**Xiangyang Shi** [1]
**István Bányai** [2]
**Keyla Rodriguez** [1]
**Mohammad T. Islam** [1]
**Wojciech Lesniak** [1]
**Peter Balogh** [1]
**Lajos P. Balogh** [1]
**James R. Baker Jr.** [1]

[1] Michigan Nanotechnology Institute for Medicine and Biological Sciences, University of Michigan, Ann Arbor, MI, USA
[2] Department of Physical Chemistry, University of Debrecen, Debrecen, Hungary

Received November 2, 2005
Accepted November 3, 2005

Research Article

# Electrophoretic mobility and molecular distribution studies of poly(amidoamine) dendrimers of defined charges

Generation 5 ethylenediamine (EDA)-cored poly(amidoamine) (PAMAM) dendrimers (E5, E denotes the EDA core and 5 the generation number) with different degrees of acetylation and carboxylation were synthesized and used as a model system to investigate the effect of charge and the influence of dendrimer surface modifications on electrophoretic mobility (EM) and molecular distribution. The surface-modified dendrimers were characterized by size-exclusion chromatography, $^{1}$H NMR, MALDI-TOF-MS, PAGE, and CE. The focus of our study was to determine how EM changes as a function of particle charge and molecular mass, and how the molecular distribution changes due to surface modifications. We demonstrate that partially modified dendrimers have much broader migration peaks than those of fully surface functionalized or unmodified E5 dendrimers due to variations in the substitution of individual dendrimer surfaces. EM decreased *nonlinearly* with increases in surface acetylation for both PAMAM acetamides and PAMAM succinamic acids, indicating a complex migration activity in CE separations that is not solely due to charge/mass ratio changes. These studies provide new insights into dendrimer properties under an electric field, as well as into the characterization of dendrimer-based materials being developed for medical applications.

**Keywords:** Capillary electrophoresis / Electrophoretic mobility / Molecular distribution / Poly(amidoamine) dendrimers DOI 10.1002/elps.200500818

## 1 Introduction

The past two decades have seen a growing scientific and technological interest in poly(amidoamine) (PAMAM) dendrimers because of their unique architecture and properties [1–3]. PAMAM dendrimers are densely branched macromolecules with well-defined (spherical or nearly spherical) geometry. Dendrimer diameters ranging from approximately 1.1 (generation 1) to 9 nm (generation 8) [3] and their physicochemical properties are intrinsically dependent on generation number and surface functionalities. Compared with standard organic or inorganic colloidal particles, the surface charge of PAMAM dendrimers is inherently relevant to the polymer molecules themselves instead of arising from ionic surfactants adsorbed onto particles during their preparation. The surface charge of these colloids plays a major role in their mobility and migration behavior, as their properties are often dominated by electrostatic interactions in aqueous solution. PAMAM dendrimers with systematically varied surface charges are expected to serve as a nearly ideal model for the investigation of electrophoretic mobility (EM).

The separation of organic and inorganic colloidal particles and biological vesicles may be of both analytical and preparative interest in environmental sciences, biomedicine, and biotechnology. Among the techniques used for the separation of colloids and biologic macromolecules, CE has been established as a reliable and widely useful analytical technique with high resolution, high efficiency, and high automation capability [4, 5]. A broad range of colloids including polystyrene lattices [6–8], silica sols [9], semiconductor clusters [10], virus and bacterial particles [11, 12], and liposomes [13, 14] have been separated by CE. The size distribution of various colloid particles, their

**Correspondence:** Dr. James R. Baker Jr., Michigan Nanotechnology Institute for Medicine and Biological Sciences, University of Michigan, Ann Arbor, MI 48109, USA
**E-mail:** jbakerjr@umich.edu
**Fax:** +1-734-936-2990

**Abbreviations: 2,3-DAP**, 2,3-diaminopyridine; **EM**, electrophoretic mobility; **MBA**, 4-methoxybenzyl alcohol; **PAMAM**, poly(amidoamine); **SEC**, size exclusion chromatography

© 2006 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

surface charge density, and relaxation effect under electric field can be effectively evaluated using CE. For charged dendrimer spheres, Dubin and co-workers [15] investigated the effect of pH, ionic strength, and electric field on the mobility of carboxyl-terminated cascade dendrimers (Z-cascade:methane [4]:(3-oxo-6-oxa-2-aza-heptylidyne):(propanoic acid)) of generations 2–5. It was found that counter-ion binding plays a major role in the separation of these Newkome-type dendrimer carboxylates. Welch and Hoagland [16] studied the EM of polypropyleneimine (PPI) dendrimers and concluded that PPI dendrimers behave as charged spheres. It is believed that surface charges of dendrimer spheres play an important role in influencing their EMs. It has been demonstrated that CE can be utilized for the separation of different generation of dendrimers and characterization of individual polycationic and polyanionic PAMAM dendrimers [17–20]. However, to our knowledge, there are no reports specifically related to EM studies of the same-generation PAMAM dendrimers with different functionalities. These surfaces are modified in many uses of dendrimers, especially in medical applications where surface amines are neutralized to prevent toxicity.

It is well established that the EM of linear polyelectrolytes is linearly proportional to their charge density at different zones [21]. In contrast, polyelectrolyte dendrimers analyzed in identical instrument and run conditions provide EM that changes due to changes in pH and surface functionality. Dendrimer surface modifications often result in a change in total surface charge, an increase in molar mass of the product, and changes in the generational, skeletal, and/or substitutional distributions. Charged PAMAM dendrimers, because of their nearly spherical shape, nanoscale size, and high concentration of nitrogen ligands in the interior and on the surface, are expected to exhibit behavior different from that of linear polyelectrolytes. Dendrimer end group modifications with multifunctional moieties (drugs, targeting agents, and imaging dyes) have been recently demonstrated to be extremely useful in biomedical applications [22, 23]. As highly positively charged macromolecules are often cytotoxic, the dendrimer surface charges have to be reduced and partially modified as intermediate materials. Charge and charge distribution of partially modified dendrimers plays an important role in determining physicochemical properties of the final products and influences their interactions with biologic entities. Therefore, the motivation of this work encompasses three aspects: (i) to seek a reliable method for analyzing various surface-modified PAMAM dendrimer materials; (ii) to investigate the influence of charge on the EM of PAMAM dendrimers after surface modification; and (iii) to evaluate the charge distribution after dendrimer end-group functionalization.

