ELSEVIER

Journal of Chromatography A, 814 (1998) 233–246

JOURNAL OF CHROMATOGRAPHY A

Slab-gel and capillary electrophoretic characterization of polyamidoamine dendrimers

Herbert M. Brothers II, Lars T. Piehler, Donald A. Tomalia*

Michigan Molecular Institute, 1910 W. Saint Andrews Road, Midland, MI 48640, USA

Received 13 February 1998; received in revised form 12 May 1998; accepted 12 May 1998

Abstract

Polyamidoamine (PAMAM) dendrimers and derivatives of these polymers were analyzed by polyacrylamide gel- (PAGE) and capillary (CE) electrophoretic methods. Slab-gel PAGE allowed characterization of several generations of PAMAM dendrimers with terminal amino or carboxylate groups. Generational separation was achieved in CE up to the fifth generation of ammonia-core PAMAM dendrimers. Furthermore, CE allowed the separation of different substitution levels of low-generation, PAMAM dendrimers in which terminal amino groups were modified with salicylaldehyde groups, or of low-generation, methyl ester PAMAM dendrimers in which some of the terminal methyl ester groups were hydrolyzed to carboxylate groups.

Keywords: Polyamidoamine dendrimers; Polymers

1. Introduction

As the number of published studies on dendrimers and related dendritic polymers [1–4] continues to increase nearly exponentially, an examination of the literature reveals the need for further refinement of analytical methodologies for these large and somewhat complex molecules. The advent of electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) mass spectroscopic (MS) methods have probably provided the most significant advancements for the structural characterization of dendritic polymers [5–8]. Even with the advancement of MS methods, supplemental methods of analysis are necessary. Mass spectrometers are not always available to investigators as an analytical strategy for obtaining information on purity, homogeneity and surface groups. In the present study, we report facile methods for the electrophoretic characterization of polyamidoamine (PAMAM) dendrimers. To our knowledge, this is the first published report on the use of polyacrylamide gel electrophoresis (PAGE) as a characterization technique for dendritic polymers. In addition, related capillary electrophoresis (CE) data are presented for PAMAM dendrimers and various PAMAM dendrimer derivatives.

Currently, the majority of routine electrophoretic analyses are performed on biomolecules. The analytes found in both gel and capillary electrophoretic protocols are commonly, but not exclusively, proteins and nucleic acid polymers. Slab-gel electrophoretic techniques [9] have been commonplace in biochemical laboratories for decades. For example, PAGE is often used to determine the homogeneity of proteins. In the presence of detergents such as sodium dodecyl sulfate (SDS), PAGE is used to

*Corresponding author.

0021-9673/98/$19.00
PII: S0021-9673(98)00419-1

determine molecular masses of proteins (e.g., SDS–PAGE), and both PAGE and agarose slab gels are used for the analysis of oligonucleotides, RNA and DNA. Since slab-gel electrophoresis offers high resolving power, it is extremely useful and applicable to naturally monodispersed molecules such as proteins or DNA. Monomers, dimers and other higher oligomers of proteins are generally easy to separate on either an analytical or preparative scale with slab electrophoretic techniques. Not only are multimers separated, but even protein variants (e.g., hemoglobin variants) or modified structures (e.g., glycosylated proteins) may be resolved with electrophoretic methods. In DNA sequencing protocols, resolution down to a single base is routinely achieved on slab gels.

With the recent advances in CE techniques, electrophoretic analytes have increasingly extended outside of the conventional biomolecule regime. However, a perusal of the literature convincingly demonstrates that most polymers analyzed by CE are usually protein or DNA biopolymers [10]. When compared to biopolymers, little has been reported on the electrophoretic analysis (gel or capillary) of abiotic polymers [11–16]. The absence of these analytical methodologies for the characterization of synthetic polymers may result largely from the incompatibility of abiotic polymer analytes to normal electrophoresis conditions. Many synthetic polymers are not water soluble and, therefore, electrophoretic analysis by conventional techniques (i.e., in aqueous media) is not straightforward. For this reason, analysis of abiotic polymers by gel electrophoretic methods has been largely limited to synthetic polyelectrolytes [14]. Furthermore, molecular mass distributions for most abiotic polymers are very broad compared to the extremely monodispersed characteristics of proteins and DNA. Typically, the latter exhibit bands on electrophoresis gels whereas the former exhibit smears. Some polymers and polymer particles have been successfully analyzed by CE techniques. Latex particles functionalized with ionizable groups [15], polystyrene nanospheres [16] and Jeffamines [12], are among those polymers analyzed by CE.

In this work, PAMAM dendrimers were analyzed by both slab-gel and capillary electrophoresis. The PAMAM dendrimers are water-soluble polymers with relatively precise macromolecular architectures [17,18]. These dendritic polymers are methodically constructed through repetitive alkylation and amidation steps in which iteration yields the next higher generation of dendrimer. Because of this controlled synthesis, these polymers have well-defined molecular masses, which increase as a function of the dendrimer generation, and have relatively low polydispersities when compared to classical linear polymers. This combination of properties makes PAMAM dendrimers particularly amenable to electrophoretic analysis. With PAGE analysis, PAMAM dendrimers exhibit interesting trends when migration distances are compared to the molecular masses of the respective dendrimers. The PAMAM dendrimers have the potential to be used as calibration standards in electrophoretic methods, as they do in size-exclusion chromatography methods [19,20], for determining radii of proteins and, perhaps, of other biological macromolecules. Using CE analyses, different PAMAM polymers may be separated as a function of generation or degrees of terminal group substitution.

Although recent reports on the use of dendritic macromolecules as a pseudostationary phase in electrokinetic chromatography [21–27] have appeared, there has been only one report on the CE analysis of dendritic polymers [28]. In that study, the authors report on the CE–MS characterization of low generation, polypropyleneimine dendrimers with nitrile terminal groups. To our knowledge, there are no reports regarding the use of slab-gel electrophoresis for the characterization of dendritic polymers other than recent papers on the electrophoresis of DNA–dendrimer complexes and protein–dendrimer conjugates [29,30].

2. Experimental

2.1. Apparatus

2.1.1. PAGE

Analysis of dendrimers by PAGE was usually performed on a Micrograd vertical electrophoresis system (Gradipore, Sydney, Australia). Precast gels were also obtained from Gradipore. Most analyses involved Hylinx polyacrylamide gels, which are 5–50% T gradient gels, or MG540 polyacrylamide gels,

which are 5–40% T gradient gels [T=(g acrylamide + g *N,N′*-methylenebisacrylamide)/100 ml solution]. Gel sizes were ordinarily 2.5 cm (l)×7 cm (w), which are hereafter referred to as microgels. A conventional power supply (Model 500/200; Bio-Rad, Hercules, CA, USA) was used. Some analyses were performed on minigels (8.4×8.4 cm) of 5% T and 3–40% T, also obtained from Gradipore. Electrophoresis experiments on conventional sized gels (18×16 cm) were run on a vertical electrophoresis unit (Model Protean I; Bio-Rad). Gels that were not prepared commercially were cast according to published procedures [31].

