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Polyacrylamide gel electrophoresis separation and detection of polyamidoamine dendrimers possessing various cores and terminal groups

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Abstract

Detection and separation of polyamidoamine dendrimers possessing various cores and surface groups was studied by polyacrylamide gel electrophoresis. Although many dyes and staining techniques were able to detect dendrimers on polyacrylamide gels, Coomassie Blue was found to be the most sensitive and convenient. Amine and hydroxyl terminated dendrimers were best separated under acidic conditions, while dendrimers with carboxyl surfaces required alkaline buffers. Some dendrimers were very susceptible to diffusion that could occur during their separation, staining or destaining steps. In the absence of an appropriate fixation step, dendrimers could be resolved by using small pore size gels and low voltage or current. Increasing core lengths did not significantly affect migration of a given dendrimer generation but exhibited improved separation and staining characteristics. Polyacrylamide gel electrophoresis was found to be a rapid, inexpensive, and reliable procedure to characterize many different water-soluble dendritic macromolecules.
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Keywords: Polyamidoamine dendrimers; Polyacrylamide gel electrophoresis; Staining

1. Introduction

Polyamidoamine or PAMAM dendrimers are synthetic polymer-based nanoparticles (with diameters in the 1–15 nm range), which are widely recognized as important quantized nanoscale building blocks in biomedical research [1,2]. Dendrimers have unique structural or architectural components, unlike other traditional polymers. The various parts of a typical dendrimer molecule include a core, an interior consisting of repetitive branch cell units and terminal functional groups. In addition, cavities are also present in the macromolecule (Fig. 1). The high density of terminal functional groups, formed as a result of branching, can be used to conjugate other molecules to the dendrimer. For example, many drug molecules may be covalently attached and carried into the blood stream by each dendrimer molecule. The cavity, which increases in size as the dendrimer grows to larger generations, may be used to encapsulate small molecules like drugs [3]. The core may be cleaved to provide pie-shaped dendrimers or dendrons, which can self-assemble to form large nanoparticles [4]. Dendrimers are versatile molecules that have many potential applications in nanotechnology and life sciences, including gene and drug delivery, contrast agents for imaging and in vitro diagnostic reagents [5].

Characterization of dendrimers and their conjugates is critical for their successful synthesis and applications. Capillary electrophoresis (CE) has been used for characterization of low generation PAMAM dendrimers [6–8]. Separation of higher generations become problematic due to their similar charge densities and interactions with the walls of the capillary [6]. The absence of any strong chromophore in PAMAM dendrimers requires the use of very low wavelengths (around

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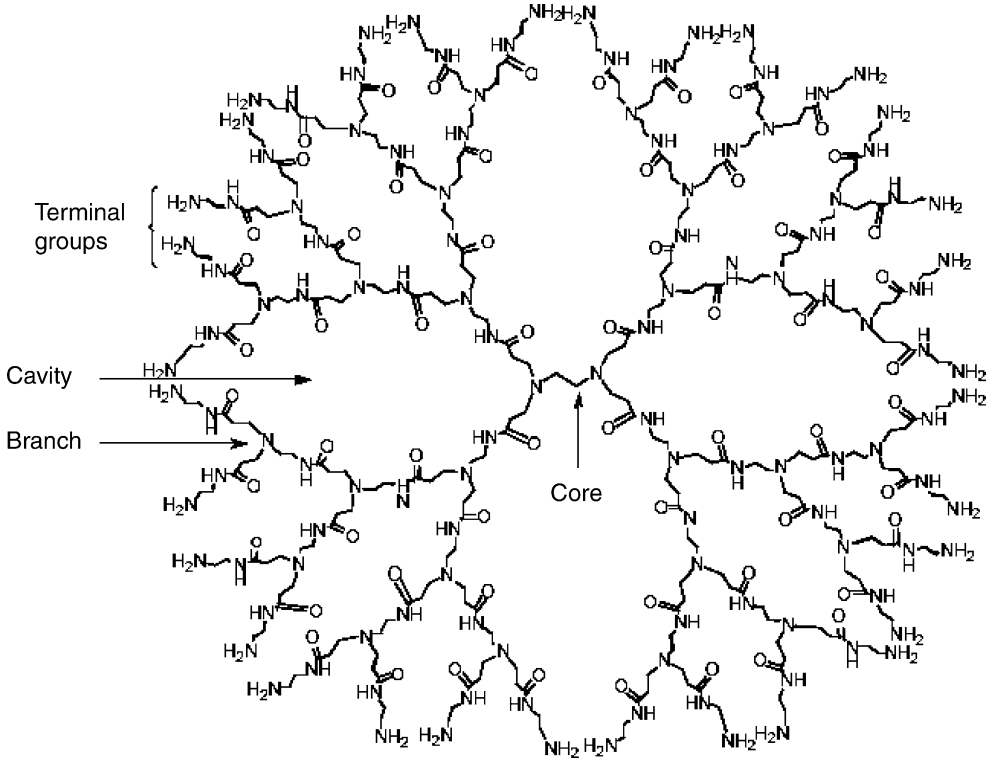