In this present study, PAMAM dendrimer derivatives were synthesized from generation 5 amine-terminated (E5.$NH_2$) materials with variations of surface acetylation and carboxylation. The resulting polycationic and polyanionic PAMAMs were characterized by a variety of techniques, including size-exclusion chromatography (SEC), $^1$H NMR, and MALDI-TOF-MS. CE and PAGE were employed to investigate the specific effect of charge on the EM of the polycationic and polyanionic dendrimer molecules. Factors influencing the EMs of both polycationic and polyanionic dendrimers are briefly discussed.

## 2 Materials and methods

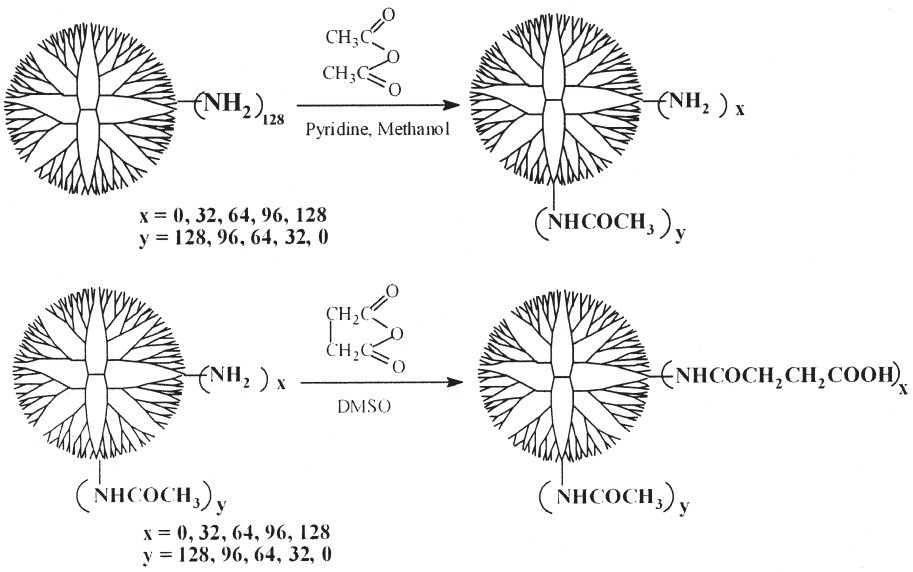
### 2.1 Materials

Ethylenediamine (EDA)-cored PAMAM dendrimer of generation 5 (E5.$NH_2$, Lot No: 0602–03-E5.0-LD) was purchased from Dendritech (Midland, MI) in methanol solution (14.17 wt%). The methanol of the E5.$NH_2$ solution was removed by rotary evaporation and the dendrimer was redissolved in water, followed by lyophilization before use. Acetic anhydride, absolute methanol, pyridine, succinic anhydride, chlorotrimethylsilane (1.0 M in THF), DMSO, and all the other chemicals and solvents were obtained from Aldrich and used as received. Water used in all of the experiments (unless otherwise stated) was purified by a MilliQ Plus 185 water purification system (Millipore, Bedford, MA) with resistivity higher than 18 MΩ·cm. Regenerated cellulose dialysis membranes (MWCO = 10 000) were acquired from Fisher.

### 2.2 Synthesis of polycationic and polyanionic E5 PAMAM dendrimers

The reactions to synthesize polycationic (with different acetylation percentages) and polyanionic (in varying carboxylation percentages) E5 PAMAMs are schematically presented in Scheme 1. The acetylation reaction procedure in this work was slightly modified from that reported in the literature [24]. The only difference is that we used pyridine to neutralize the by-product acetic acid instead of triethylamine. Both pyridine and triethylamine worked equally well in the acetylation reaction. Briefly, 0.5 mL of pyridine was added to a 10 mL of methanol solution containing 100 mg E5.$NH_2$ dendrimer. Methanolic solutions (10 mL) of acetic anhydride with different calculated molar ratios (25, 50, 75, and 100% of the total primary amines of E5.$NH_2$) were in parallel added into the dendrimer/pyridine mixture solutions while vigorously stirring and the mixture was allowed to react for 24 h. Methanol was then removed from the reaction mixture on a rotary evaporator.

© 2006 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 




**Scheme 1**. Schematic representation of acetylation and carboxylation reactions with E5.$NH_2$ PAMAM dendrimer.

The oily crude product was diluted with $H_2O$ and dialyzed against water (6 × 4L) for 3 days to remove the excess of reactants and byproducts. Water was removed from the retentate, and the product was redissolved in water and then lyophilized. Yields were in the range of 91.4–94.2%. The final E5 acetamides are denoted by using their theoretical composition numbers as E5.25Ac, E5.50Ac, E5.75Ac, and E5.100Ac (for practical substitution degrees see Table 1). For the carboxylation reaction, 50 mg dry polycationic E5 acetamide derivative (acetylation percentages 0, 25, 50, and 75%) was dissolved in 10 mL of DMSO. Into each of the E5 acetamide solution was added 10 mL of DMSO solution containing succinic anhydride with two to three times molar excess of the remaining primary amine groups of the particular E5 acetamide under vigorous stirring. The reaction was maintained at room temperature for 24 h. Then, the final DMSO solution was dialyzed against water (6 × 4 L) to remove the excess succinic anhydride as well as the organic solvent. The aqueous retentate was filtered and then lyophilized. The final E5 succinamic acids are denoted as E5.25SAH, E5.50SAH, E5.75SAH, and E5.100SAH, and their yields were in the range of 53.6–75.2% (for practical substitution degrees see Table 2).

## 2.3 SEC analysis

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

## 2.4 Potentiometric acid-base titration

Acid-base titrations were performed manually using a Beckman Φ10 pH meter (Fullerton, CA 92634–3100) at room temperature (23 ± 1°C). Fifty milligrams of E5.$NH_2$ dendrimer was dissolved in 30 mL of water containing 0.1 M NaCl to give a solution concentration of 1.66 mg/mL. This solution was titrated by a standard HCl solution (0.0951 N), and then back titrated using a standard NaOH (0.1021 N) solution. The numbers of primary and tertiary amine groups of E5.$NH_2$ were calculated using back titration data and the absolute molecular weight measured by SEC.

