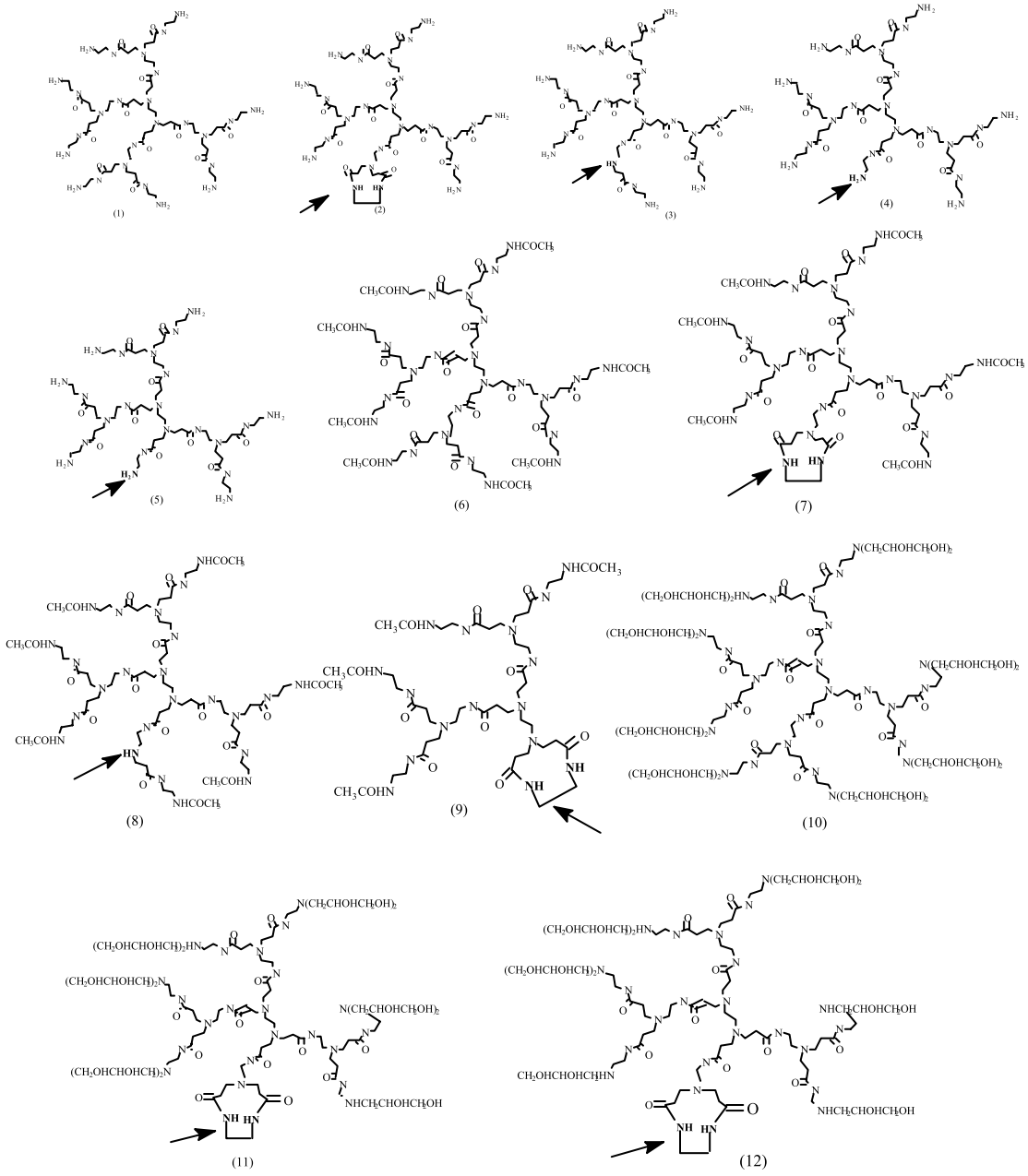


Fig. 7. Typical generation 1 PAMAM dendrimer structures as suggested by mass spectra. (Based on structures from Ref. [12]).

and E1.N(Gly)OH dendrimer molecules with slightly different structures are hidden in a single peak because their differences are too small. The same explanation applies to the comparison of MS data with GPC data. Molecular mass distribution of E1.N(Gly)OH is influenced by the imperfect substitution of the secondary amino groups by glycidol molecules (that may be due to steric hindrance). Structures related to missing arms and loops shown in the MS spectra of E1.NH_2 are also present in those of E1.NHAc and E1.N(Gly)OH materials (Fig. 6 and Supporting Information), indicating that their structural diversities are essentially similar (Table 3 and Fig. 7) and were 'inherited' from the parent material (repeated purification by dialysis may lead to slight differences in the products). The results of ESI are similar to MALDI-TOF. The only important difference for E1.N(Gly)OH is that the molecules are double-charged in the ESI-MS spectrum, hence the molecular weight of fragments appear to be half of those shown in MALDI-TOF (Fig. 6).

3.6. NMR analysis

We investigated the three different E1 PAMAM dendrimers by means of ^{1}H and ^{13}C, ^{1}H COSY, ^{1}H–^{1}H NOESY and ^{13}C–^{1}H HETCOR NMR spectra spectroscopy. For E1.NHAc, a product close to ideal is expected because a

small molecule, which is not expected to experience any steric hindrance, was used as a reactant in high molar excess. For the E1.N(Gly)OH, reaction of the second glycidol group with the secondary amines is sterically hindered; therefore, defects occur in the form of missing terminal glycidol groups. Since this dendrimer is more complex than the starting material E1.NH_2, a complete assignment of E1.N(Gly)OH is given (See Supporting Information). This is helpful for the understanding of NMR analysis results of E1.NH_2 and E1.NHAc.

3.6.1. E1.NH_2

The protons are numbered after the carbons to which they are attached (Scheme 2(a)). The EDA core is indicated with letter *c*, while the CO carbons hold their generation number. Fig. 8 displays the ^{1}H NMR spectrum with the proton numbering as seen from Scheme 2(a), while Table 4 contains the numerical results. The assignment was done on the basis of ^{1}H COSY and ^{13}C–H HETCOR experiments. According to the integration of different proton signals (within the $\pm$3–5% experimental uncertainty of the quantitative ^{1}H NMR), this E1.NH_2 has no defects at the intramolecular level detectable by ^{1}H NMR.

3.6.2. E1.NHAc

This dendrimer appear to be also monodispersed at the molecular level from an NMR point of view (short range nuclear interactions). Scheme 2(b) refers to the numbering of the carbons. The complete assignment is collected in Table 5. The assignment of the three carbonyls was verified by long-range proton-carbon 2D correlation (COLOC). Protons on carbons 7 and 8 are equivalent, therefore, their peaks are singlets at the same chemical shift and so are their respective ^{13}C signals. Similar to the E1.NH_2, the scalar ^{1}H–^{1}H coupling constants indicate that the protons occupy the usual *trans*-gauche position, i.e., there is no hindrance of free rotation at this generation level.

3.6.3. E1.N(Gly)OH

Complete assignment of E1.N(Gly)OH is provided in the Supporting Information. As it is expected, missing terminal

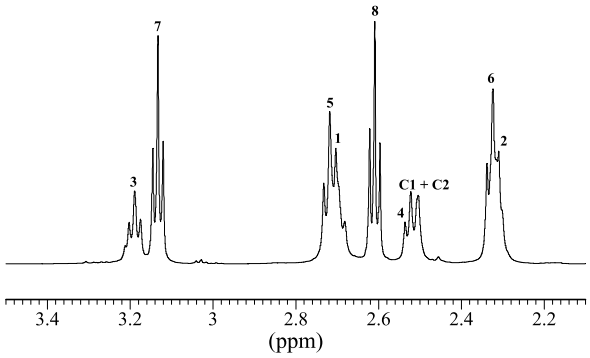
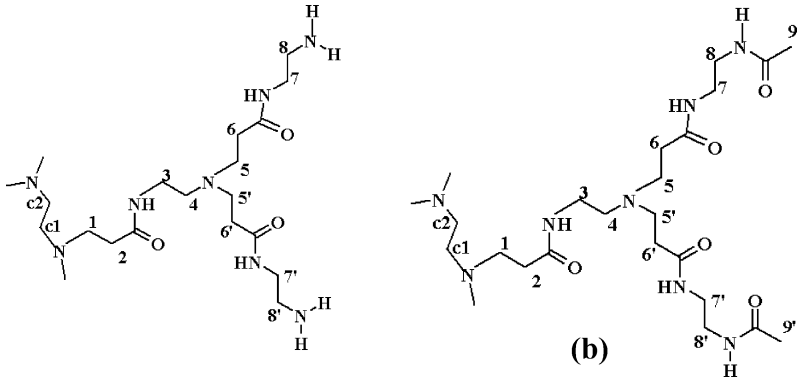


Fig. 8. ^{1}H NMR spectrum of E1.NH_2 dendrimer.

groups cause a change in the chemical shifts of the neighborhood protons, which provides a way to quantify the degree of substitution observed in ES-MS and MALDI-TOF. According to the ^{1}H NMR integrals, approximately 3 CH protons are missing. This level of defects has an 81% effective yield for the E1.N(Gly)OH dendrimer, i.e. 81% of the E1.N(Gly)OH product contains tertiary amines only, but in 19% of the molecules secondary amines are also present. The ^{13}C NMR (see Supporting Information) confirms the same result. First, three different CH (labeled as number 10 shown in Scheme S1 in Supporting Information) are present in almost equal intensity. Second, three carbonyl signals are observed, thus the E1 CO groups are in two different chemical environments.