Fig. 1. Structure of an amine-terminated G3 PAMAM dendrimer with an EDA core.

214 nm) for their detection in CE. The buffer and sample therefore has to be very clean to avoid false peaks due to light scattering effects. In addition, CE requires expensive instrumentation and a trained operator. A much less expensive alternative is polyacrylamide gel electrophoresis (PAGE), which has been used to separate PAMAM dendrimers of various generations [6,9]. PAGE has many potential advantages for dendrimer characterization. It allows separation under physiological conditions, requires small sample size, yields a visual end signal, and is a mild, non-destructive technique that does not cause fragmentation of the dendrimer sample [10].

PAMAM dendrimers can be successfully separated by PAGE [6,9]. Most of this work has been directed to PAMAM dendrimers with amine surfaces and ethylenediamine (EDA) cores. However, there are many other kinds of PAMAM dendrimers that are commercially available today. These include dendrimers possessing various dimensions (generations) and cores like diaminobutane (DAB), diaminohexane (DAH), diaminododecane (DAD) and cystamine. Besides amines, other surface functional groups include carboxyls, hydroxyls and hexylamides. The physicochemical variations found in these dendritic structures may require modified electrophoresis conditions for their separation, as in the case for separation of acidic and basic proteins. The main objective of this report is to study the effect of the various dendrimer cores and their surfaces on electrophoretic migration and to find suitable conditions for their separation and detection.

2. Experimental

2.1. Apparatus

2.1.1. PAGE

Electrophoresis was performed on polyacrylamide gels in acidic or basic conditions. Mini gels (10 cm × 8 cm × 0.75 mm) were used during the study. The electrophoresis system was a Dual Mini-Vertical Slab Gel Electrophoresis Unit from Sigma (St. Louis, MO, USA). This apparatus allows gel cooling by running water. Gels were photographed with a Kodak DC 40 digital camera and images analyzed with Kodak Digital Science 1D image analysis software (Eastman Kodak, Rochester, NY).

2.2. Procedures

2.2.1. PAGE

Electrophoresis under acidic or basic conditions was performed as described before [7]. Amine-terminated dendrimers were fixed with glutaraldehyde. Unless specified, all electrophoresis steps were performed at 4 °C.

2.2.2. Dendrimer purification from polyacrylamide gels

After electrophoresis, the desired dendrimer band was excised from the gel and minced in an appropriate solvent (e.g., water if the dendrimer is very water soluble). The gel was then subjected to three cycles of freeze and thaw (15 min

each). It was incubated overnight at room temperature in a shaker. The sample was then centrifuged at 16,000 × *g* on an Eppendorf centrifuge 5415*C* for 10 min. The supernatant was removed and lyophilized.

2.2.3. Staining of amine surface PAMAM dendrimers

Coomassie Blue R-250 (0.2%, w/v) was prepared in 50% methanol/10% acetic acid solution. After electrophoresis, the gel was carefully removed from between the glass plates and placed in a small dish for staining. The gel was placed in the cold (4 °C) staining solution overnight (about 16 h). After staining, the gel was destained for 10–15 min in cold 50% methanol/10% acetic acid and then placed in cold 10% methanol/10% acetic acid until the background was clear.

Dendrimer staining was also performed with the amine reagent, naphthoquinone. Ponceau S, Brilliant Blue G perchloric acid (Sigma), 1% (w/v) Fast Green, Silver stain (Sigma procedure P3040), and Colloidal Coomassie Blue (Sigma). A carbon disulphide–silver nitrate (CS_2/$AgNO_3$) staining was also carried out. In this procedure, the post-electrophoresis gel was stained in a CS_2 solution (made 1:1 in absolute ethanol) for about 5 min and then excess of CS_2 was removed by rinsing the gel with deionized water for 15 s, five times. The gel was then placed in a 1% aqueous $AgNO_3$ solution for 24 h.