## 2.5 MALDI-TOF measurements

MALDI-TOF mass spectra were acquired using a Micromass TofSpec-2E spectrometer (Beverly, MA). Linear mode was selected as the operation mode. Ten milligrams *per* milliliter β-indoleacrylic acid in ACN/$H_2O$ (v/v 70:30) was used as the matrix. One milligram of dendrimer samples was dissolved in 1 mL of methanol, and

© 2006 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

**Table 1.** Physicochemical parameters of E5 PAMAMs of different acetylation degree

| Dendrimer | E5.$NH_2$ | E5.25Ac | E5.50Ac | E5.75Ac | E5.100Ac |
|---|---|---|---|---|---|
| Calculated acetylation percentage (%) | 0 | 25 | 50 | 75 | 100 |
| Theoretical number of terminal amino groups | 128 | 96 | 64 | 32 | 0 |
| Practical acetylation degree[a] (%) | 0 | 19 | 45 | 65 | 95 |
| Practical number of terminal amino groups[a] | 110 | 89 | 61 | 39 | 6 |
| Tertiary amine groups[a] | 116 | 116 | 116 | 116 | 116 |
| Total amine groups | 226 | 205 | 177 | 155 | 122 |
| Theoretical $M_r$ | 28 826 | 30 170 | 31 514 | 32 858 | 34 202 |
| Practical $M_n$ (SEC) | 26 010 | 27 510 | 28 680 | 29 240 | 30 990 |
| Practical $M_r$ (SEC) | 28 730 | 29 880 | 31 150 | 32 150 | 32 840 |
| Polydispersity (SEC) | 1.104 | 1.086 | 1.086 | 1.100 | 1.060 |
| Practical $M_r$ (MALDI-TOF) | 26 462 | 26 778 | 27 667 | 28 889 | 30 065 |
| $N$[b]/$M_n$[c] | 8.689E-3 | 7.452E-3 | 6.172E-3 | 5.301E-3 | 3.937E-3 |
| Relative migration time ratio (Td/Ts) (RSD, $n$ = 3) | 1.18 (2.75%) | 1.25 (1.31%) | 1.35 (4.22%) | 1.48 (3.86%) | 1.80 (8.07%) |
| Normalized migration time[d] (min) | 14.32 | 15.18 | 16.39 | 17.97 | 21.85 |
| Apparent EM ($10^{-4} \times cm^2/V \cdot s$) | 3.2 ± 0.07 | 3.02 ± 0.02 | 2.79 ± 0.09 | 2.55 ± 0.08 | 2.10 ± 0.12 |
| Temporal width at half height[e] (min) | 0.552 | 1.981 | 2.384 | 2.162 | 0.95 |
| Spatial width at half height (cm)[f] | 2.7 | 9.1 | 10.2 | 8.4 | 3.0 |

a) Calculated by NMR and potentiometric acid-base titration.
b) $N$ represents the number of total amine groups under CE conditions.
c) Determined using molar mass measured using SEC.
d) Average migration time of 2,3-DAP is 12.14 min.
e) Temporal width at half height was obtained from Fig. 3.
f) Spatial width at half height was calculated by multiplying the temporal width at half height by the average velocity of the peak.

**Table 2.** Physicochemical parameters of E5 PAMAMs of different carboxylation degree

| Dendrimer | E5.25SAH | E5.50SAH | E5.75SAH | E5.100SAH |
|---|---|---|---|---|
| Calculated carboxylation degree (%) | 25 | 50 | 75 | 100 |
| Theoretical surface carboxyl groups | 32 | 64 | 96 | 128 |
| Practical carboxylation degree[a] (%) | 41 | 61 | 82 | 100 |
| Practical surface carboxyl groups[a] | 45 | 67 | 90 | 110 |
| Theoretical $M_r$ | 36 058 | 37 914 | 39 770 | 41 626 |
| Practical $M_n$ (SEC) | 32 910 | 36 050 | 37 650 | 40 330 |
| Practical $M_r$ (SEC) | 34 390 | 38 660 | 41 250 | 42 500 |
| Polydispersity (SEC) | 1.045 | 1.072 | 1.096 | 1.054 |
| Practical $M_r$ (MALDI-TOF) | 31 222 | 32 333 | 33 889 | 35 111 |
| $N$[b]/$M_n$[c] | 1.367E-3 | 1.859E-3 | 2.390E-3 | 2.727E-3 |
| Relative migration time ratio (Td/Ts) (RSD, $n$ = 3) | 1.57 (1.94%) | 1.74 (3.68%) | 1.91 (4.14%) | 2.04 (5.15%) |
| EM ($10^{-4} \times cm^2/V \cdot s$) | −2.77 ± 0.06 | −3.27 ± 0.13 | −3.27 ± 0.21 | −3.84 ± 0.13 |
| Temporal width at half height[d] (min) | 1.300 | 1.139 | 0.502 | 0.277 |
| Spatial width at half height[e] (cm) | 14.6 | 13.2 | 4.6 | 2.4 |

a) Calculated by NMR and acid-base titration.
b) $N$ represents the number of total carboxylic acid groups.
c) Determined using molar mass measured using SEC.
d) CE peak width at half height was obtained from Fig. 4.
e) Spatial width at half height was calculated by multiplying the temporal width at half height by the average velocity of the peak.

© 2006 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

then diluted with methanol to get the final concentration of 0.2 mg/mL. Equal volumes of 0.2 mg/mL dendrimer solution and matrix solution were well mixed. Then, 1 μL solution of this mixture was injected on the spots of target. Five picomol protein standard of cytochrome *c* (12 359 g/mol), myoglobin (16 951 g/mol), and trypsinogen (23 976 g/mol) were used as external standards.

## 2.6 $^1$H NMR characterization

$^1$H NMR spectra of PAMAM dendrimer samples were recorded on a Bruker DRX 500 instrument (Billerica, MA). Dendrimer samples were dissolved in $D_2O$ at the concentration of 5 mg/mL before NMR measurements.