2.1.2. CE

All CE experiments were performed on a P/ACE 2050 system equipped with System Gold software (Beckman, Palo Alto, CA, USA). Open-tube analyses were completed with 57 cm×75 μm I.D. uncoated fused silica capillaries that were obtained from Beckman Instruments. The distance of the capillary from the point of injection to the detector window was 50 cm.

2.2. Procedures

2.2.1. PAGE

The buffer system primarily used for both the full-generation PAMAM dendrimers (with primary amine groups on the surface) and the half-generation PAMAM dendrimers (with carboxylate groups on the surface) was 90 m*M* Tris, pH 8.3, 80 m*M* boric acid and 2.5 m*M* EDTA (TBE). Some analyses of full-generation PAMAM dendrimers were performed using a 0.1-*M* citrate buffer, pH 3.0. Electrophoresis typically required 30 min at 200 V on the microgels. Minigels were normally run at 200 V for 90 min. For full-sized conventional gels, a run time of 8 h was typical. Normal polarity (with the anode at the bottom of the gel) was used for analysis of the negatively charged half-generation carboxylate salts of the PAMAM dendrimers. Reverse polarity was used for analysis of the positively charged full-generation PAMAM dendrimers.

Sample sizes were 1 to 10 μg (10 μl of a 0.1 to 1 mg/ml solution was applied per sample well). A small aliquot (10% of the sample volume) of 50% sucrose (which also contained tracking dye, i.e. 0.1% bromophenol blue for anionic analytes and 1% methylene blue for cationic analytes) was added to samples for ease of loading. Gels were stained overnight with 0.025% Coomassie Blue R-250 in 40% methanol and 7% acetic acid aqueous solution. The gels were destained with 7% (v/v) acetic acid and 5% (v/v) methanol in water.

2.2.2. CE

Before initial use, the uncoated, fused-silica capillaries were rinsed with 0.1 *M* NaOH for at least 15 min, then with deionized water (resistivity of 18 MΩ cm; Milli-Q water purification system, Millipore, Bedford, MA, USA) for at least 15 min, then with the run buffer for 15 min. Before each injection, the capillary was rinsed with deionized water for 2 min, then with the run buffer for 2 min. At the beginning of each day, the capillary was rinsed with 0.1 *M* NaOH for 5 min. The instrument was configured so that injection occurred at the anodic end of the capillary, with migration towards the cathodic end of the capillary. Detection was carried out with UV light at 214 nm. The voltages used were 30 kV, for separations run in borate buffer, and 15 kV, for separations run in phosphate buffer. All separations were performed at 25°C.

The buffer used in the analysis of the half-generation PAMAM dendrimers (both the neutral ester-terminated dendrimers and the negatively charged carboxylate-terminated dendrimers) was 0.4% H_3BO_3 and 0.3% $Na_2B_4O_7$ (w/w) buffer, pH 8.2, in water. The buffer was filtered through a 1-μm nylon membrane (Acrodisc, Gelman, Ann Arbor, MI, USA) before use. The buffer used in the analysis of full-generation PAMAM dendrimers (with amine surface groups) was 0.1 *M* phosphate buffer, pH 2.7. Once a capillary was used with a given buffer, it was committed to use only with that buffer system.

Samples were typically prepared by dissolving 1 mg of dendrimer in 1 ml of the run buffer or 1 ml of the run buffer diluted tenfold with deionized water. Samples were run as soon as possible after dissolution, to avoid any possible decomposition. Samples were introduced into the capillary by pressure injection (usually 5 s, 0.5 p.s.i.; 1 p.s.i.=6894.76 Pa).

2.2.3. Dendrimer surface modification

A 1.0 *M* solution of generation zero ethyl-

enediamine (EDA)-core PAMAM dendrimer (250 mg of dendrimer in 500 ml of methanol) was made 3.6 *M* in salicylaldehyde (4:1, salicylaldehyde–dendrimer). After an overnight incubation at room temperature, the reaction mixture was treated with 120 mg of sodium cyanoborohydride. After standing overnight, a CE sample was prepared by diluting 10 μl of the reaction mixture with 5 ml of 0.1 *M* NaH_2PO_4, pH 2.7. In another modification reaction, a 0.35-*M* solution of generation one EDA-core PAMAM dendrimer (250 mg of dendrimer in 500 μl of methanol) was made 2.8 *M* in salicylaldehyde (8:1, salicylaldehyde–dendrimer). After 1 h, the reaction mixture was treated with 88 mg of sodium cyanoborohydride. After 2 h, a sample for CE analysis was prepared as described above.

2.3. Chemicals

Starburst PAMAM dendrimers (Starburst is a trademark of Dendritech) were obtained from Dendritech (Midland, MI, USA). Some PAMAM dendrimer samples were prepared according to general procedures described in the literature [17,32]. In the figures, EDA-core PAMAM dendrimers are designated with the letter E preceding the generation number. For example, E5 denotes a fifth-generation EDA-core PAMAM dendrimer. Ammonia-core PAMAM dendrimers are designated with the letter N. For example, N5 denotes a fifth-generation ammonia-core PAMAM dendrimer. Dendrimers composed of L-lysine repeat units (Denkewalter-type dendrimers) were prepared by published procedures [33]. Denkewalter dendrimers are designated in the figures with a letter D. For example, D5 denotes a fifth generation Denkewalter-type dendrimer. Tris-(hydroxymethyl)aminomethane (Tris), sodium borate, boric acid, disodium ethylenediaminetetraacetic acid (EDTA), monobasic sodium phosphate, acrylamide, bisacrylamide, ammonium persulfate, tetramethylenediamine (TEMED), sucrose, bromophenol blue, polylysine, sodium cyanoborohydride (Sigma, St. Louis, MO, USA), Coomassie Blue R-250 (Pierce, Rockford, IL, USA), methylene blue, salicylaldehyde (Aldrich, Milwaukee, WI, USA), citric acid and sodium hydroxide (Fisher Scientific, Pittsburgh, PA, USA) were employed.