2.2.4. Mass spectrometry

Mass spectra were recorded using a Bruker Daltonics MALDI-TOF mass spectrometer (Billerica, MA, USA) operating in a pulse ion extraction linear mode. The sample (1 μl) was mixed with 10 μl of matrix (10 mg/ml of 3-hydroxyphenyl acetic acid). Solvent was allowed to evaporate under ambient conditions. The dendrimer-matrix was then spotted on the target plate.

2.3. Chemicals

All routine chemicals were obtained from Aldrich (Milwaukee, WI, USA). All dendrimers were either purchased from Aldrich or Dendritic NanoTechnologies (Mt. Pleasant, MI, USA; URL: http://dnanotech.com).

3. Results and discussion

3.1. Dendrimer staining

Previous work has clearly demonstrated the usefulness of polyacrylamide gel electrophoresis for the separation of ammonia or EDA-core PAMAM dendrimers possessing various surfaces [6,9]. Optimum electrophoresis conditions were described for dendrimer separation under acidic or basic conditions with various buffers (Tris–glycine, Tris–borate, acetate or citrate). The common protein stain Coomassie Blue R-250 was used to detect the separated dendrimers on the gel. Other than the reported use of Coomassie Blue, we could not find any previous reports on the staining characteristics of PAMAM dendrimers on PAGE gels. Staining is an important part of electrophoresis. This step allows visualization of the separated components on the gel.

Using EDA core PAMAM dendrimers with amine surface, a number of other stains used for proteins and or amines in electrophoresis or histochemistry were evaluated in this part of the investigation. The objective was to study the staining characteristics of dendrimers towards various dyes and to determine if there was a more sensitive staining method for amine-terminated PAMAM dendrimers than Coomassie Blue R-250.

The dye is slightly soluble in water because of its hydrophobic side-chains. The exact mechanism of Coomassie Blue staining for proteins or dendrimers is uncertain, but electrostatic interactions between the dye's sulfonic acid groups and amine groups in proteins and van der Waals forces have been implicated [11,12].

Coomassie Blue R-250 was prepared in methanol/acetic acid. Staining of dendrimers was performed in the cold. Various incubation times were tested (30, 180 min and overnight) in order to optimize staining time. Results from this study revealed that the best results were obtained by overnight staining of the gel (results not shown). After staining, the gel was destained for 10–15 min in cold 50% methanol/10% acetic acid and then placed in cold 10% methanol/10% acetic acid until the background was clear. Staining and destaining in the cold helped reduce diffusion, especially for lower generation dendrimers [9]. The sensitivity of Coomassie Blue R-250 for amine surface EDA-core PAMAM dendrimers was 1.5 μg for G0, under optimized acidic conditions [9]. For proteins, the reported sensitivity is 50 ng [13].

Many primary amines develop a blue color when treated with ortho-quinones. The preferred reagent is the sodium salt of 1,2-naphthoquinone-4-sulphonic acid [14]. Although amine-terminated PAMAM dendrimers could be stained with naphthoquinone, the sensitivity of this technique was over 10 times less than that of Coomassie Blue (results not shown). Dendrimers could also be detected with Ponceau S, Brilliant Blue G perchloric acid, and Fast Green staining [15,16]. However, all of these stains displayed much lower sensitivity than Coomassie Blue. Silver staining is a highly sensitive method for visualization of proteins in electrophoresis gels [12]. However, unlike Coomassie Blue, it could not even detect 10 μg EDA-core amine surface G0. A spot test used in organic functional group analysis for amines involves the reaction of amines with carbon disulphide to form dithiocarbamates. The mixture is then reacted with 1% silver nitrate (in nitric acid) to give black silver sulphide [17]. This reaction was evaluated for detection of PAMAM amines on PAGE gels. This staining methodology was quite sensitive for amine-terminated PAMAM dendrimers. The sensitivity of the stain was compared with Coomassie Blue R 250. Different concentrations of G5–G9 and G0–G4 mixtures were run on gels that were stained with either Coomassie Blue R 250 or CS_2/$AgNO_3$ stain. Both staining procedures could detect down to 1 μg

of G2–G9 (results not shown). Attempts to further improve the sensitivity of the stain for dendrimers were unsuccessful. Although sensitivity of this method was comparable to Coomassie Blue R 250, it required more steps and involved the use of hazardous materials. Another protein stain, Brilliant Blue G-colloidal has been utilized in the staining of proteins in polyacrylamide and agarose gels. As a protein stain, this dye is reported to be five times more sensitive than traditional Coomassie Blue, takes only 1 h to stain and clear background is achieved in 7 h [18]. However, it could not stain dendrimers. The lack of staining may be due to the inability of the large stain particles to penetrate the cavities present in a dendrimer.