## 2.7 PAGE analysis

Analysis of PAMAM dendrimers by PAGE was performed on a Micrograd vertical electrophoresis system (Model FB-VE10–1, FisherBiotech, Pittsburgh, PA). Precast 4–20% gradient express gels for PAGE were obtained from ISC BioExpress (Kaysville, UT). A commercial power supply (Model EC135–90; Thermo Electron, Milford, MA) was used. Tris-glycine buffer (pH 8.3) was purchased from Invitrogen (Carlsbad, CA). It was diluted ten times to prepare the running buffer. PAGE separations typically required 40–50 min at 200 V. Reverse polarity was used for the analysis of the polycationic PAMAM dendrimers. Into each sample well 2 μL of a sample solution containing 1 μL of 1 mg/mL polycationic PAMAM dendrimers and 1 μL of methylene blue sucrose dye (50% sucrose, 1% methylene blue) was injected. For polyanionic PAMAM dendrimers, a mixture of 1 μL of 1 mg/mL PAMAM dendrimers and 1 μL of bromophenol blue sucrose dye (50% sucrose, 1% bromophenol blue) was injected. Developed gels were stained overnight with 0.025% CBB R-250 in 40% methanol and 7% acetic acid aqueous solution. The gels were destained with an aqueous solution containing 7% v/v acetic acid and 5% v/v methanol.

## 2.8 CE analysis

An Agilent Technologies (Waldbronn, Germany) CE instrument was used in this work. Unmodified quartz capillaries were purchased from Polymicro Technologies (Phoenix, AZ). Different voltages (10, 15, 20, and 25 kV) were applied for separation. The migration time ratios between dendrimer samples and internal standards are essentially constant as a function of voltage. Therefore, in most cases, the voltage was kept at 20 kV. An on-capillary UV diode array detector (DAD) was used, operating at wavelengths of 200, 210, 250, and 300 nm. Samples were introduced by hydrodynamic injection at a pressure of 50 mbar for 3 s.

### 2.8.1 Separation of polycationic PAMAMs

For the characterization of polycationic E5 dendrimers, silanized capillaries [19] (id 100 μm) with total length of 78.5 cm and effective length of 70 cm were employed. For the silanization of capillary, briefly, the bare fused-silica capillary was pretreated by rinsing with a 1.0 M NaOH solution for 30 min to activate the hydroxyl groups on the silica surface, followed by an aqueous wash with water for 30 min, then with methanol for 30 min, and subsequently with THF for 20 min. The internal wall of the capillary was silanized by filling up the capillary with the mixture of chlorotrimethylsilane (1.0 M in THF) and pyridine (10:1 v/v) for 24 h. The capillary was extensively rinsed using THF (for at least 20 min) and then washed with water and equilibrated with the running buffer. Rinsing and flushing were performed at room temperature under syringe-induced vacuum. We have previously compared the CE performance using both bare fused-silica capillary and silanized capillary [19]. We show that for polycationic PAMAM dendrimers, the separation reproducibility is significantly improved using silanized capillary because dendrimer adsorption onto the capillary are significantly decreased. The capillary temperature was maintained at 40°C. Before use, the silanized capillary was initialized by rinsing with 0.2 M $H_3PO_4$ for 15 min, then washed with deionized water (Purchased from Agilent) for 15 min, and conditioned with the running buffer for another 15 min. Before each injection, the capillary wall was rinsed with a sequence of 0.2 M $H_3PO_4$ (3 min), deionized water (3 min), and then the running buffer (10 min). Phosphate buffer (pH 2.5, 50 mM) was obtained from Agilent Technologies and used as received. Polycationic E5 PAMAMs were dissolved in pH 2.5 phosphate buffer and the sample solutions were adjusted to pH 2.5 using 0.1 M phosphoric acid to give a concentration of 1 mg/mL. All polycationic dendrimer samples contained 0.05 mg/mL 2,3-diaminopyridine (2,3-DAP) as an internal standard to normalize the migration times of polycationic E5 PAMAMs (E5.$NH_2$, E5.25Ac, E5.50Ac, E5.75Ac, and E5.100Ac) [19]. The normalization is necessary because the absolute migration times of polycationic PAMAMs often change due to their dynamic adsorption/desorption onto capillary surface during separation, while the migration time ratio between PAMAMs and internal standands are always constant in different runs. The apparent EM was calculated according to the following equation:

$$\mu = \frac{lL}{Vt_s} \quad (1)$$

where $\mu$ is the apparent EM ($cm^2/V \cdot s$), $l$ is the effective length of capillary (cm), $L$ is the total length of capillary (cm), $V$ is the applied voltage (V), and $t_s$ is the migration time of PAMAM (s).

© 2006 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

### 2.8.2 Separation of polyanionic PAMAMs

Bare silica capillaries (id 75 μm) with total length of 64.5 cm and effective length of 56 cm were used for the characterization of polyanionic E5 succinamic acid dendrimers. The capillary temperature was maintained at 20°C. Before use, the uncoated silica capillary was pretreated by rinsing with 1 M NaOH (15 min), deionized water (purchased from Agilent) (15 min), and running buffer (15 min) [20]. Before each injection, the capillary was rinsed with a similar sequence of each eluent (each for 5 min). Twenty millimolar borate buffer (pH 8.3) was used as the running buffer. E5 PAMAM succinamic acid dendrimers were dissolved in the running buffer and the sample's pH was adjusted to 8.3 with 20 mM sodium tetraborate solution to get a final concentration of 1 mg/mL. 4-Methoxybenzyl alcohol (MBA) (0.05 mg/mL) was used as a neutral marker to calculate the EMs of dendrimers according to the following equation:

$$\mu_E = lL/V(1/t_m - 1/t_s) \quad (2)$$

where $\mu_E$ is the EM ($cm^2$/Vs), $l$ is the effective length of capillary (cm), $L$ is the total length of capillary (cm), $V$ is the applied voltage (V), $t_m$ is the migration time of the neutral marker, and $t_s$ is the migration time of PAMAM (s).

## 3 Results

### 3.1 Structural characterization of E5 PAMAM derivatives

#### 3.1.1 Molecular weight determination

Absolute molar masses ($M_n$) of the synthesized E5 PAMAM derivatives were measured using SEC equipped with a multi-angle laser light scattering (MALLS) detector. After the acetylation and carboxylation reactions, the $M_n$ of E5 PAMAM dendrimer consistently increased with increased degree of surface modification. The detected practical $M_n$ of E5.$NH_2$ (26 010 g/mol, see Table 1) is lower than the theoretical $M_n$ value (28 826 g/mol), *i.e.*, exhibits a slight deviation from the theoretical structure due to structural imperfections. The starting material contained >95% E5.$NH_2$, although traces of E4.$NH_2$ and some dimers were also detected (see PAGE results, vide infra). The generational purity and polydispersities ($M_r$w/$M_n$) of E5 PAMAM derivatives (Table 1) were almost identical regardless of the modification degree and surface functionalities. The acetylated and carboxylated PAMAM dendrimers were also analyzed by MALDI-TOF-MS. Their molecular weights from this analysis are listed in Tables 1 and 2.