3. Results and discussion

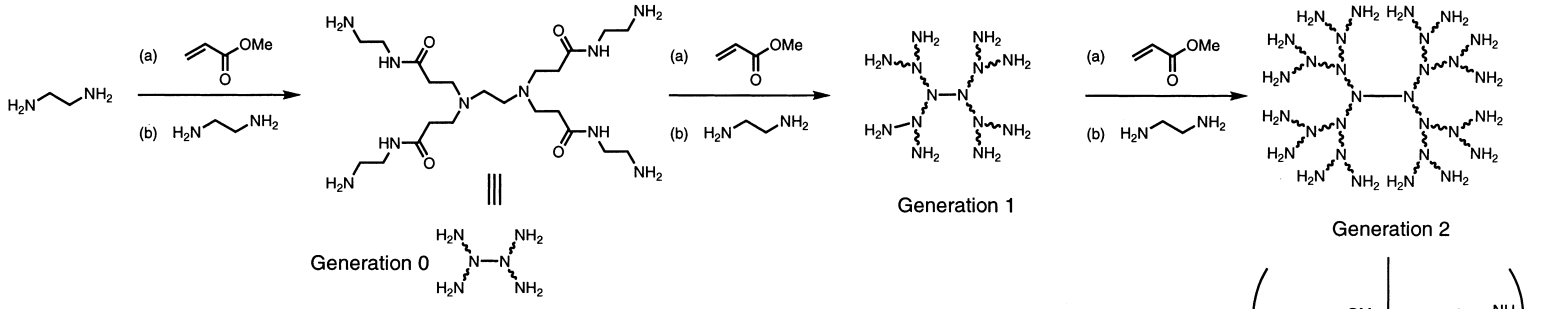
3.1. Description of PAMAM dendrimers

Dendrimers are generally built by a reiterative method which yields precision macromolecules that increase in molecular mass according to dendritic rules and mathematically driven principles [3,4]. The PAMAM dendrimers are dendritic polymers constructed from an initiator core, such as ammonia or EDA. The first step in their preparation is to conduct an exhaustive Michael addition to the amine with methyl acrylate. This is a rapid, high yield reaction, producing a polyester-terminated intermediate that is referred to as a ‘half generation’. The next step is to add the multiester intermediate to a large excess of EDA to produce a molecule with several amine groups on its surface. The resultant molecule is referred to as a ‘full generation’. These steps are repeated with each iteration yielding a higher generation after each series of reactions and, hence, a larger dendrimer molecule. Dendrimers with carboxylate terminal groups can be formed by hydrolyzing the methyl ester terminal groups on the half generation dendrimers. Details of the synthesis of PAMAM dendrimers and a description of growth have been presented elsewhere [3,17,32]. Fig. 1 summarizes the reaction chemistry and schematically depicts the growth of PAMAM dendrimers.

With each successive iteration of the reaction sequence (i.e., formation of the next generation), the molecule enhances its diameter by approximately 10 Å and the number of terminal groups, *Z*, doubles. Furthermore, the molecular mass approximately doubles as a function of generation. As a result, the formal charge-to-mass ratio (assuming all *Z* groups are carrying a charge) from generation to generation essentially remains constant. Therefore, the PAMAM dendrimers provide a series of molecules of nearly equivalent charge density that span a molecular mass range from a few hundred to nearly a million Daltons.

These spherical dendritic macromolecules may be cationic or anionic polyelectrolytes, or neutral, depending on the functional groups on the surface and in the interior of the molecule and on the pH of the medium. This, in conjunction with their monodispersity [3,5] and globular shape (especially at the

PAMAM Dendrimers



G X (EDA)

Generation	molecular mass	Primary Amino Groups
0	517	4
1	1430	8
2	3256	16
3	6909	32
4	14215	64
5	28826	128
6	58048	256
7	116493	512
8	233383	1024
9	467162	2048
10	934720	4096

Fig. 1. Schematic illustration of the synthesis and general structure of PAMAM dendrimers. The calculated molecular masses and surface group numbers are based on ideal molecular structures.

higher generations), makes these macromolecules reminiscent of folded, three-dimensional proteins [34].

3.2. Polyacrylamide gel electrophoretic analysis

Fig. 2 shows generations two through ten of EDA-core PAMAM dendrimers on a gradient polyacrylamide gel. Each generation of dendrimer was run in its own lane. The full generation dendrimers possess primary amine terminal groups and migrate towards the cathode, much as a basic protein would migrate towards the cathode in a buffer with a pH below that of the isoelectric point (p*I*) of the protein. Titration data of the full generation PAMAM dendrimers [3,17] show that there are two types of protonation events for the amines. The terminal primary amines have a pK_a of ca. 9–10, whereas the interior tertiary amines exhibit a pK_a of ca. 4–5. When PAGE experiments are conducted at pH 8.3, the primary amines are protonated whereas the tertiary amines remain unprotonated. Bands of the dendrimers were relatively narrow at pH 8.3, however, experiments run at pH 3.0 exhibited even tighter bands. At the acidic pH (well below the two pK_a regimes), protonation of all amines, both primary and tertiary, is favored. The protonation–deprotonation behavior of the polyamines is relatively complex since there are numerous microenvironments that affect the equilibria of subsequent protonations [35].

The smaller sized lower generations clearly migrated farther than their higher congeners. Upon examining the gel, some general correlations between migration distance and size properties of the dendrimer analytes can be made. It was found empirically that when the logarithm of the experimentally determined radius of a dendrimer (determined by size-exclusion chromatography) [3] is plotted against migration distance, the correlation between these two parameters is approximately linear. We caution that the correlations observed for the gels used in our experiments may not hold true for different gels with different gradient equations and, for this reason, we only qualitatively state these observations.

The carboxylate salts of the PAMAM dendrimers migrate towards the anode when placed in an electric field. The carboxylate-terminated dendrimers were analyzed at pH 8.3, wherein the carboxylate terminal groups are not protonated. Fig. 3 shows generations 3.5 through 7.5 of ammonia-core PAMAM dendrimers on a gradient polyacrylamide gel. Again, an empirical plot of the logarithm of the radii plotted against the migration distance is linear for this particular gel system.

A given generation of a PAMAM (either an amine surface full generation or a carboxylate surface half generation) dendrimer is separated from its flanking generations on the gradient gels and, therefore, a given generation of dendrimer is separated from its dimer, which has approximately the same molecular

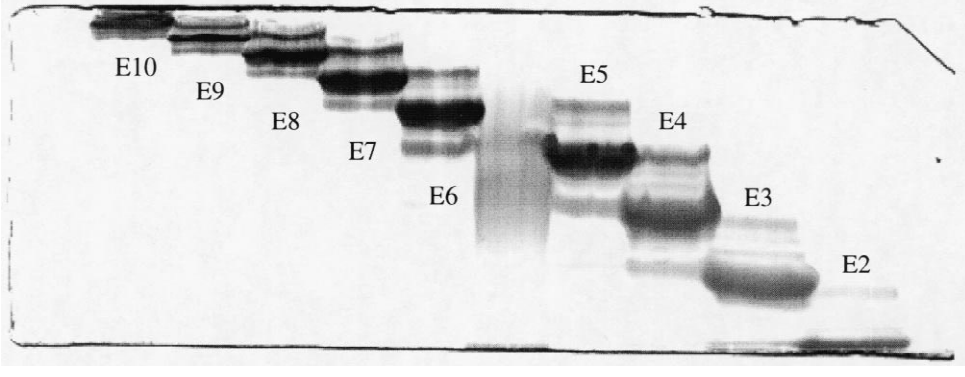


Fig. 2. Electrophoretogram of several generations of EDA-core PAMAM dendrimers analyzed on a 5–40% T polyacrylamide gel. The letter E before the generation number indicates an EDA-core PAMAM dendrimer. A 0.1 *M* citric acid buffer, pH 3.0, was used as the run buffer in both the upper and lower tanks. The generations descend from ten through two from left to right. The unlabelled smear in the middle of the gel is a proprietary sample.