3.2. Electrophoretic separation of dendrimers

Sharma et al. showed that PAMAM dendrimer mixtures could be separated in non-gradient gels [9]. Employing conditions that reduced diffusion, even low generation dendrimers (G0–G2) could be clearly resolved. These conditions included carrying out all steps (separation, staining and destaining) at 4 °C, running the separation at low current or voltage to reduce heat generation, and whenever possible, fixation of the dendrimer samples after electrophoresis. PAMAM dendrimers with neutral or positively charged surfaces have higher charge-mass ratios at low pH while those with negative charges have higher ratios at basic pH. Therefore, the former were separated under acidic conditions while the latter were separated under basic conditions [6,9]. In the last few years, many new PAMAM dendrimers have been synthesized. Currently, PAMAM dendrimers of many different cores and surfaces are commercially available. Compared to the commonly used EDA core, dendrimers with two-fold (DAB core), three-fold (DAH core) and six-fold (DAD core) increased core lengths have been prepared. The increased core lengths may have some interesting effects on the architecture and physicochemical properties of these dendritic macromolecules [19]. Electrophoresis of dendrimers with various cores and surfaces is discussed below.

3.2.1. Separation of dendrimers with amine surfaces

Amine-terminated PAMAM dendrimers have higher charge/mass ratios under acidic conditions (Table 1). Their separation is therefore better at low pH [9]. Using conditions described before [9], amine-terminated PAMAM dendrimers with various cores were separated on polyacrylamide gels with *T* and *C* values of 15 and 2.6%, respectively.

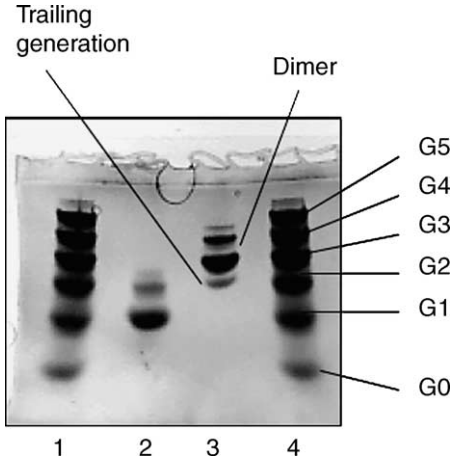

Fig. 2. Electrophoresis of amine surface PAMAM dendrimers with EDA and cystamine cores. Acidic electrophoresis was performed on 15% gel for 55 min at 200 V. Gel was stained with Coomassie Blue R-250. Lanes 1 and 4, G0–G5 EDA core ladder; lane 2, G1 cystamine core; lane 3, G3 cystamine core.

Separation of a mixture of G0–G5 EDA core PAMAM dendrimers is shown in Fig. 2 (lanes 1 and 4). Staining was performed with Coomassie Blue R 250. In accordance with previous reports, acidic electrophoresis under conditions that reduced diffusion and inclusion of a glutaraldehyde-fixation step led to sharp, well-resolved bands. The largest difference in charge/mass ratio is between G0 and G1. The difference in ratio between two consecutive generations then progressively decreases with increasing generation number (Table 1). This effect is clearly observed on PAGE gels. The difference in migration is greatest between G0 and G1 and then it progressively decreases (Fig. 2, lanes 1 and 4). A similar phenomenon is observed under alkaline separations [6,9]. Separation of higher generation dendrimers involves more than just differences in their charge/mass ratios. Molecular sieving and possible sample-gel interactions may also aid in their separation. The acidic separation procedure also works very well in separation of dendrimers with other cores. As shown in Fig. 2 (lanes 2 and 3), cystamine dendrimers with

Table 1
Theoretical molecular characteristics of amine surface PAMAM dendrimers (EDA core)