#### 3.1.2 Potentiometric acid-base titration

Back titration data reflect full protonation and deprotonation states of PAMAM dendrimers [25], which we used to calculate the practical number of terminal and tertiary amine groups. Titration data revealed that the number of terminal and tertiary amine groups were 110 and 116 (Table 1), respectively, which confirmed the observed slight deviation from the theoretical structure in agreement with the SEC measurements (theoretical numbers are 128 and 126, respectively [3]). Combined with $^1$H NMR analysis data, exact number of terminal acetyl and carboxyl groups of the synthesized E5 PAMAM derivatives can be obtained.

#### 3.1.3 PAGE analysis

PAGE analysis supplies information regarding both the purity and EM of these substituted PAMAM dendrimers [17, 19, 20, 26, 27]. Shown in Figs. 1a and 1b are the PAGE electropherograms of acetylated and carboxylated PAMAM dendrimers, respectively. From all analyses, it can be seen that the trailing generation and dimers appear in all the dendrimers (the fully acetylated E5 dendrimer, E5–100Ac, is neutral under the PAGE pH condition). The bands of trailing generation and dimers originate from the starting material and are byproducts of the divergent synthesis. In addition, EM increases with the increase of the number of the primary amine and carboxyl groups.

### 3.2 EM and molecular distribution studies of E5 PAMAM derivatives

#### 3.2.1 CE analysis of acetylated E5 PAMAMs

The average migration time of 2,3-DAP (12.14 ± 0.06 min, 5 runs) was used to normalize the peak positions of the acetylated E5 PAMAMs. Figure 2 shows the normalized electropherograms of the polycationic PAMAMs. The migration time increases with the increase of acetylation degree, which is attributed to the decreased charge/mass ratio (summarized in Table 1). It is found that the peaks of partially acetylated E5 PAMAMs are much broader than those of the nonacetylated (E5.$NH_2$) and fully acetylated E5.100Ac (see both the temporal width at half height and spatial width at half height in Table 1). In order to evaluate the charge distribution as a function of dendrimer surface substitution, we utilized the approach described by Zare *et al.* [28] to elucidate the CE peak width. Both the temporal width at half height ($w_t$, min) and spatial width at half height ($w_s$, cm) were used. $w_s$ is calculated by simply multiplying $w_t$ with the average zone velocity. The average

© 2006 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

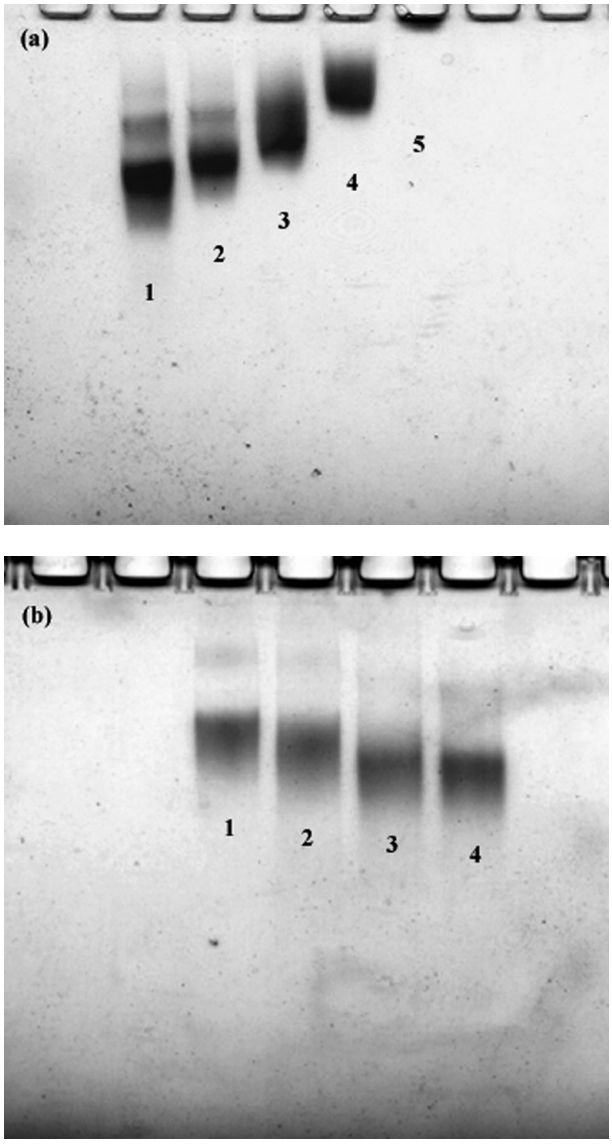



**Figure 1.** PAGE electropherogram of acetylated (a) and carboxylated (b) E5 PAMAM dendrimers analyzed on a 4–20% express gel. Tris-glycine native buffer (pH 8.3) was used as the running buffer. Reverse polarity was applied for acetylated PAMAM dendrimers. One microgram for each dendrimer was injected. (a) Lane 1: E5–0Ac; Lane 2: E5–25Ac; Lane 3: E5–50Ac; Lane 4: E5–75Ac; Lane 5: E5–100Ac. (b) Lane 1: E5–25SAH; Lane 2: E5–50SAH; Lane 3: E5–75SAH; Lane 4: E5–100SAH.

zone velocity is equal to *l*/*t*, where *l* is the effective length of capillary (cm), and *t* is the average migration time of dendrimers. From Table 1, assuming the normalized migration times are close to the actual average absolute migration times, conversion of $w_t$ to $w_s$ interestingly shows that the width for E5.100Ac peak is just slightly larger than that for E5.$NH_2$ peak, rather than being twice as large as suggested by $w_t$.