4. Conclusion

In summary, structural (i.e., generational, skeletal and substitutional) deviations were identified in generation one PAMAM dendrimers through the extensive characterization of E1.NH_2, E1.NHAc, and E1.N(Gly)OH materials using combined analytical techniques. According to PAGE, ESI-MS and MALDI-TOF, about 14% of the starting material contained loops. We found that at pH = 8.3 the E1.N(Gly)OH was not neutral, which was attributed to the incomplete substitution reaction of the terminal secondary amine groups of E1.NH_2 with glycidol (even using 2.5-fold



Scheme 2. Expected formulas and numbering for E1.NH_2 (a) and E1.NHAc (b). Only a quarter of the respective molecules are shown.

Table 4
NMR data for E1.NH_2

No. carbons	^{13}C NMR		^{1}H NMR		
	Chem. shift (δ/ppm)	Notes	Chemical shift (δ/ppm)	Coupling const. (J/Hz)	Integral[a]
c(1,2)	50.34		2.50	*s*	3.97 (3)
1	49.45	Same as 5	2.70	*t* ($\approx$7.8)	8.1 (5)
2	32.95		2.31	*t* ($\approx$7.8)	8.1 (6)
3	37.13		3.19	*t* (6.85)	7.8 (8)
4	51.60		2.53	*t* (6.85)	7.9 (core)
5	49.45		2.72	*t* (7.17)	16.3 (1)
6	33.17		2.32	*t* (7.17)	16.2 (2)
7	42.05		3.13	*t* (6.29)	15.9
8	40.16		2.61	*t* (6.29)	15.7 (4)
CO(E0)	174.94	$I=I$(E1)			
CO(E1)	175.43				

[a] In parenthesis, we note the numbers of protons, which were integrated together. In the notes column, 'I' refers to the NMR integrals.

excess of the reactant). CE studies show that the electrophoretic mobility (effective charge/mass) decreases after the surface modification of E1.NH_2 and the electrophoretic mobility at pH 2.5 is in the order of E1.NH_2 > E1.N(Gly)OH > E1.NHAc. GPC analysis shows that the studied dendrimer materials are relatively monodispersed. Average numbers of primary and tertiary amine groups, was measured by acid–base titration and indirectly with NMR. Potentiometric titration studies indicated that the numbers of terminal and tertiary amine groups deviate from the theoretical numbers. NMR integral values of E1.N(Gly)OH dendrimer indicated the presence of 19% secondary amines, the presence of which enables the product to migrate in PAGE and be stained by a dye. Based on mass spectrometry and NMR, E1.NH_2 and E1.NHAc PAMAM dendrimers have only minor structural defects at the generation 1 level. Acetamide and hydroxyl-terminated E1 PAMAM dendrimers had essentially the same skeletal defects as the amine-terminated starting material did. Amine and acetamide-terminated PAMAMs had narrow polydispersities, while the glycidol-terminated E1 had a broader substitutional diversity due to distribution of substituents on their amine termini centered around 14 ± 3 glycidol. This was verified also by MS and NMR (1D and 2D) techniques. We conclude that none of the single techniques are able to completely characterize PAMAM dendrimer material composition and all the three distributions (*generational, skeletal, and substitutional*) are present. The use of combined characterization techniques in a frame of a systematic investigation is required even at the generation 1 level to clarify and determine the molecular composition of PAMAM dendrimer materials. This study provides new insights for the characterization of higher generation PAMAM-based multifunctional nanodevices.

Acknowledgements

This work was financially supported in part by the

Table 5
NMR data for E1.NHAc

No. carbons	^{13}C NMR		^{1}H NMR		
	Chemical shift (δ/ppm)	Notes	Chemical shift (δ/ppm)	Coupling const. (J/Hz)	Integral[a]
c(1,2)	49.58		2.73	*s*	3.96 (5)
1	48.79		2.86	*t* (7.03)	7.4
2	31.67		2.41	*t* (7.03)	8.0 (6)
3	51.30		2.59	*t* (6.55)	7.9
4	36.55		3.23	*t* (6.55)	8.1 (7 and 8)
5	49.04		2.77	*t* (7.05)	15.8 (core)
6	32.25		2.34	*t* (7.05)	15.9 (2)
7	38.63		3.19	*s*	16.2
8	38.63		3.19	*s*	16.2 (4)
9 (CO)	174.23				
10	21.90		1.87	*s*	24.6
CO(E0)	173.92	I=I(E1).			
CO(E1)	174.71				

[a] In parenthesis, we note the numbers of protons, which were integrated together. In the notes column, 'I' refers to the NMR integrals.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.polymer.2005.01.081

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