Dendrimer	G0	G1	G2	G3	G4	G5	G6	G7	G8	G9
Molecular weight	517	1430	3256	6909	14215	28826	58048	116493	233383	467162
Diameter (Å)	15	22	29	36	45	54	67	81	97	114
Number of primary amines	4	8	16	32	64	128	256	512	1024	2048
Number of tertiary amines	2	6	14	30	62	126	254	510	1022	2046
Total number of amine groups	6	14	30	62	126	254	510	1022	2046	4094
Charge/mass ratio at pH 8.8 ($\times 10^{-4}$) (C/g)[a]	77	56	49	46	45	44.4	44.1	43.9	43.87	43.83
Charge/mass ratio at pH 2.9 ($\times 10^{-4}$) (C/g)[b]	116	97.9	92.1	89.7	88.6	88.1	87.8	87.3	87.66	87.63

[a] Charge/mass ratio was calculated assuming theoretical MW values and full protonation of only terminal primary amines.
[b] Charge/mass ratio was calculated assuming theoretical MW values and full protonation of all amines (terminal primary and interior tertiary amines).

surface amine groups show sharp bands in which trailing generations, dimers and other contaminants (which are produced during synthesis) were clearly resolved.

The acidic electrophoresis procedure was also utilized for separation of amine-terminated PAMAM dendrimers of varying core lengths. Changing the core length has been reported to dramatically alter the morphology and properties of these macromolecules [19]. Amine terminated PAMAM dendrimers with diaminododecane core, diaminohexane core, and diaminobutane core were run under acidic conditions. In general, dendrimers with higher chain length cores gave sharper bands than EDA core dendrimers, although their charge/mass ratios were not significantly different. It is likely that the increased core length made it more hydrophobic, which resulted in decreased dendrimer diffusion during electrophoresis, fixing, staining and destaining steps, especially for the lower generations that are more susceptible to diffusion artifacts. It is also possible that the increased core length may have led to enhanced interaction of Coomassie Blue dye with the dendrimer.

3.2.2. Separation of dendrimers with carboxyl surfaces

Brothers et al. used alkaline conditions to resolve 3.5 and higher generation carboxyl terminated dendrimers [6]. We used similar conditions on 15% gels, except that all steps were performed at 4 °C. Although sharp bands were obtained for various EDA core, carboxyl-terminated PAMAM dendrimer, G1.5 could not be visualized (results not shown). These dendrimers seem to be very sensitive to diffusion probably due to their excellent water solubility. Some fixative methodologies were tried (crosslinking by EDC/diamine, use of chromium salts) but did not yield any improvements. Cationic dyes like alcian blue and toluidine were used to stain the carboxyl-terminated dendrimers. However, they did not perform as well as Coomassie Blue (results not shown). Therefore, smaller pore size gels and reduced voltage (or current) was used to reduce diffusion. In addition, destaining time was kept to a minimum (less than 30 min instead of 3–4 h). Once the bands were clearly visible, the gels were photographed. The use of 20% polyacrylamide gel, separation at a lower voltage (less than 100 V), and reduced destaining time, resulted in a better separation. G1.5 could be clearly seen and a mixture of G1.5–4.5 was well resolved with sharp bands. However, G1.5 and G2.5 have very similar migrations, and cannot be resolved in mixtures. Trailing generations and higher molecular weight species could be clearly seen and variations in core lengths did not appreciably affect migration times of dendrimers under these conditions (results not shown). Another carboxyl-containing class of dendrimers contains succinamic acid surface groups. These dendrimers are commercially available with various core lengths and types, and can also be well-resolved under these conditions. An example of this is shown in Fig. 3. Cystamine core PAMAM G4 dendrimer with succinamic acid surface (MW 20 711, lane 1, Fig. 3A and B) was denatured with 10-fold excess DTT for 24 h at room temperature. The products of this cleavage (dendrons with half the molecular weight of the dendrimer) migrated similar to a G3 dendrimer (MW 10 203) (lane 2, Fig. 3A). Higher generation contaminants in the G4 sample were broken down into species that migrated like G4 dendrimers. Purification of G4 gave a single band (lane 3, Fig. 3A and lane 2, Fig. 3B). Reduction of the purified G4 dendrimer under similar conditions, showed the original band and a new band that migrated like a non-reduced G3 (lanes 3–5, Fig. 3B). Some G4 was still visible even after 24 h reduction under these conditions (Fig. 3C). As with proteins, PAGE is a convenient way to monitor dendrimer cleavage reactions.