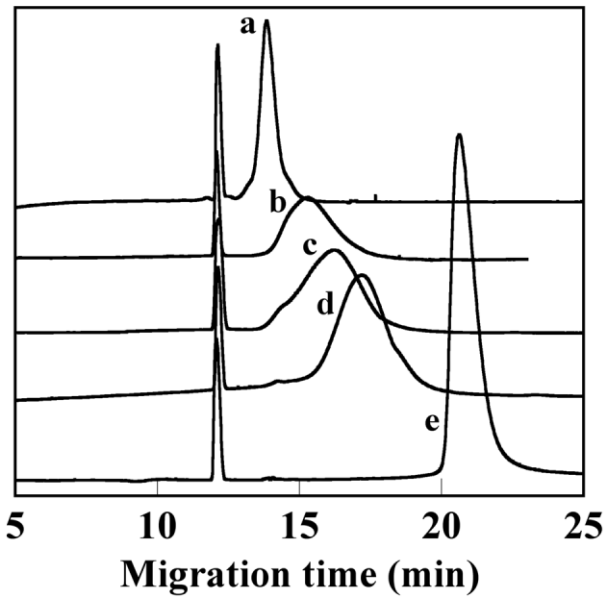



**Figure 2.** Normalized electropherograms of acetylated E5 PAMAMs with acetylation percentage of 0% (a), 25% (b), 50% (c), 75% (d), and 100% (e) analyzed using a silanized silica capillary (id 100 μm, total length 78.5 cm and effective length 70 cm) under phosphate buffer (pH 2.5, 50 mM) condition. Injection time: 3 s. First peak corresponds to the internal standard 2,3-DAP.

We have also found that although the practical charge/mass ratio of these polycationic dendrimers (determined by $^1$H NMR and titration) decreases linearly (Fig. 3, curve a) with the increase of acetylation degree, their EMs decrease nonlinearly, in a convex upward shape (Fig. 3, curve b), which indicates that the difference in their charge/mass ratios is not solely responsible for the migration rate differences of the PAMAM dendrimer derivatives. If the changes in the EMs are plotted as a function of charge/mass ratio, the same nonlinear change is observed (see Fig. 3, curve c). Note that the EM data were calculated from the migration time of E5 PAMAMs at maximum peak height. Considering the asymmetric nature of the electrophoretic profiles, this value may not represent the centroid of the electrophoretic behavior of a given dendrimer. This parameter may be more adequate for comparisons with the average charge/mass ratio values. Same situation applies to polyanionic E5 PAMAM succinamic acids (vide infra).

### 3.2.2 CE analysis of carboxylated E5 PAMAM dendrimers

A bare silica capillary was used to separate carboxylated E5 PAMAMs with mixed acetyl and carboxyl groups. The average migration time of MBA (3.96 min, 25 runs) was

© 2006 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

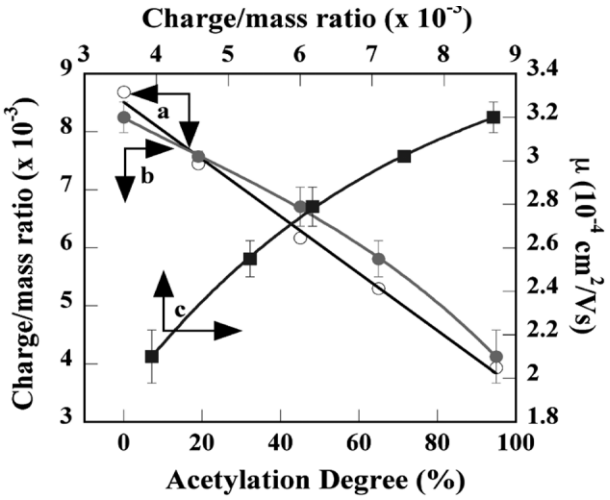



**Figure 3.** Measured charge/mass ratios of surface acetylated E5 PAMAM dendrimers as a function of the degree of acetylation (a), the dependence of EM on the degree of acetylation (b) and the EM of acetylated E5 PAMAMs as a function of practical charge/mass ratio (c). Arrows indicate the appropriate axis.

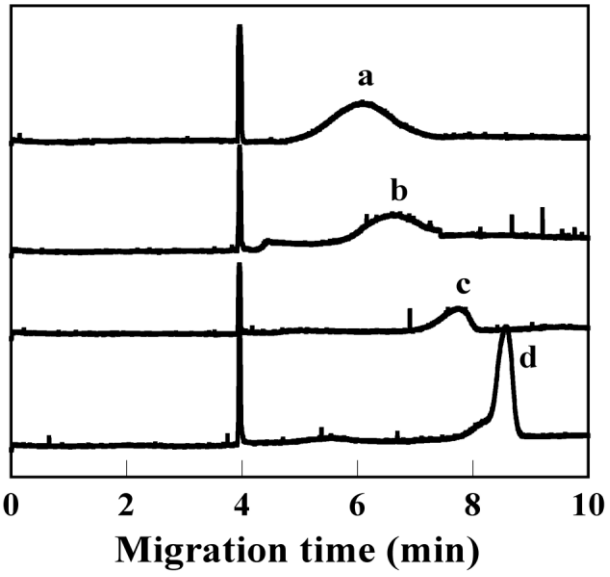



**Figure 4.** Normalized electropherograms of carboxylated E5 PAMAMs having carboxylic acid termini of 25% (a), 50% (b), 75% (c), and 100% (d) of the available end groups collected using a bare silica capillary (id 75 μm, total length 64.5 cm and effective length 56 cm) under borate buffer (pH 8.3, 20 mM) condition. Injection time: 3 s. First peak corresponds to MBA, the neutral marker.

used to normalize the peak positions of the carboxylated E5 PAMAMs. Figure 4 shows the normalized electropherograms of these polyanionic PAMAMs. It is clear that the migration time of these PAMAMs increases with the increase of carboxylation degree, which is ascribed to the