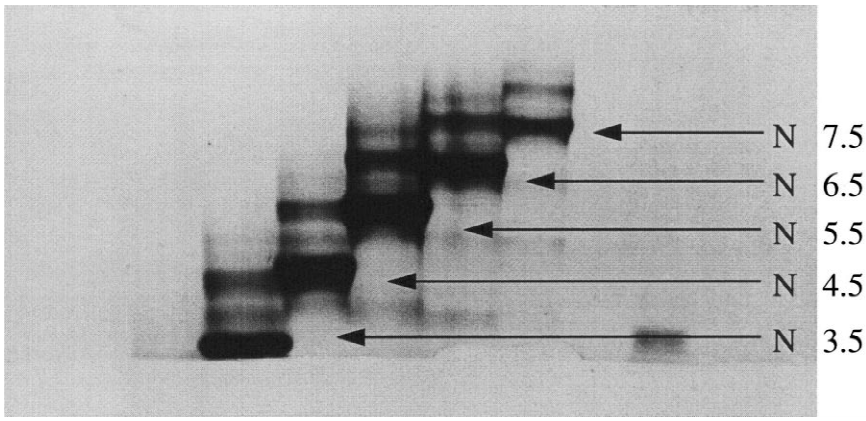


Fig. 3. Electrophoretogram of generation 3.5 through 7.5 of the sodium carboxylate salts of ammonia-core PAMAM dendrimers. The letter N before the generation number indicates an ammonia-core PAMAM dendrimer. The gel is a 5–40% T polyacrylamide gel. A TBE buffer, pH 8.2, was used as the run buffer in both the upper and lower tanks.

mass as the next higher generation since the molecular mass approximately doubles with each successive generation. Furthermore, higher oligomers are also separated from the desired monodendrimer species. It can also be seen on the gels that dendrimer samples may have trailing generations. These materials are undoubtedly generated during the PAMAM dendrimer synthesis by incomplete removal of the excess EDA after the amidation stage. In this case, residual EDA acts as a nucleophilic initiator core or seed for parallel growth of new generation regressed PAMAM dendrimers. For example, when ammonia-core PAMAM dendrimers are synthesized, ammonia is first alkylated with methyl acrylate to form a half-generation methyl ester-terminated material. A large excess of EDA is necessary for complete amidation of the material, to avoid crosslinking of dendrimer molecules and to drive the reaction to completion. If all EDA is not removed, residual EDA may act as a new initiator core and react with methyl acrylate in the next step. This is clearly evident on the polyacrylamide gels when ammonia is used as the initiator core. The ammonia core has a valency of three, to which three methyl acrylate molecules add. EDA as a core has a valency of four, to which four methyl acrylate molecules add. This difference in valencies of the core molecules leads to different molecular masses at successive generations such that an EDA-core material of a given generation will have a higher molecular mass than that of the same generation of the ammonia-core PAMAM dendrimer. When a series of ammonia-core PAMAM dendrimers are analyzed on a polyacrylamide gel, the trailing generations fall in between the migration distances of the parent dendrimers, since the trailing generations are EDA-core PAMAM dendrimers.

A key benefit gained by PAMAM dendrimer analyses on polyacrylamide gels is that this method of analysis can be used to either qualitatively or semi-quantitatively assess purity at a given generation. Densitometric analysis of these gels can yield quantitative information as long as appropriate linearity studies and other precautions, such as uniform incubation times in stain, are performed. Another benefit offered by gel electrophoretic analysis is that it potentially can be scaled to preparative electrophoretic separations where relatively pure material (i.e., monodendrimers without dendrimeric dimers and higher oligomer contamination) can be obtained for downstream studies and applications.

For comparative purposes, PAMAM dendrimers were analyzed side by side with linear polylysine on a common gradient gel. As evident in Fig. 4, the polylysine, with a reported polydispersity of 1.3 and a molecular mass of 37 200 (supplier information), exhibits a broad smear, whereas the PAMAM dendrimers exhibit well-defined bands. The higher polydispersity of the polylysine undoubtedly contributes to the smeared migration of the linear polymer on the gel. In addition, the linear primary structure of

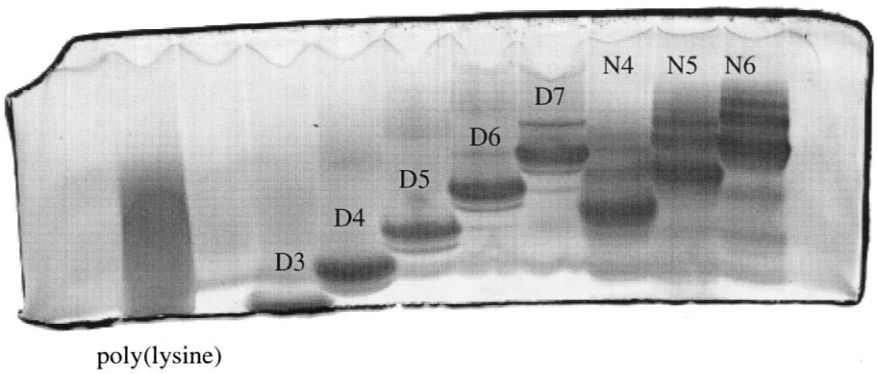


Fig. 4. An electrophoretogram that compares the electrophoretic behavior of polylysine at the left (molecular mass of ca. 37 200) with polylysine dendrimer generations three through seven in the center (Denkewalter dendrimers) and PAMAM dendrimer generations four through six at the right. The letter D before the generation number indicates a Denkewalter-type dendrimer. The Denkewalter dendrimers are actually monodendrons grown from a benzhydrylamine (BHA) core, where D3 is (BHA)LysLys$_2$Lys$_4$Lys$_8$, D4 is (BHA)LysLys$_2$Lys$_4$Lys$_8$Lys$_{16}$, D5 is (BHA)LysLys$_2$Lys$_4$Lys$_8$Lys$_{16}$Lys$_{32}$, D6 is (BHA)Lys$_{63}$Lys$_{64}$, and D7 is Lys$_{127}$Lys$_{128}$. The Denkewalter-type dendrimers have been deprotected and the surface groups are primary amines.

polylysine may lead to migration mechanisms, such as reptation, which are different from migration mechanisms of the structurally globular dendrimers. As a further comparison, monodisperse asymmetric dendrons derived from polylysine branch cell units, the so-called 'Denkewalter dendrimers' [33], are also analyzed on the gel shown in Fig. 4. These macromolecules, which are constructed according to dendritic rules and principles, exhibit dramatically narrow bands when compared to traditional linear polylysine. The growth sequence for 'Denkewalter dendrimers' is depicted in Fig. 5.

PAGE analysis of PAMAM dendrimers was also performed on full-size (18×16 cm) nongradient gels of 5% T to 15% T and 3–17% T gradient gels, whereas other analyses were performed on mini gels (8.4×8.4 cm) of 5% T and gradients up to 3–40% T. Separations by generation were achieved on all gels, however, the bands were generally more diffuse and fine bands were not as well resolved as in the 5–40% T and 5–50% T micro gels (7.0×2.5 cm).

3.3. CE of unmodified PAMAM dendrimers

It is readily apparent that PAMAM dendrimers should be amenable to CE analysis. They are water-soluble, carry charge and are mimics of proteins, which are routinely analyzed with CE. Furthermore, it is well known that PAMAM dendrimers bearing terminal amino groups adsorb tenaciously to a variety of solid media [36]. This property presents difficulties for analysis by conventional chromatography.

In CE, dendrimers migrate through a fused-silica capillary as a result of electroosmotic flow (EOF) of the bulk fluid and electrophoretic mobility of the analyte. The EOF is towards the cathodic buffer chamber and the direction of electrophoretic mobility depends on the net macromolecular charge. Positively charged dendrimers will migrate towards the cathode, in the same direction as the EOF, and negatively charged dendrimers will migrate against the EOF.

Full-generation PAMAM dendrimers (positively charged) are run at acidic pH and at a relatively high ionic strength (0.1 *M* Na_2HPO_4), to minimize interaction with the walls of the capillary. Fig. 6 shows that low generation PAMAM dendrimers are separated, however, the resolution decreases between the higher generations under these conditions. This is not surprising in that the charge densities of the molecule become nearly uniform at higher generations. In an effort to separate higher generations from each other, we have performed several experiments utilizing hydroxylpropylmethyl cellulose and polyethylene oxide as soluble sieving matrices. We did notice some improvement with respect to generational separation, but analysis times were relatively long

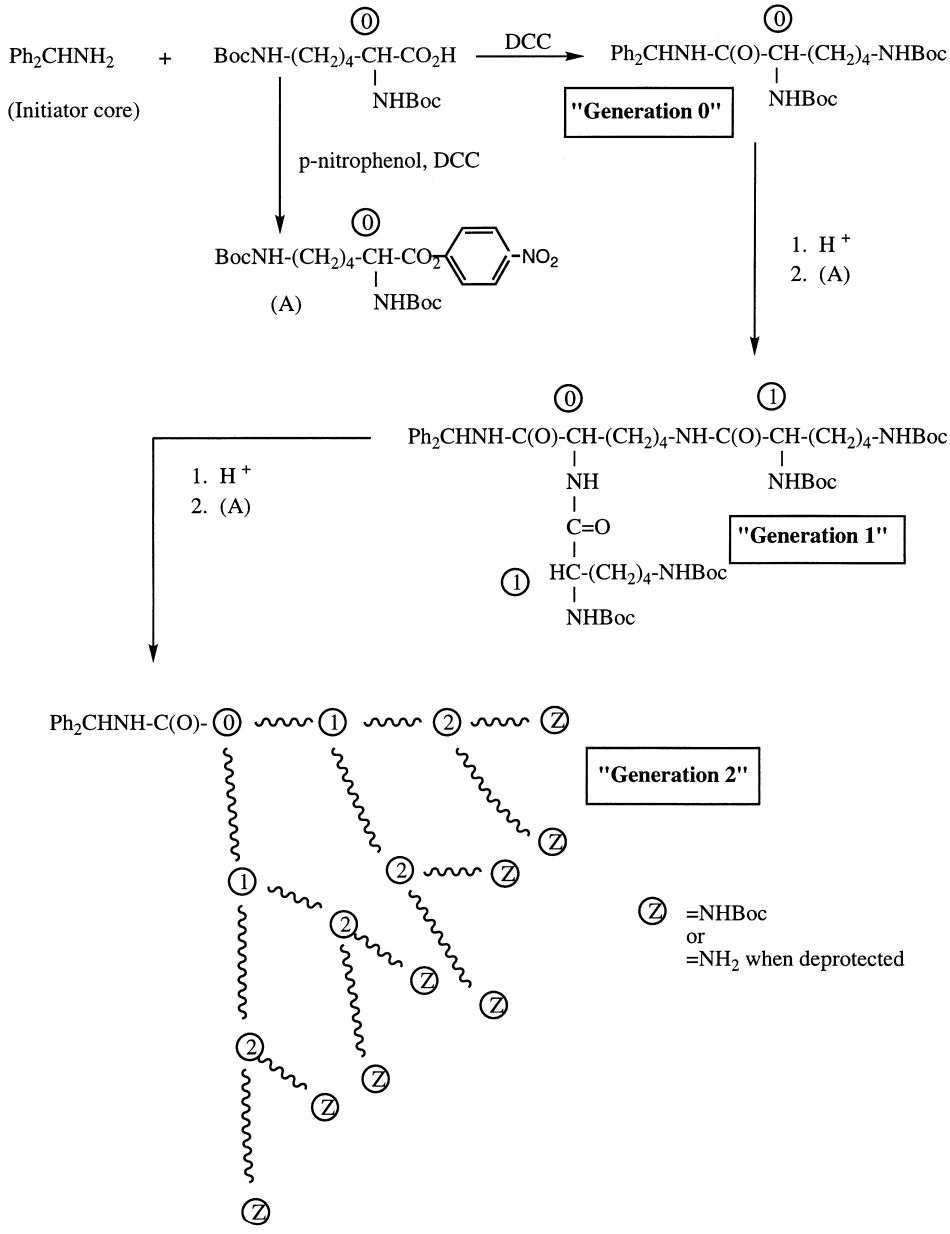


Fig. 5. Synthetic scheme for Denkewalter dendrimers.

and peaks were relatively broad. Although they show promise, further refinement is required in the development of these methods with soluble polymer as a sieving medium, and they are not further elaborated upon in this report.

Under the conditions of Fig. 6, it is somewhat unexpected that the lower generations have a higher apparent electrophoretic mobility than the higher generations. If a simple charge/$(\text{mass})^{2/3}$ calculation is performed, assuming a perfect dendrimer structure and all amine groups (primary and tertiary) are protonated, the higher generations should be ex-

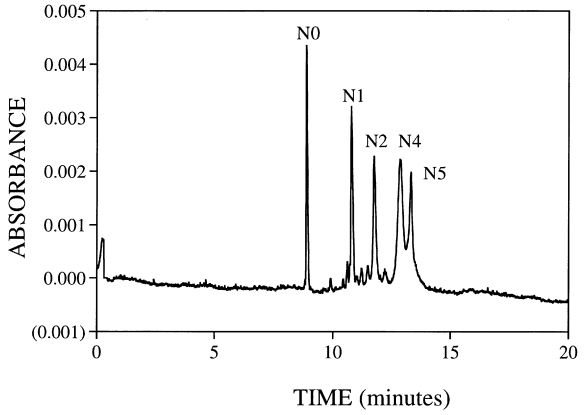


Fig. 6. Capillary electropherogram of low generation ammonia-core PAMAM dendrimers analyzed in an open-tube capillary filled with 0.1 *M* NaH_2PO_4 buffer, pH 2.7. The mixture does not contain a generation three PAMAM dendrimer.

pected to have a higher theoretical electrophoretic mobility (based on formal charge) than the lower generations. A possible explanation for the observed reverse in the trend is that the effective charge is less than the formal charge for the larger generations, because of incomplete protonation of the amine groups. Alternatively, ion-pairing of the dendrimer molecules with phosphate ions in the buffer may not allow straightforward comparisons of relative electrophoretic mobilities with the formal charge and calculated mass of these analytes.

The EOF under the conditions of Fig. 6 is negligible and analyte movement predominately results from electrophoretic migration. Attempts were made to measure EOF at this pH by injecting 10% acetone. No acetone peak was seen to pass the detector even after 3 h of monitoring. The low pH suppresses the EOF, and the high ionic strength minimizes solute adsorption to the capillary wall. For comparison, acetone passes the detector at 2.2 min in a capillary of the same length when the run buffer is 0.4% (w/w) boric acid, 0.3% (w/w) sodium borate, pH 8.2, and the voltage is 30 kV.

Half-generation carboxylate-terminated PAMAM dendrimers are run at pH 8.2, to ensure deprotonation of the charge-carrying carboxylate groups. At this pH, the EOF is relatively high and the analytes still migrate towards the cathode. Electropherograms of generations 0.5, 1.5 and 2.5 ammonia-core

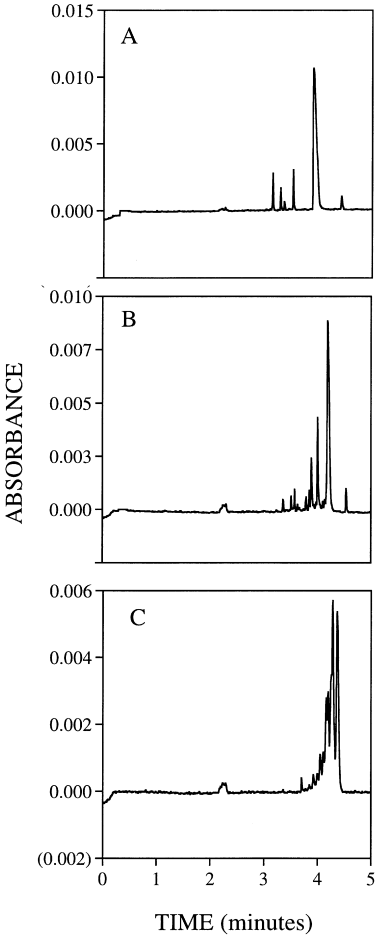


Fig. 7. Capillary electropherograms of generations 0.5, 1.5 and 2.5 EDA-core PAMAM dendrimers with carboxylate surface groups. (A) Generation 0.5, (B) generation 1.5 and (C) generation 2.5. All analyses were performed in a borate buffer, pH 8.2, containing 0.4% boric acid–0.3% sodium borate (w/w).

PAMAM dendrimers are shown in Fig. 7. These data indicate several peaks that are most likely due to defective structures or incomplete hydrolysis of the methyl esters to the carboxylate groups in the synthesis of these compounds. No tandem method of analysis has been performed on these peaks; therefore, positive identification of individual peaks has not been made at this time.

3.4. CE of surface-modified PAMAM dendrimers

The PAMAM dendrimers can be synthesized with

reactive terminal functional groups, which are easily derivatized with a wide number of reagents. The infinite possibilities for terminal group modification make dendritic macromolecules excellent candidates for signal amplification schemes, time-release drug delivery, magnetic resonance imaging, ternary and higher complexity conjugation schemes, and numerous other applications [37–39]. For modified dendrimers, CE provides an excellent analytical strategy for separating various degrees of terminal group substitution. If separation is achieved, preparative methods may allow discrete isolation of a single substituted species for more detailed experimentation.

An experimental demonstration of this potential involves the in situ conversion of a PAMAM dendrimer possessing methyl ester surface groups to carboxylate surface groups. A typical reiteration sequence for PAMAM dendrimers yields half-generation dendrimers with the methyl ester surface groups derived from the Michael addition of methyl acrylate to the preceding amine-terminated full generation. When these half-generation esters are placed in alkaline, aqueous media, the ester groups hydrolyze readily, even at room temperature. They do not all hydrolyze simultaneously, but follow a statistically driven hydrolysis pattern, such that the first predominant species formed is a dendrimer possessing only one hydrolyzed ester group. It is then this species that most probably forms a molecule possessing two hydrolyzed terminal groups and so on.

Specifically, when a generation 1.5 ester-terminated ammonia-core PAMAM is placed in an alkaline borate buffer of pH 8.2, the dendrimer is at first neutral. The tertiary amines are not protonated at this pH and all methyl esters are intact. A capillary electropherogram of this freshly dissolved material shows only one predominant peak, which migrates with an EOF marker, thus indicating that the molecule is indeed neutral. When the sample is examined a few minutes later, new peaks appear, which have a lower velocity than the neutral marker, thus indicating that they are anionic. As time passes, more peaks continue to grow until, eventually, the total number of major peaks observed matches the number of end groups on the molecule. In the case of generation 1.5 PAMAM (ammonia-core) dendrimer, twelve new prominent peaks appear over the course of several hours. Fig. 8 shows representative electropherograms of this phenomenon. When peak heights are plotted as a function of time, the classical kinetic scheme of the reaction type A→B→C→D→ etc. is apparent. As B (the monocarboxylate species) is formed, A (the neutral starting material, the dodecamethylester) decays. Since C (the dicarboxylate species) arises from B, there is an inductance period before any formation of C is observed. Since B is being formed at the same time as it is being consumed, B goes through a maximum at a time *t* before it begins to decay. A plot of peak heights versus time for the

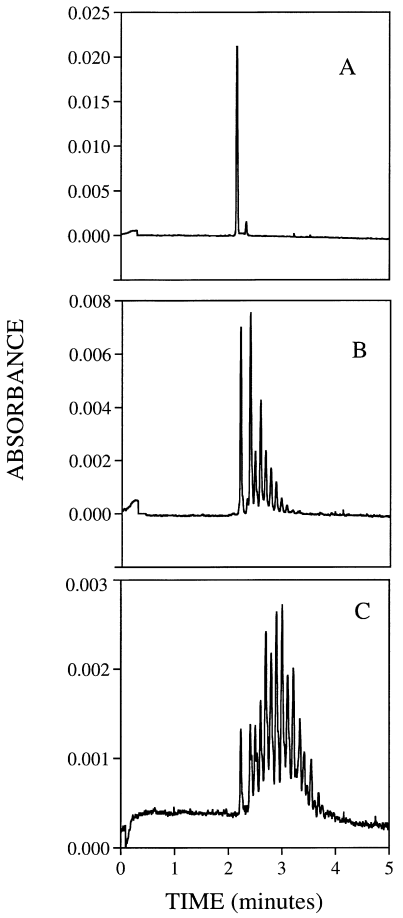


Fig. 8. Capillary electropherograms showing hydrolysis as a function of time of a generation 1.5 methyl ester-surface ammonia-core PAMAM dendrimer. (A) Sample in buffer of pH 8.2 for 9 min; (B) sample in buffer of pH 8.2 for 242 min; and (C) sample in buffer of pH 8.2 for 645 min.

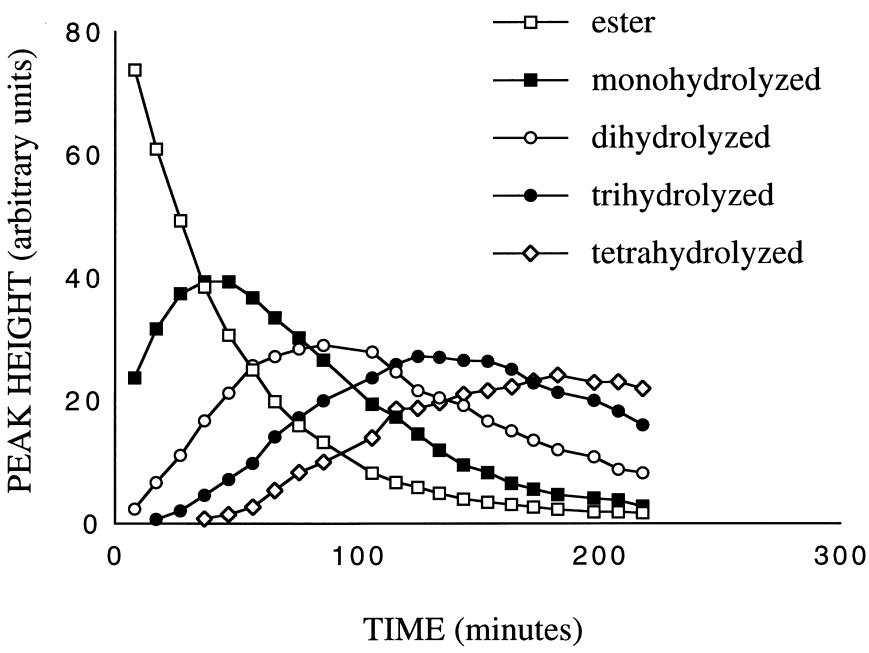


Fig. 9. Plot of peak heights as a function of time of individual products resultant from the hydrolysis of a generation 3.5 methyl ester-surface ammonia-core PAMAM dendrimer.

hydrolysis of a generation 3.5 PAMAM dendrimer with ester surface groups is shown in Fig. 9.

A more classical modification scheme is observed when the terminal groups of a full-generation amino-surface PAMAM dendrimer are modified with salicylaldehyde in methanol. In this reaction scheme, the terminal amino groups react with the aldehydic function to form a Schiff base. When the reaction mixture is reduced with sodium cyanoborohydride, the imine linkage is reduced to a stable secondary amine. The capillary electropherogram of a generation zero EDA-core PAMAM modified with salicylaldehyde yielded four peaks, presumably one for each degree of substitution of the tetrafunctional molecule. The capillary electropherogram of a generation one EDA-core PAMAM modified with salicylaldehyde showed at least eight new partially resolved peaks, again presumably one for each degree of substitution of the octafunctional molecule. Electropherograms of these surface-modified PAMAM dendrimers are shown in Fig. 10. Analysis of higher generations were increasingly more difficult, due to the inability to separate the larger number of discretely substituted species.

4. Conclusion

Analyses of PAMAM dendrimers by PAGE and CE are described. The separation of dimers, oligomers and other generations from a given monodendrimer has been demonstrated by PAGE. Under the appropriate conditions, different generations of dendrimers, and also dendrimers with different degrees of terminal group substitution, can be separated by CE.

The potential for further development of PAGE and CE in the analysis of dendrimers is indeed promising. Further progress with these methodologies may allow the separation of defective structures from perfect structures. Such separations may be achievable by the use of gel-filled capillaries. This capability will undoubtedly allow in-depth characterization of mutant or defective dendrimer species

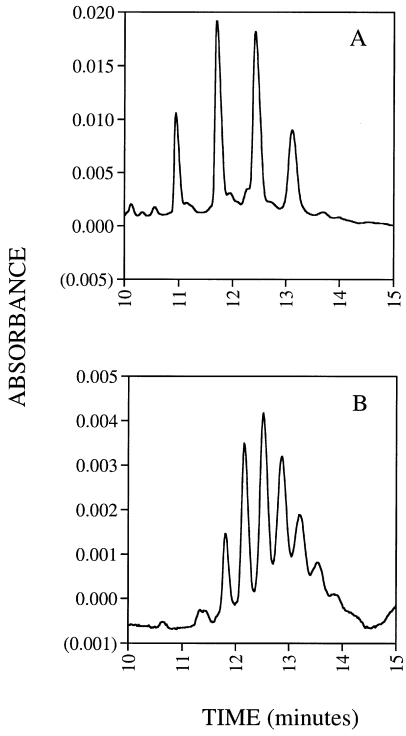


Fig. 10. Expanded regions from the capillary electropherograms of generation zero EDA-core PAMAM modified with salicylaldehyde (A) and generation one EDA-core PAMAM modified with salicylaldehyde (B). The separations were performed in a 57-cm fused-silica capillary with 0.1 *M* NaH_2PO_4 buffer, pH 2.7, at 15 kV and 25°C.

that are of interest in various amplified, genealogically directed dendrimer syntheses [40]. Furthermore, since both PAGE and CE are nondestructive techniques, micropurification of dendrimer derivatives by these techniques is certainly feasible.

Acknowledgements

The authors wish to thank Dr. H. Dupont Durst (ERDEC) for his many invaluable discussions. We would like to acknowledge ERDEC (Edgewood Research, Development and Engineering Center), ARO (Army Research Office) and Dendritech Inc. (Midland, MI, USA) for financial support.

References

[1] D.A. Tomalia, H.D. Durst, in E. Weber (Editors), Topics in Current Chemistry, Springer Verlag, Berlin, 1993, p. 193.
[2] G.R. Newkome, C.N. Moorefield, F. Vögtle, in Dendritic Molecules, Verlag Chemie, Weinheim, 1996.
[3] D.A. Tomalia, A.M. Naylor, W.A. Goddard III, Angew. Chem., Int. Ed. Engl. 29 (1990) 138.
[4] D.A. Tomalia, Adv. Mater. 6 (1994) 529.
[5] G.J. Kallos, D.A. Tomalia, D.M. Hedstrand, S. Lewis, J. Zhou, Rapid Commun. Mass Spectrom. 5 (1991) 383.
[6] D.A. Tomalia, Aldrichim. Acta 26 (1993) 91.
[7] B.L. Schutz, A.L. Rockwood, R.D. Smith, D.A. Tomalia, R. Spindler, Rapid Commun. Mass Spectrom. 9 (1995) 1552.
[8] L.P. Tolic, G.A. Anderson, R.D. Smith, H.M. Brothers II, R. Spindler, D.A. Tomalia, Int. J. Mass Spectrom. Ion Processes 165–166 (1997) 405.
[9] A.T. Andrews, in A.R. Peacocke, W.F. Harrington (Editors), Electrophoresis: Theory, Techniques, and Biochemical and Clinical Applications, Oxford University Press, New York, 1987.
[10] R.L. St. Claire III, Anal. Chem., 68 (1996) 569R and references cited therein.
[11] D.A. Hoagland, M. Muthukumar, Macromolecules 25 (1992) 6696.
[12] L.N. Amankwa, J. Scholl, G. Kuhr, Anal. Chem. 62 (1990) 2189.
[13] J.B. Poli, M.R. Schure, Anal. Chem. 64 (1992) 896.
[14] J.L. Chen, H. Morawetz, Macromolecules 15 (1982) 1185.
[15] H.K. Jones, N.E. Ballou, Anal. Chem. 62 (1990) 2484.
[16] B.B. Van Orman, G.L. McIntire, J. Microcol. Sep. 1 (1989) 289.
[17] D.A. Tomalia, H. Baker, J. Dewald, M. Hall, G. Kallos, S. Martin, J. Roeck, J. Ryder, P. Smith, Polymer J. 17 (1985) 117.
[18] D.A. Tomalia, J.R. Dewald, M.J. Hall, S.J. Martin, P.B. Smith, Preprints of the First SPSJ Int. Polym. Conf., Kyoto, August 1984, p. 65.
[19] P.L. Dubin, S.L. Edwards, J.I. Kaplan, M.S. Mehta, D.A. Tomalia, J. Xia, Anal. Chem. 64 (1992) 2344.
[20] P.L. Dubin, S.L. Edwards, M.S. Mehta, D.A. Tomalia, J. Chromatogr. 635 (1993) 51.
[21] N. Tanaka, T. Tanigawa, K. Hosoya, K. Kimata, T. Araki, S. Terabe, Chem. Lett. (1992) 959.
[22] S.A. Kuzdzal, C.A. Monnig, G.R. Newkome, C.N. Moorefield, J. Chem. Soc., Chem. Commun., (1994) 2139.
[23] S.A. Kuzdzal, J.J. Hagen, C.A. Monnig, J. High Resolut. Chromatogr. 18 (1995) 439.
[24] S.A. Kuzdzal, C.A. Monnig, G.R. Newkome, C.N. Moorefield, J. Am. Chem. Soc. 119 (1997) 2255.
[25] N. Tanaka, T. Fukutome, K. Hosoya, K. Kimata, T. Araki, J. Chromatogr. A 716 (1995) 57.
[26] N. Tanaka, H. Iwasaki, T. Fukotome, K. Hosoya, T. Araki, J. High Resolut. Chromatogr. 20 (1997) 529.
[27] C.P. Palmer, J. Chromatogr. A 780 (1997) 75.

[28] D. Stockigt, G. Lohmer, D. Belder, Rapid Commun. Mass Spectrom. 10 (1996) 521.
[29] J.F. Kukowska-Latallo, A.U. Bielinska, J. Johnson, R. Spindler, D.A. Tomalia, J.R. Baker Jr., Proc. Natl. Acad. Sci. USA 93 (1996) 4897.
[30] J.C. Roberts, Y.E. Adams, D.A. Tomalia, J.A. Mercer-Smith, D.K. Lavallee, Bioconjugate Chem. 2 (1990) 305.
[31] U.K. Laemmli, Nature 227 (1970) 680.
[32] A.D. Meltzer, D.A. Tirrell, A.A. Jones, P.T. Inglefield, D.M. Hedstrand, D.A. Tomalia, Macromolecules 25 (1992) 4541.
[33] R.G. Denkewalter, J.F. Kole, W.J. Lukasavage, US Pat., 4 410 688 (1983).
[34] Y. Sayed-Sweet, D.M. Hedstrand, R. Spindler, D.A. Tomalia, J. Mater. Chem. 9 (1997) 1199.
[35] Y. Mengerink, M. Mure, E.M.M. de Brabander, Sj. van der Wal, J. Chromatogr. A 730 (1996) 75.
[36] G.R. Killat, D.A. Tomalia, US Pat., 4 871 779 (1989).
[37] P. Singh, F. Moll III, S.H. Lin, C. Ferzli, K.S. Yu, R.K. Koski, R.G. Saul, P. Cronin, Clin. Chem. 40 (1994) 1845.
[38] R.F. Barth, D.M. Adams, A.H. Soloway, F. Alam, M.V. Darby, Bioconjugate Chem. 5 (1994) 58.
[39] E.C. Wiener, M.W. Brechbiel, H.M. Brothers, R.L. Magin, O.A. Gensow, D.A. Tomalia, P.C. Lauterbur, Magn. Reson. Med. 31 (1994) 1.
[40] M.K. Lothian-Tomalia, D.M. Hedstrand, D.A. Tomalia, A.B. Padias, H.K. Hall Jr., Tetrahedron 53 (1997) 15495.