3.2.3. Separation of dendrimers with hydroxyl surfaces

A major advantage of acidic electrophoresis is the separation of PAMAM dendrimers that possess uncharged terminal groups [9]. The interior tertiary amines (with p*K* around 4–5) are protonated under acidic conditions. This charge enables their electrophoretic migration. However, their charge/mass ratios are almost half that of amine surface PAMAM dendrimers. Electrophoresis times would be very long (2–3 h) if low voltage (or currents) or small pore gels were used to minimize diffusion (as in case of carboxyl-terminated dendrimers). Thus, the diffusion problem is the worst for separation of hydroxyl surface dendrimers. Nevertheless, acidic electrophoresis can provide adequate separation and detection when all steps are carried out in the cold and staining

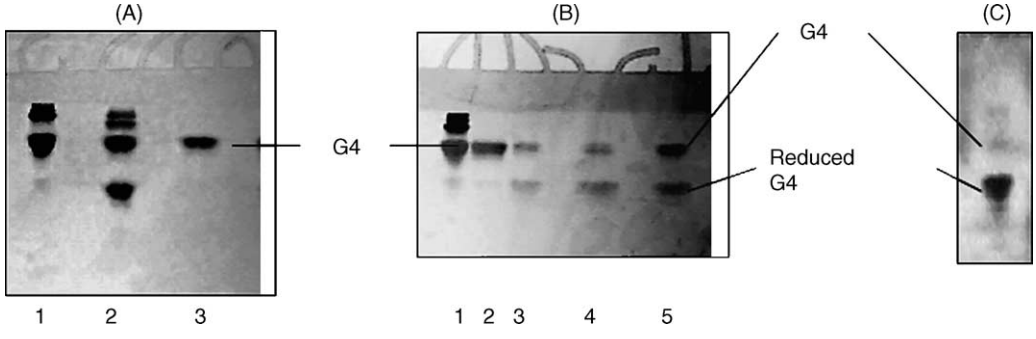

Fig. 3. Reduction and separation of succinamic acid surface PAMAM dendrimers with cystamine core. Alkaline electrophoresis was performed on 20% gel for 30 min at 150 V. Gel was stained with Coomassie Blue R-250. (A) Lane 1, unpurified G4; lane 2, G4 reduced with DTT for 24 h; lane 3, purified G4. (B) Lane 1, unpurified G4, lane 2, purified G4; lanes 3–5, purified G4 reduction for 5, 3, and 1 h, respectively. (C) Purified G4 reduction for 24 h.

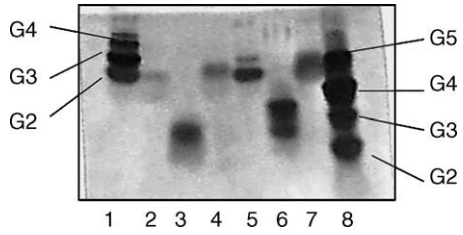

Fig. 4. Separation of hydroxyl surface PAMAM dendrimers with EDA and cystamine cores. Acidic electrophoresis was performed on 15% gel for 90 min at 200 V. Gel was stained with Coomassie Blue R-250. Lane 1, G2–G4 TRIS surface, cystamine core; lane 2, G2 TRIS surface cystamine core; lane 3, G2 EDA core AEEA surface; lane 4. G2 EDA core TRIS surface; lane 5, G3 TRIS surface cystamine core; lane 6, G3 EDA core AEEA surface; lane 7, G3 EDA core TRIS surface; lane 8, G0–5 EDA core amine surface.

and destaining times are kept to the minimum possible. Separation of various hydroxyl dendrimers is presented in Fig. 4. For comparison, a mixture of G0–G5 EDA core amine surface dendrimers was also run (lane 8; the diffuse bands are likely due to lack of glutaraldehyde fixation. G0 and G1 were not visible since they migrated out of the gel). The difference between migration rates of amine and hydroxyl surface dendrimers is clearly evident from these results. For example, a G2 (EDA core) tris dendrimer (MW 4233, lane 4) travels similar to a G5 (EDA core) amine (MW 28 826, lane 8). Amidoethylethanolamine (AEEA) surface dendrimers (lanes 3 and 6) have faster migrations than the tris dendrimers (lanes 4 and 7), although their molecular weights are not very different (molecular weights of EDA core G2 and G3 AEEA surface are 3961 and 8319, respectively; EDA core G2 and G3 tris surface dendrimers have MW of 4233 and 8862, respectively). This is due to the extra positively charged secondary nitrogen available on each branch of the AEEA dendrimer. Cystamine core hydroxyl surface dendrimers were also well resolved on acidic gels (lane 1, Fig. 4).

The reliability of the electrophoresis procedure for separating various dendrimer species in a given sample was evaluated by comparing the bands observed on PAGE gels with MALDI-TOF. A typical result is shown in Fig. 5. An unpurified G6 TRIS surface, cystamine core PAMAM dendrimer (theoretical MW 73 764) was subjected to 10-fold excess DTT reduction for 24 h. After reduction, the sample was immediately run under acidic electrophoresis on a 10% gel and simultaneously analyzed by mass spectrometry. Four bands (A–D) could be resolved with PAGE. MALDI also showed four peaks. As expected, the major fraction (band B) was the G6 dendrimer split into dendrons, with half the molecular weight. This fraction appeared as the darkest band on the gel (MW 33 916 by MALDI). A lighter-stained, faster moving band showed a MW of 17 463 (band A). This band was very sensitive to diffusion, especially during destaining. The third band (C) was also sharp and well-stained like band B and corresponded to a MALDI peak of MW 64 747. A narrow, sharp band that was the slowest moving band (D) showed a MALDI MW of 95 844. This band was also detected

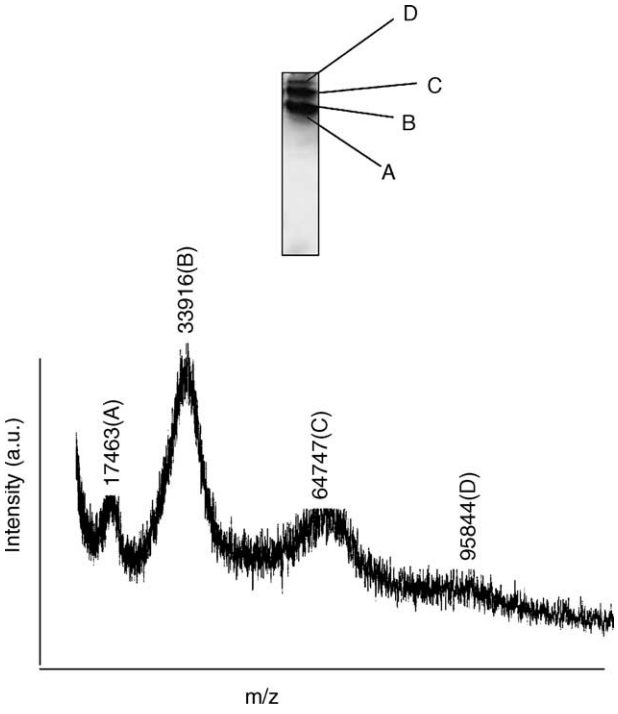

Fig. 5. Electrophoresis and MALDI-TOF mass spectrum of reduced G6 TRIS surface PAMAM dendrimer with cystamine core. Acidic electrophoresis was performed on 10% gel for 90 min at 90 V. Gel was stained with Coomassie Blue R-250.

by PAGE and MALDI in the unreduced G6 dendrimer sample.

4. Conclusion

In this study, a number of detection and separation methodologies used in protein research have been applied to PAMAM dendrimers. These synthetic well-defined macromolecules display many properties that are similar to proteins. They show discreet bands on electrophoresis gels and react with many dyes and reagents used for protein detection. However, there are some important differences between PAMAM dendrimers and globular proteins. The extensive folding and unfolding displayed by proteins is absent in PAMAM dendrimers. Unlike proteins, dendrimers therefore cannot be fixed easily after their electrophoretic separation are thus more susceptible to diffusion during various staining and destaining steps. In addition, the decreased hydrophobic effect caused by a lack of denaturation leads to decreased binding of dendrimers to stains and dyes. Nevertheless, the results presented above demonstrate the use of PAGE as a useful tool not only for dendrimer analysis but also for their purification to obtain microgram quantities of highly pure dendrimer. This study also shows that simple modifications of the basic PAGE protocols will allow one to use PAGE for analysis and purification of any water-soluble dendrimer with a low polydispersity.

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