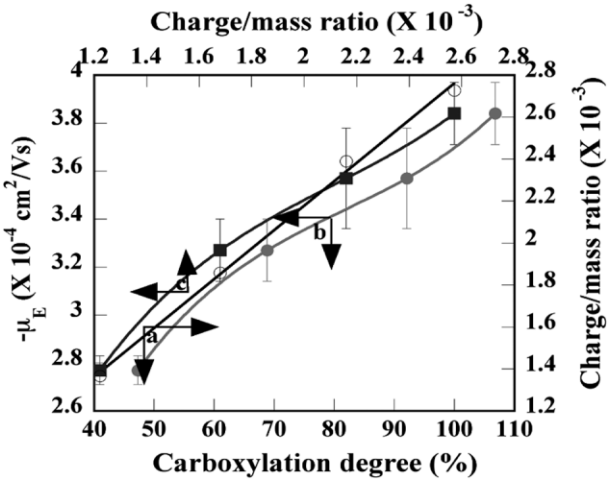



**Figure 5.** Measured charge/mass ratios of carboxyl surface substituted E5 PAMAM dendrimers as a function of the degree of carboxylation (a), the dependence of EM of carboxylated E5 PAMAMs on the degree of carboxylation (b), and the EM of carboxylated E5 PAMAMs as a function of charge/mass ratio (c). Arrows indicate the appropriate set of axis.

increased charge/mass ratio, resulting in their increased EMs (shown in Table 2). We found that the peak of partially carboxylated E5 PAMAMs is much broader than 100% carboxylated E5 dendrimer (see the CE peak width at half height in Table 2). In Table 2, both the temporal half-widths and spatial half-widths suggest that partially carboxylated E5 PAMAMs exhibit broader charge distribution than fully carboxylated E5 PAMAM dendrimer. This is consistent with the broader substitutional distribution of acetylated PAMAMs as described above (note that carboxylate groups of dendrimers do not interact with the negatively charged capillary surface). We also found that the practical charge/mass ratio *versus* carboxylation degree is linear (Fig. 5, curve a), while their EMs as a function of both carboxylation degree (Fig. 5, curve b) and charge/mass ratio (Fig. 5, curve c) are nonlinear, in an "S" shape, indicating that beyond from the charge/mass ratio, additional mechanisms are also involved in their separation. Please note that the EMs shown in Table 2 were not calculated using the normalized migrations times of carboxylated E5 PAMAMs as they were calculated for polycationic E5 PAMAMs (see Table 1). Instead, the EM reflects the average values calculated according to Eq. (2) from three individual runs.

## 4 Discussion

PAMAM dendrimers are monodispersed synthetic polymers, which have molecular similarities to proteins [29]. A variety of characterization techniques confirmed the suc-

© 2006 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

cessful synthesis of E5 PAMAM derivatives with defined surface substitutions. Generational and skeletal polydispersities do not change significantly after surface modification, as verified by SEC measurements (this technique is based on polymer hydrodynamic size distribution). However, the molecular charge distribution changes significantly after dendrimer surface modifications as confirmed by CE analysis (Figs. 2 and 4). Partially modified E5 PAMAMs have a much broader overall distribution than those of the fully modified or nonmodified E5 dendrimers due to an increase in the substitutional distribution. The reason this occurs may be because full generation, amine-terminated PAMAM dendrimers are a mixture of at least three major components; the idealized structure (>90%) and several related materials (trailing generation dendrimers and dimers, usually totaling <10%). Partially derivatized dendrimers have a secondary distribution of surface substitutions across each of these primary components, because of the random nature of this substitution reaction. Consequently, the highest charge distribution ensues when 50% of the surface functionalities of each primary component are substituted. In addition, the acetylation percentage determined by $^1$H NMR and titration studies reflects the average or apparent ratios of populations of individual PAMAM dendrimer molecules, and does not give information on individual dendrimers. The same explanation could also apply for partially modified E5 PAMAM succinamic acids. In support of this concept, similar results showing broad distribution for partially modified E2.$NH_2$ PAMAM dendrimers with 1,2-epoxyhexane have also been demonstrated by using ESI-MS analysis [30].

EM is usually linearly dependent on the charge/mass ratio of materials; however, it changes when different mechanisms are involved in the separation process. The nonlinear change of EM (convex upward shape shown in Fig. 3) of E5 PAMAM acetamides may be related to several factors influencing the separation of these molecules. One factor might be adsorption–desorption of dendrimers onto capillary surface that occurs despite silanization coating on the capillary internal quartz surface [19, 31]. We have compared the performance of both bare fused-silica and silanized-silica capillaries, and found that silanization surface significantly improved the reproducibility and stability of separation due to decreased adsorption of PAMAMs [19]. Despite this, we believe that the differential adsorption/desorption process of PAMAM polycations onto the capillary surface still could be responsible for nonlinear changes in EM. E5 PAMAM acetamides with lower degrees of acetylation (higher charge/mass ratio) display much stronger adsorption on the capillary surface than E5 PAMAM dendrimers with higher acetylation degree. This would reduce the relative EM of E5 PAMAM acetamides with higher charge/mass ratio. This explanation is supported by the analysis of PAMAM succinamic acids of different generations [20], which indicates very similar mobilities (having almost identical charge/mass ratios) because these polymers lack electrostatic interactions with the capillary surface. Counter-ion binding onto charged dendrimer spheres is another explanation for the nonlinear change of the EM of substituted dendrimers. As demonstrated by Manning's theory [32] and confirmed later by Dubin's [15, 21] studies on linear and dendrimeric polyelectrolytes using CE, counter-ion condensation around polyelectrolytes strongly influences their electrokinetic mobility. In our situation, acetylated E5 PAMAMs bearing more terminal amine groups may experience higher counter-ion binding force during electrophoresis, which also could contribute to the convex, upward nonlinear increase of EMs as a function of charge/mass ratio. Finally, dendrimer hydrodynamic size may change due to substitution. Previous work has shown that an increasing number of acetyl termini leads to the shrinkage of the dendrimer molecule [24]. We propose that larger dendrimer spheres experience resistance to migration during the electrophoresis. In contrast, highly surface acetylated E5 PAMAMs assume smaller diameters due to less charge interference between terminal amine groups and migrate more readily. Thus, we believe that a combination of these three factors may contribute to the nonlinear increase in EM of acetylated E5 PAMAMs with increased charge/mass ratios.

For E5 PAMAM succinamic acids, the same nonlinear (S shape) increase of their EM as a function of charge/mass ratio was observed. The latter two factors discussed above may also result in the nonlinear change of the EM of these polymers. It might also be expected that E5 PAMAM with high numbers of surface succinamic acids (and therefore carboxyl surface groups) have larger hydrodynamic sizes and are hindered during electrophoretic migration. However, as mentioned above, surface-carboxylated E5 PAMAM have no electrostatic interaction with the capillary surface at the running buffer of pH 8.3 [20]. This means that counter-ion binding onto charged dendrimer spheres and dendrimer hydrodynamic size may play a major role in influencing the nonlinear increase of EM of anionic-surfaced dendrimers with increasing charge/mass ratio.

