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Acetylation of Poly(amidoamine) Dendrimers

István J. Majoros,* Balázs Keszler, Scott Woehler, Tricia Bull, and James R. Baker, Jr.

Center for Biologic Nanotechnology, University of Michigan, Ann Arbor, Michigan 48109-0533

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ABSTRACT: The precise stoichiometry required for the acetylation of surface amines of a poly(amidoamine) (PAMAM) dendrimer generation 5 (G5) was verified by using potentiometric titration, gel permeation chromatography, and nuclear magnetic resonance spectroscopy. The average number of primary amine groups, absolute molecular weight, and molecular weight distribution of G5 PAMAM were determined by potentiometric titration and GPC. These fundamental parameters were used to design the stoichiometry of an acetylation reaction that yielded acetylation fractions from 0 to 100% of the primary amines on the macromolecule. GPC refractive index detector confirmed that the diameter of the dendrimer related inversely to the degree of acetylation. The acetylated dendrimers do not follow the elution behavior of the conventional polymer molecules most probably because of their spherical shape and polycationic nature. This study clarifies the nature of the acetylation reaction and provides a well-defined acylated macromolecule, which can serve as a scaffold for the development of complex dendrimeric structures.

Introduction

Dendrimers are spherical, highly branched macromolecules that have a central core with emanating dendrons and terminal end groups. Each branched layer of macromolecule makes up a single generation, which defines the size and characteristics of the dendrimer.[1,2] The dendrimers exhibit an exponential increase of surface groups as a function of generation, while the volume of the sphere increases with the cube of the generation.[3] The chemistry of the outer functional groups provides many of the unique properties of dendrimers such as solubility and reactivity.

The dendrimer surface groups can be functionalized with molecules of biological importance. It has been reported that 5-fluorouracil, an antitumor agent, reacted with surface amino groups to form dendrimer−5FU conjugates. The drug could be freely released after the conjugate was placed in a phosphate buffer solution having a near neutral pH.[4] As well, complexation of plasmid DNA with dendrimers has led to high transfection efficiency in a number of systems.[5] Further studies show that with appropriate reaction conditions the end groups on dendrimers can be modified for applications including gene therapy, protein receptors, catalysts, and drug delivery devices.[6−9]

Acetylation of G5 poly(amidoamine) (PAMAM) dendrimers' reactive end groups has been a route to specifically functionalize the primary surface amines.[4] When these dendrimers are acetylated, they become more water-soluble, and this quality is vital for biomedical applications that require solubility in aqueous solutions.[10] However, no systematic study of the chemistry involved in the acetylation of ethylenediamine (EDA) core G5 PAMAM dendrimers has been performed. By defining the exact percentage of acetylated end groups and the structure of the dendrimer, one can produce polymers that are soluble and serve as a scaffold for further functionalization. These studies were designed to investigate the nature of the acetylation reaction used and to provide an analysis of well-defined dendrimer conjugates for use in the biomedical field. Full characterization of a series of acetylated PAMAM G5 dendrimers was performed with several techniques including potentiometric titration, GPC, ^{1}H NMR, and ^{13}C NMR.

* Corresponding author: e-mail majoros@umich.edu.

Experimental Section

Materials. All chemicals were purchased from Aldrich Co. and used as received (acetic anhydride, Et_3N, MeOH, NaCl, HCl, NaOH). All solvents were HPLC grade. The PAMAM G5 dendrimers were synthesized at the Center for Biologic Nanotechnology, University of Michigan.

Potentiometric Titration. Titration was carried out manually using a Mettler Toledo MP230 pH meter and MicroComb pH electrode at room temperature, 23 ± 1 °C. A 10 mL solution of 0.1 M NaCl was added to approximately 100 mg of PAMAM dendrimer to shield amine group interactions. Titration was performed with 0.1028 N HCl, and 0.1009 N NaOH was used for back-titration. The number of primary amines was determined from the back-titration data.

Gel Permeation Chromatography. GPC experiments were performed on an Alliance Waters 2690 separation module equipped with 2487 dual wavelength UV absorbance detector (Waters Corp.), a Wyatt Dawn DSP laser photometer, 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 temperature was maintained at 25 ± 0.1 °C by a Waters temperature control module. The isocratic mobile phase was 0.1 M citric acid and 0.025% sodium azide, pH 2.74, at a flow rate of 1 mL/min. Sample concentration was 10 mg/5 mL with an injection volume of 100 μL. Molecular weight, molecular weight distribution, and root-mean-square radius of the PAMAM dendrimers were determined using Astra 4.7 software (Wyatt Technology Corp.).

Acetylation. The ratio between the acetic anhydride and the dendrimer was adjusted to achieve various degrees of acetylation, with 20, 40, 60, 80, and all 120 (100%) primary amine groups converted. The amount of acetic anhydride was calculated on the basis of the number of primary amines determined by potentiometric titration to ensure a 1:1 stoichiometric relationship between the acetic anhydride and the primary amino groups of G5 PAMAM dendrimer. (When complete (100%) acetylation of dendrimer was planned, 20 mol % excess of acetic anhydride was used.) Triethylamine (10%

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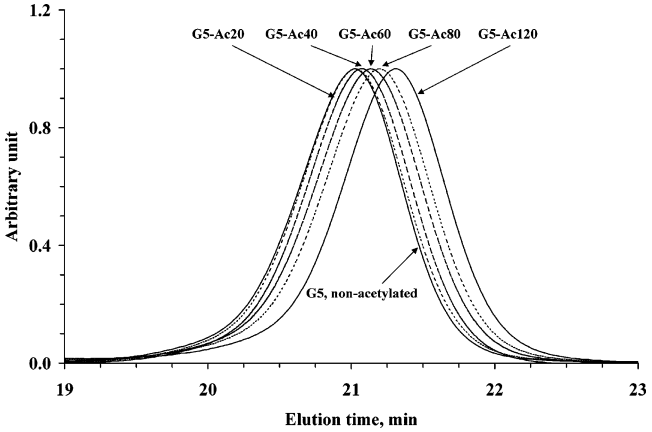

Figure 1. GPC RI eluograms of G5 PAMAM nonacetylated and acetylated to different degree dendrimers.

excess based on the amount of acetic anhydride) was added to quench acetic acid formed as a side product during the reaction. The reactions were carried out in a glass flask in anhydrous methanol solution at room temperature for 24 h. The reaction mixture was dialyzed first in phosphate buffer at pH = 8.0 and then in deionized water. The purified samples were lyophilized and stored at −20 °C.

Nuclear Magnetic Resonance Spectroscopy. ^{1}H and ^{13}C and HETCOR NMR spectra were taken in D_2O and were used to provide integration values for structural analysis by means of a Brueker AVANCE DRX 500 instrument.

Results and Discussion

Theoretically, G5 dendrimer has 128 primary amine groups on its surface. Potentiometric titration revealed 120 primary amines of the dendrimer used for acetyl derivatization. The measured molecular weight of the G5 dendrimer, 27 250 g/mol, is somewhat lower than the theoretical one, 28 826 g/mol. These results indicate a slight deviation from the theoretical structure. In an attempt to correlate the structure of the dendrimer to the experimental data, a model was assumed with eight "missing arms" from a fifth-generation level, corresponding to the eight missing primary amine groups.

A series of acetylation reactions were conducted converting 20, 40, 60, 80, and all of 120 primary amines. The eluograms (normalized RI detector signals) of the acetylated and nonacetylated dendrimers are shown in Figure 1. The retention volume (time) increases with the decreasing number of available free primary amino groups. (In the pH = 2.74 mobile phase all primary and tertiary amines are protonated.) That is, the size of the acetylated dendrimers is smaller despite their increasing molecular weight. The GPC data indicate that the acetylated dendrimer molecules do not follow the behavior of the conventional polymer molecules most probably because of their spherical shape and polycationic nature. Therefore, the conventional molecular weight determination method based on calibration cannot be used in such cases. Determination of molecular mass (absolute molecular mass) of such molecules can be established by GPC equipped with MALLS. Additionally, this setup allows determining root-mean-square radius.

The GPC data of the acetylated dendrimers shown in Table 1 indicate the molecular weight increases during acetylation. The measured molecular weights are in good agreement with the calculated weights. The molecular weight distribution (MWD) of all acetylated dendrimers remained very narrow, similar to the MWD

Table 1. M_n, M_w/M_n, Root-Mean-Square Radius, and $M_{n,theoretical}$ Determined by GPC and Calculated Based on the Result of Potentiometric Titration

	molecular weight			
sample	theoretical[b]	measured	MWD	rms radius,[c] Å
G5 0Ac[a]	27 914	27 250	1.037	25.3
G5 20Ac	28 754	28 530	1.032	24.7
G5 40Ac	29 594	29 470	1.035	24.4
G5 60Ac	30 434	30 360	1.041	23.4
G5 80Ac	31 274	31 030	1.038	22.9
G5 fully Ac	32 954	32 710	1.054	22.3

[a] Number stands for number of acylated primary amines. [b] Molecular weight was calculated assuming eight missing arms from generation 5 level. [c] Root-mean-square radius.

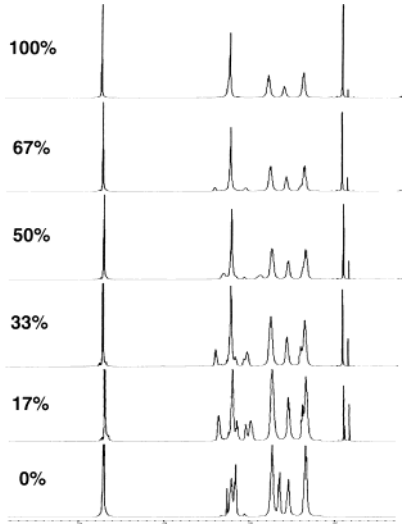

Figure 2. ^{1}H NMR spectra of nonacetylated dendrimers and acetylated dendrimers to different degrees.

of the nonacetylated dendrimer (see Table 1). The calculated root-mean-square radius decreased during acetylation. The decreasing radius and the increasing elution volume with an increasing degree of acetylation indicate a more compact structure for the acetylated molecules. Increasingly compact structure is due to the decreasing number of available primary amines for protonation, resulting in less repulsion by charges, which expand the volume (size) of the dendrimer.

The ^{1}H NMR spectra of the acetyl-derivatized dendrimers show the peak at 1.87 ppm related to the $-CH_3$ protons of the acetyl group (Figure 2). The increasing intensity of this signal reflects the increasing degree of acetylation. The reaction product is a mixture of different partially acetylated dendrimers. The number of acetyl groups is represented by a mean value (or number-average number of acetyl groups) and can be calculated by formula $\overline{N_a} = \sum n_i N_i / \sum n_i$. (This formula is analogous to the formula of number-average molecular weight in polymer chemistry.)

The acetic acid, which is a side product of the acetylation, may also react with the amines. (Et_3N and free $-NH_2$ compete in forming the alkylammonium acetate salt.) The unexpected peak at 1.81 ppm in each spectrum is related to the protons of the $-CH_3$ group in the salt**.** In a model reaction salt formation between dendrimer and acetic acid was simulated. The peak at 1.81 ppm in the ^{1}H NMR spectra was assigned to the protons of the methyl groups in the salt. On the other hand, the methyl proton peak of the acetic acid is at 2.1 ppm (from NMR handbook of Aldrich). So, it can be

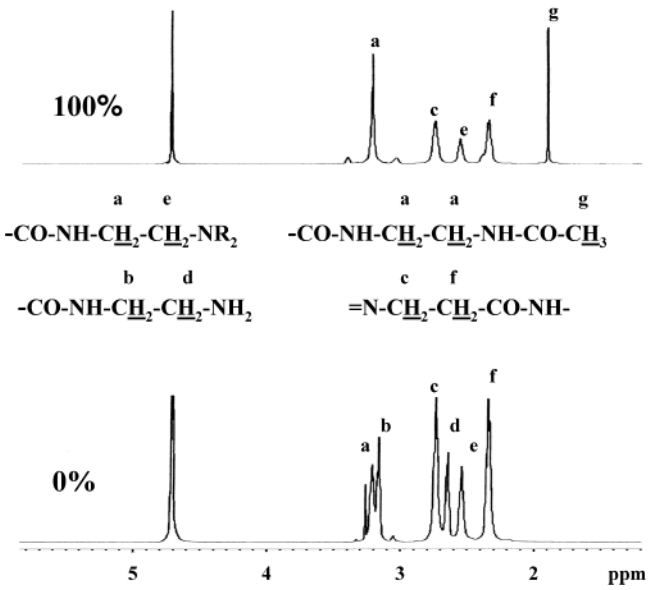

Figure 3. ^{1}H NMR spectra of acetylated (after dialysis) and nonacetylated dendrimers.

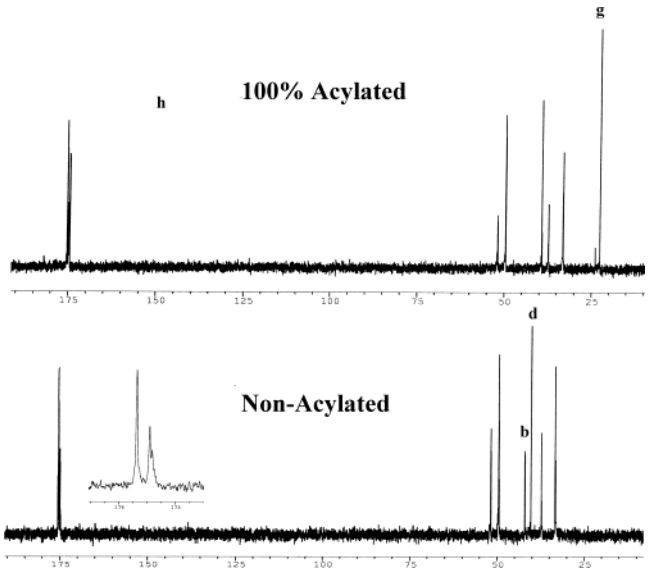

Figure 4. ^{13}C NMR spectra of acetylated (after dialysis) and nonacetylated dendrimers.

safely stated that the peak at 1.81 ppm (Figure 2) represents the methyl protons of the salt generated by the side reaction. As experience shows, this salt can be removed by careful dialysis under appropriate conditions (Figure 3).

Converting all primary amines to acetyl derivatives alters the structure of the dendrimer. The peaks of the protons related to the two $-CH_2-$ groups next to the primary amine at 3.16 and 2.63 ppm (corresponding to the "b" and "d" type of proton in Figure 3, respectively) disappear. On the other hand, the intensity of the peak due to $-CH_2-$ (the "a" type of proton in Figure 3) adjacent to the amide group at 3.20 ppm increases, indicating the conversion of "b" and "d" protons to "a".

The ^{13}C NMR and HETCOR NMR spectra show that the peak at 22.4 ppm corresponds to the $-CH_3$ carbon of the acetyl group (Figures 4 and 5). The ^{13}C NMR spectrum also shows a new peak at 174.4 ppm due to the $=$CO of the acetyl group.

The comparison of the intensity of the peak at 1.87 ppm, representing the protons of the $-CH_3$ of the acetyl groups to the sum of intensity of all $-CH_2-$ protons may be used to determine the completion of the acetylation reaction. The measured $-CH_3/-CH_2-$ ratios represent 21, 44, 66, 85, and 114 acetylated primary amino groups and are in good agreement with the calculated values (based on the "eight missing arms" model) for the acetylation of the 20, 40, 60, 80, and 120 (all) primary amine groups (Figure 6). The slope of the theoretical $-CH_3/-CH_2-$ ratios vs the number of acetylated primary amines plots decreases with increasing deviation from the theoretical structure. We have found that the measured ratio is lower than the calculated one in the case of 100% acetylation. During the acetylation the acetic anhydride reacts with the primary amino groups; however, the generated acetic acid can also react in a competing side reaction with the amines, producing acetate salt.

The rate of amide formation is apparently lower than the rate of amine consumption because of the acetate salt formation in a competing reaction. The acetate formation also competes with the triethylamine acetate salt formation (a designed reaction to eliminate free acetic acid from the system). The complex nature of the reaction explains why the actual degree of acetylation

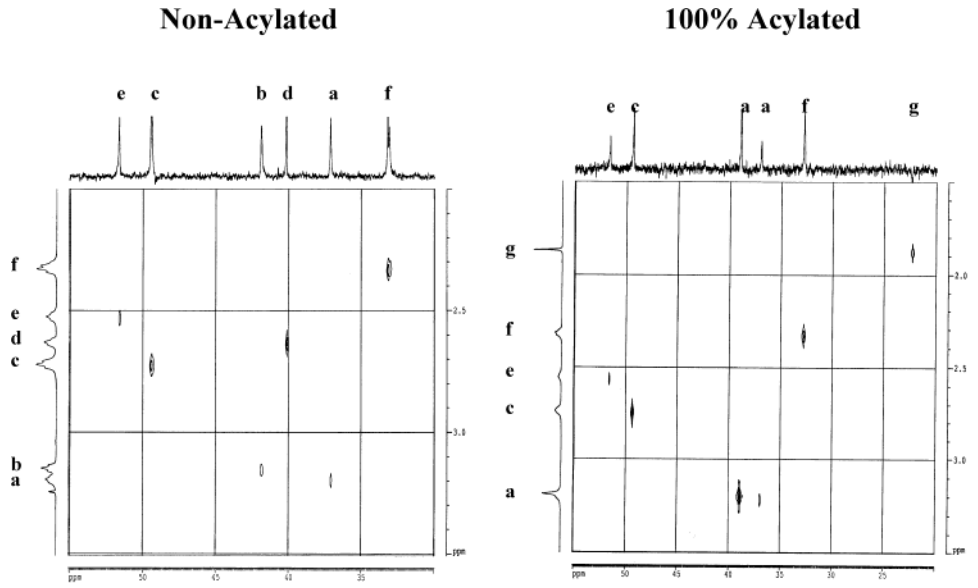

Figure 5. HETCOR NMR spectra of nonacetylated and fully acetylated G5 PAMAM dendrimer.

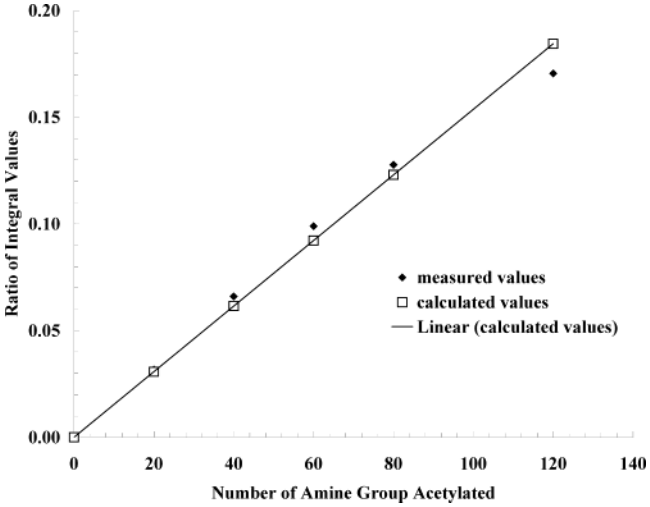

Figure 6. Number of proton ratio of $-CH_3$ in the acetyl groups and all $-CH_2-$ groups in the dendrimer structure vs number of primary amine groups acetylated: (□) the ratio of number of protons in methyl groups generated by acetylation (theoretical reaction) vs number of protons in methylene groups in the interior of dendrimer ($-CH_3/-CH_2-$); (■) the ratio of number of protons in methyl groups generated by acetylation (actual reaction) vs number of protons in methylene groups in the interior of dendrimer ($-CH_3/-CH_2-$, calculated from NMR data).

is different from the theoretical one in the case of 100% acetylation.

Conclusion

The acetylated dendrimers unexpectedly exhibit smaller molecular size despite their increasing molecular weight. They represent a more compact structure than the nonacetylated G5 PAMAM dendrimer. On the basis of the experimental results, the acetylation of G5 PAMAM dendrimers may be considered as a controlled, stoichiometric reaction where the ratio of the primary amine to the acetic anhydride is 1:1. The stoichiometric nature of the acetylation reaction with the knowledge of the exact number of the primary amine surface groups ensures a well-defined chemical structure for further dendrimer conjugates.

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