In summary, we have synthesized and characterized a set of acetylated and carboxylated E5 PAMAM dendrimers with defined surface charges. These polymers were used to investigate the EM of dendrimer molecules as a function of charge and surface functionality. CE was extensively used to analyze and evaluate the effect of charge density on the EM and the molecular distributions of these

© 2006 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 

two groups of PAMAM dendrimers of the same generation synthesized from the same starting material. Partially modified dendrimers exhibit a broader peak in CE analysis than fully modified or unmodified dendrimers, and this appeared to be due to variations in surface substitution. The nonlinear EM changes of both the acetamide-substituted polycationic E5 PAMAMs and succinamic acid-substituted polyanionic E5 PAMAMs with varied charge/mass ratio indicates complex migration activity governed by multiple factors. CE studies of PAMAM dendrimers are important for characterizing dendrimers with different and/or multiple surface functionalities, especially since these molecules are proposed for use in drug delivery and other biomedical applications.

*We thank Dr. Xiangdong Bi, Dr. Rameshwer Shukla, and Maria C. Muñiz for their valuable remarks and suggestions. This work is financially supported in part by the National Cancer Institute (NCI), and National Institute of Health (NIH), under the contract # NOI-CO-97111 and by the Environmental Protection Agency, EPA Nanotechnology Award R829626 as part of the STAR program.*

## 5 References

[1] Tomalia, D. A., Baker, H., Dewald, J. R., Hall, M. *et al.*, *Polym. J.* 1985, *17*, 117–132.

[2] Tomalia, D. A., Baker, H., Dewald, J. A., Hall, M. *et al.*, *Macromolecules* 1986, *19*, 2466–2468.

[3] Tomalia, D. A., Naylor, A. M., Goddard, W. A. III., *Angew. Chem. Int. Ed. Engl.* 1990, *29*, 138–175.

[4] Radko, S. P., Chrambach, A., *J. Chromatogr. B* 1999, *722*, 1–10.

[5] Linhardt, R. J., Toida, T., *Science* 2002, *298*, 1441–1442.

[6] Huff, B. V., McIntire, G. L., *J. Microcol. Sep.* 1994, *6*, 591–594.

[7] Jones, H. K., Ballou, N., *Anal. Chem.* 1990, *62*, 2484–2490.

[8] Petersen, S. L., Ballou, N. E., *Anal. Chem.* 1992, *64*, 1676–1681.

[9] McCormick, R. M., *J. Liq. Chromatogr.* 1991, *14*, 939–952.

[10] Wang, Y., Harmer, M., Herron, N., *Israel J. Chem.* 1993, *33*, 31–39.

[11] (a) Pfetsch, A., Welsch, T., *Fresenius' J. Anal. Chem.* 1997, *359*, 198–201; (b) Kremser, L., Blaas, D., Kenndler, E., *Electrophoresis* 2004, *25*, 2282–2291.

[12] Baygents, J. C., Glynn, J. R., Jr., Albinger, O., Biesemeyer, B. K. *et al.*, *Environ. Sci. Technol.* 1998, *32*, 1596–1603.

[13] Radko, S. P., Stastna, M., Chrambach, A., *Anal. Chem.* 2000, *72*, 5955–5960.

[14] Duffy, C. F., Gafoor, S., Richards, D. P., Admadzadeh, H. *et al.*, *Anal. Chem.* 2001, *73*, 1855–1861.

[15] (a) Huang, Q. R., Dubin, P. L., Moorefield, C. N., Newkome, G. R., *J. Phys. Chem. B* 2000, *104*, 898–904. (b) Seyrek, E., Dubin, P. L., Newkome, G. R., *J. Phys. Chem. B* 2004, *>108*, 10168–10171.

[16] Welch, C. F., Hoagland, D. A., *Langmuir* 2003, *19*, 1082–1088.

[17] Brothers, H. M. II, Piehler, L. T., Tomalia, D. A., *J. Chromatogr. A* 1998, *814*, 233–246.

[18] Ebber, A., Vaher, M., Peterson, J., Lopp, M., *J. Chromatogr. A* 2002, *949*, 351–358.

[19] Shi, X., Bányai, I., Lesniak, W., Islam, M. T. *et al.*, *Electrophoresis* 2005, *26*, 2949–2959.

[20] Shi, X., Patri, A. K., Lesniak, W., Islam, M. T. *et al.*, *Electrophoresis* 2005, *26*, 2960–2967.

[21] Gao, J. Y., Dubin, P. L., Sato, T., Morishima, Y., *J. Chromatogr. A* 1997, *766*, 233–236.

[22] Quintana, A., Raczka, E., Piehler, L., Lee, I. *et al.*, *Pharm. Res.* 2002, *19*, 1310–1316.

[23] Patri, A. K., Majoros, I., Baker, J. R., *Curr. Opin. Chem. Biol.* 2002, *6*, 466–471.

[24] Majoros, I. J., Keszler, B., Woehler, S., Bull, T., Baker, J. R. Jr., *Macromolecules* 2003, *36*, 5526–5529.

[25] Cakara, D., Kleimann, J., Borkovec, M., *Macromolecules* 2003, *36*, 4201–4207.

[26] Sharma, A., Mohanty, D. K., Desai, A., Ali, R., *Electrophoresis* 2003, *24*, 2733–2739.

[27] Zhang, C., Tomalia, D. A., in: Fréchet, J. M. J., Tomalia, D. A. (Eds.), *Dendrimers and Other Dendritic Polymers*, John Wiley & Sons, New York 2001, pp. 239–253.

[28] Huang, X., Coleman, W. F., Zare, R. N., *J. Chromatogr.* 1989, *480*, 95–110.

[29] Esfand, R., Tomalia, D. A., *Drug Discov. Today* 2001, *6*, 427–436.

[30] Zhang, C., O'Brien, S., Balogh, L., *J. Phys. Chem. B* 2002, *106*, 10316–10321.

[31] Betley, T. A., Holl, M. M. B., Orr, B. G., Swanson, D. R. *et al.*, *Langmuir* 2001, *17*, 2768–2773.

[32] Manning, G. S., *J. Chem. Phys.* 1969, *51*, 924–933.

© 2006 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim 