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Received June 26, 2007
Revised July 19, 2007
Accepted July 19, 2007

Research Article

CE of poly(amidoamine) succinamic acid dendrimers using a poly(vinyl alcohol)-coated capillary

Various generations (G1–G8) of negatively charged poly(amidoamine) (PAMAM) succinamic acid dendrimers (PAMAM-SAH) were analyzed by CE using a poly(vinyl alcohol)-coated capillary. Due to its excellent stability and osmotic flow-shielding effect, highly reproducible migration times were achieved for all generations of dendrimer (*e.g.*, RSD for the migration times of G5 dendrimer was 0.6%). We also observed a reverse trend in migration times for the PAMAM-SAH dendrimers (*i.e.*, higher generations migrated faster than lower generation dendrimers) compared to amine-terminated PAMAM dendrimers reported in the literature. This reversal in migration times was attributed to the difference in counterion binding around these negatively charged dendrimers. This reverse trend allowed a generational separation for lower generation (G1–G3) dendrimers. However, a sufficient resolution for the migration peaks of higher generations (G4–G5) in a mixture could not be achieved. This could be due to their nearly identical charge/mass ratio and dense molecular conformations. In addition, we show that dye-functionalized PAMAM-SAH dendrimers can also be analyzed with high reproducibility using this method.

Keywords:
CE / Poly(amidoamine) succinamic acid dendrimers / Poly(vinyl alcohol)-coated capillary
DOI 10.1002/elps.200700454

1 Introduction

Poly(amidoamine) (PAMAM) dendrimers have generated immense interest, both scientifically and technologically, owing to their unique structural properties. These synthetic macromolecules display a high degree of molecular uniformity, narrow molecular weight distribution, well-defined structure, and a tailored surface chemistry [1, 2]. Because of these properties, there is growing application of PAMAM dendrimers in the nanomedicine field [3–10]. For example, PAMAM dendrimers have been covalently conjugated with targeting, imaging, and drug molecules for their subsequent use in imaging, and targeted drug delivery to tumor cells [3, 6, 7, 9].

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Abbreviations: 6T, 6-carboxytetramethylrhodamine succinimidyl-ester; **EM,** electrophoretic mobility; **PAMAM-SAH,** poly(amidoamine) succinamic acid dendrimers; **PVA,** poly(vinyl alcohol)

PAMAM-succinamic acid (PAMAM-SAH) dendrimers are a new class of polyanionic dendrimers derived from a reaction of succinic anhydride with amine-terminated PAMAM counterparts (Fig. 1). The synthesis of these dendrimers is relatively simple and usually offers a less polydisperse sample when compared to the PAMAM "half generation" (carboxylate) dendrimers. These PAMAM-SAH dendrimers have been previously used in the synthesis of tecto-dendrimers [11]. More importantly, the synthesis of multifunctional dendrimer-based nanodevices often requires a partial carboxylation reaction with succinic anhydride to facilitate further conjugation of drug molecules [3] or peptides [12] for medical applications. Therefore, appropriate characterization of PAMAM-SAH dendrimers is crucial in the quality control of the final dendrimer-based biomedical nanodevices.

As efforts are being directed to synthesize a variety of dendritic structures, characterization becomes an important factor for checking the uniformity of these novel dendritic structures. Dendrimers have been analyzed using a range of different analytical methods, such as potentiometric titrations [13–15], dynamic light scattering [16], size-exclusion chromatography (SEC) [3, 4], HPLC [8, 17, 18], mass spectrometric methods (MALDI-MS, ESI-MS) [19, 20], and PAGE [21–23].

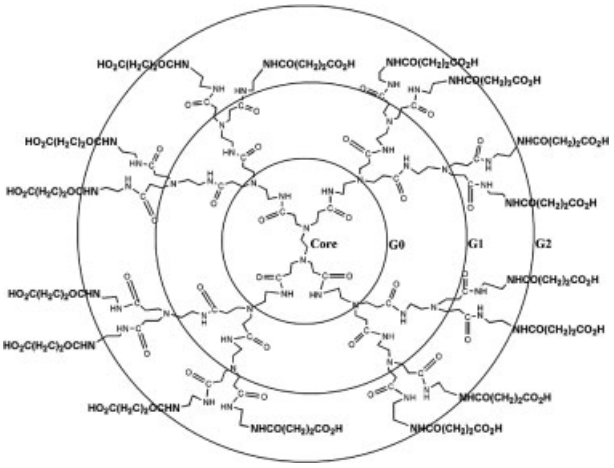

Figure 1. Schematic representation of the molecular structure of G2.SAH.

CE is one such powerful bioanalytical technique and has been successfully applied to characterize biomolecules like DNA [24] and proteins [25]. Due to its high efficiency, high sensitivity, automation, and fairly short run times, CE is being routinely used as a quality control tool in the characterization of dendrimers [14, 15, 23, 26–36]. The separation principle is mainly based on the difference in the charge/mass ratios. Other factors such as the hydrodynamic radius, counter ion binding, and the adsorption/desorption of the samples onto the surface of the capillary also influences the migration of the samples under study. It was previously reported that separation of polycationic PAMAM dendrimers using a bare fused-silica capillary lacks reproducibility because of the strong interaction between the dendrimers and the capillary surface [30, 34]. In order to improve the separation reproducibility, the surface of the capillary was modified through silanization [26–29], dynamic polymer coating [35], or permanent polymer coating [31] to decrease the interaction of the polycationic dendrimers with the capillary surface. A recent report shows that separation of polycationic PAMAM dendrimers using a poly(vinyl alcohol) (PVA)-coated capillary can achieve excellent reproducibility [31].

As opposed to polycationic dendrimers, negatively charged PAMAM-SAH dendrimers do not adsorb onto the bare silica capillary surface, and thus the migration of these dendrimers is largely dependent on the EOF and the charge/mass ratio [23, 26]. Due to the relatively similar charge/mass ratio for PAMAM-SAH dendrimers of different generations, separation of a mixed dendrimer sample with different generations was not possible [23].

In the present study, we investigated the separation of polyanionic dendrimers on a PVA-coated capillary. Our main objectives were (i) to achieve better reproducibility in the analysis of PAMAM-SAH dendrimers and (ii) to check whether counter-ion binding plays a significant role in achieving a generational separation (*i.e.*, better resolution) for these polyanionic PAMAM dendrimers when separated on a permanently coated (neutral surface) capillary. To the best of our knowledge, this is the first effort to reproducibly characterize this class of polyanionic PAMAM dendrimers using a permanently coated capillary.

2 Materials and methods

2.1 Materials

A PVA-coated capillary (id: 50 μm, total length: 64.5 cm, and effective length: 56 cm), 50 mM phosphate buffer (pH 7.0), phosphoric acid solution, water, polypropylene vials, and polyurethane snap caps were purchased from Agilent Technologies (Waldbronn, Germany). DMSO, succinic anhydride, and all the other chemicals and solvents were obtained from Aldrich (Milwaukee, WI) and were used as received. The 6-carboxytetramethylrhodamine succinimidylester (6T) was purchased from Molecular Probes (Eugene, OR). Water used in all the other experiments was purified by a Milli-Q 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 (Fairlawn, NJ).

Amine-terminated PAMAM dendrimers with different generations were obtained from Dendritech (Midland, MI). Polyanionic PAMAM-SAH dendrimers were synthesized and characterized according to our previous literature procedures [23]. 6T-labeled G5 dendrimers with succinamic acid surfaces were prepared according to a reported procedure with slight modification [37]. Instead of a final acetylation of the remaining amines of G5 dendrimers, a carboxylation using succinic anhydride was performed to convert the remaining amines of the G5 dendrimers to the succinamic acid groups, according to our previous report [6, 23]. Characterization of the product by ^{1}H NMR and UV–Vis spectroscopy showed the number of 6T molecules *per* dendrimer to be three.

2.2 Methods

CE was performed on an Agilent's CE system. The capillary temperature was maintained at 20°C, and the separation voltage was kept at −20 kV for all the measurements. On-capillary UV diode-array detection 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 4 s. Dendrimer samples were dissolved into phosphate buffer (50 mM, pH 7.0) with a concentration of 1 mg/mL. Prior to injection of the sample, the capillary was sequentially preconditioned by flushing it with phosphoric acid (0.1 M) for 10 min, followed by washing with water for 3 min and then further conditioning by the running buffer (phosphate buffer, 50 mM, pH 7.0) for 15 min. Between each injection, the conditioning steps were

repeated. At the end, the capillary was flushed with water and air for storage. The apparent electrophoretic mobility (EM) was calculated according to the following equation

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

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

3 Results and discussion

Since the use of a PVA-coated capillary achieved a reproducible separation for polycationic PAMAMs [31], we attempted to characterize polyanionic dendrimers using CE on a similar PVA-coated capillary. Figure 2a shows the separation of a generation 5 PAMAM-SAH dendrimer (G5.SAH). Under the conditions employed, the migration times obtained for the sample were very reproducible, with a mean migration time of 9.85 min and an SD of 0.056 min ($n = 6$). Between-day reproducibility (Fig. 2b) for G5.SAH was checked occasionally for over 3 months and the RSD was less than 0.6%.

We also analyzed various generations (G1–G8) of polyanionic PAMAM-SAH dendrimers under similar conditions (Table 1). Each generation was run at least five times to check for reproducibility in the migration times. As seen in Fig. 3, lower generation (G1–G3) polyanionic dendrimers showed multiple peaks. This could be attributed to imperfections in the dendrimer architecture (starting material) since the commercial amine-terminated lower generation dendrimers (below G3) are not subjected to purification by ultrafiltration, thereby containing generational, skeletal, substitutional diversities, and isomes [14]. These impurities or imperfections could have eventually been converted to succinamic acid derivatives and is separated under an electric field because of the relatively significant differences in their charge/mass ratios. For G4 and higher generations PAMAM-SAHs, it seems that the method does not have enough selectivity/efficiency to resolve the dispersity of the dendrimers, especially for the impurities arising from substitutional diversity. The broad peaks indicate that there might be a dispersity hidden beneath the peaks. It might be possible to achieve better chromatographic/electrophoretic selectivity for higher generation dendrimers that are synthesized using convergent synthetic approach, which is different from the divergent approach used to synthesize commercially available PAMAM dendrimers used in this work. The impurities arising from the convergent approach may have a more pronounced structural and physicochemical difference and may be easier to be separated.

A remarkably interesting point as shown in Fig. 3 and Table 1 is that the migration times of PAMAM-SAH dendrimers decreases with the number of the dendrimer generation, which is opposed to the separation profiles of polycationic PAMAM dendrimers in other studies [28, 31]. These experiments were repeated and, on each trial, highly reproducible results were obtained (*e.g.*, the SDs of migration times for all generations were less than 0.23 min (Table 1)). Consequently, the apparent EM of PAMAM-SAHs increases with the number of dendrimer generations (Table 1).

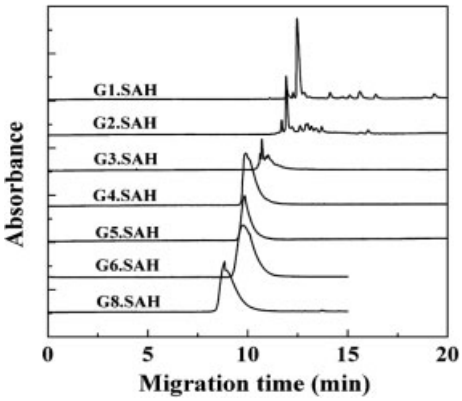

Figure 3. Capillary electropherograms of PAMAM-SAHs of generation 1, 2, 3, 4, 5, 6, and 8 analyzed using a PVA-coated capillary (id: 50 μm, total length: 64.5 cm, and effective length 56 cm). Injection time: 4 s.

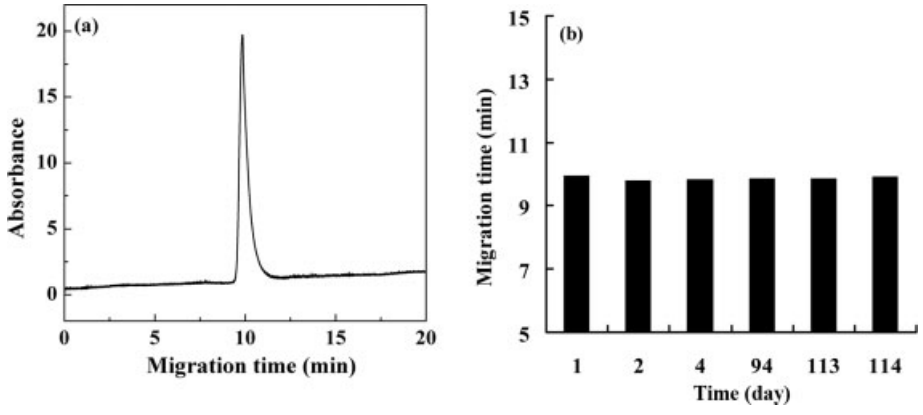

Figure 2. (a) Capillary electropherogram of polyanionic G5 PAMAM-SAH dendrimer analyzed using a PVA-coated capillary (id: 50 μm, total length: 64.5 cm, and effective length 56 cm). Injection time: 4 s. (b) The change of migration time of G5.SAH dendrimers as a function of run time shows the remarkable reproducibility of the analysis using a PVA-coated capillary.

Table 1. Physicochemical parameters of PAMAM-SAHs of different generations

PAMAM	G1.SAH	G2.SAH	G3.SAH	G4.SAH	G5.SAH	G6.SAH	G8.SAH
Mw[a)]	2230	4856	10 109	20 615	41 626	83 648	3 35 783
Mn[b)]	2063	4602	9790	19 000	38 960	70 321	3 97 500
Mw[b)]	2208	4653	10 000	20 300	40 710	84 432	4 03 400
Polydispersity[b)]	1.070	1.011	1.022	1.071	1.045	1.201	1.015
Theoretical no. of end groups[a)]	8	16	32	64	128	256	1024
Practical no. of end groups[c)]	7.1	15.3	30.6	58.8	113.2	226.6	998.6
Charge/mass (mol/g)[a,d)]	−3.59E-3	−3.30E-3	−3.18E-3	−3.11E-3	−3.08E-3	−3.06E-3	−3.05E-3
Charge/mass (mol/g)[b–d)]	−3.44E-3	−3.32E-3	−3.12E-3	−3.09E-3	−2.90E-3	−3.22E-3	−2.51E-3
Mean migration time (min)[e)]	12.47 ± 0.12	11.93 ± 0.12	10.71 ± 0.23	9.92 ± 0.13	9.85 ± 0.06	9.76 ± 0.04	8.69 ± 0.16
EM ($10^{-4} \times cm^2/V \cdot s$)[e)]	−(2. 41 ± 0.03)	−(2. 52 ± 0.03)	−(2. 81 ± 0.01)	−(3. 03 ± 0.02)	−(3. 05 ± 0.04)	−(3.09 ± 0.03)	−(3.47 ± 0.12)

a) Theoretical molecular weight assuming complete conversions.
b) As measured by SEC.
c) Measured by potentiometric titration.
d) The charge/mass ratio assumes that all dendrimer terminal carboxyl groups are deprotonated.
e) As measured by CE (n = 3–5).

Carboxyl-terminated dendrimers often experience a counterion binding effect when subjected to separation under an electric field [26, 32, 33]. The counterion binding effect, in certain circumstances, could play an important role in the separation of dendrimers. For instance, using a bare silica capillary and a positive polarity condition, the EM of a G5 PAMAM dendrimer with different carboxylation degrees does not display a linear relationship as a function of the charge/mass ratio [26]. Dubin and co-workers [32] studied the effects of pH and ionic strength on the mobility of Newkome's carboxyl-terminated dendrimers using CE. They observed that because of counterion binding, the effective charge density on the dendrimer surface is different from the geometrical charge density. When compared with a bare silica capillary, a PVA-coated capillary with a permanent coating of hydrophilic polymer on its surface can completely shield the effects of EOF. Therefore, the migration of the PAMAM-SAH dendrimer samples is dependent on both their charge/mass ratios and the effects of counterion binding. According to a previous study [23], PAMAM-SAH dendrimers display a relatively similar charge/mass ratio, regardless of the change of the number of dendrimer generations (Table 1). Therefore, they show relatively similar migration times under a bare silica capillary. The reverse trend of the migration times of PAMAM-SAH dendrimers under a PVA-coated capillary suggests that counterion binding plays a major effect during the electrophoretic separation. Smaller generations (G1–G3) exhibit a relatively open molecular structure and behave as a linear polymer, thereby receiving a higher inhibition force because of the counterion binding effect; while high generations (G4–G10) exhibit a densely close globular structure and behave as a colloidal sphere, thus receiving a smaller inhibition force.

The differences in the counterion binding effect for the separation of PAMAM-SAH dendrimers with different generations allows the separation of mixed generations for small generations of PAMAM-SAH dendrimers (Fig. 4a), which are impossible to be separated using a bare silica capillary [23]. Because of their dense spherical molecular conformation, close migration times, and broadness of the eluting peak, it is hard for higher generation dendrimers (G4 and G5) to be separated under these conditions (Fig. 4b). It should also be noted that under the separation buffer condition (pH 7.0), it is likely that lower generation dendrimers could have a different pI, compared to higher generation dendrimers, thus the percentage of ionization for various generations at pH 7.0 could be slightly different. This could affect the overall charge/mass ratio of the dendrimer from generation to generation. This change in charge/mass ratio is larger between lower generations, compared to higher generations (G4 and G5), resulting in a better resolution (generational separation) for lower generation dendrimers (Table 1).

Dye-functionalized dendrimers offer many opportunities for imaging purposes [37]. The purity and homogeneity of the dye-functionalized PAMAM dendrimers are important for their biological performance [29]. The PVA-coated capillary also allows for analysis of functionalized PAMAM-SAH dendrimers. Figure 5 shows the electropherograms of G5.SAH-6T and G3.SAH-6T dendrimers in comparison with those without 6T conjugation, respectively. Both G5.SAH-6T and G3.SAH-6T dendrimers display a decreased EM when compared with the G5.SAH and G3.SAH dendrimers, respectively. This is presumably due to the increased mass and decreased number of carboxyl groups after the conjugation modification, which results in a decreased charge/mass ratio. The analysis of both G5.SAH-6T and G3.SAH-6T dendrimers is also very reproducible with migration times of 10.03 ± 0.084 and 11.71 ± 0.14 min, respectively (n = 3). The broad peaks of G5.SAH-6T and G3.SAH-6T in the electropherograms also suggest that there might be some substitutional dispersity hidden beneath the peaks, which are not capable to be resolved using this technique. This is

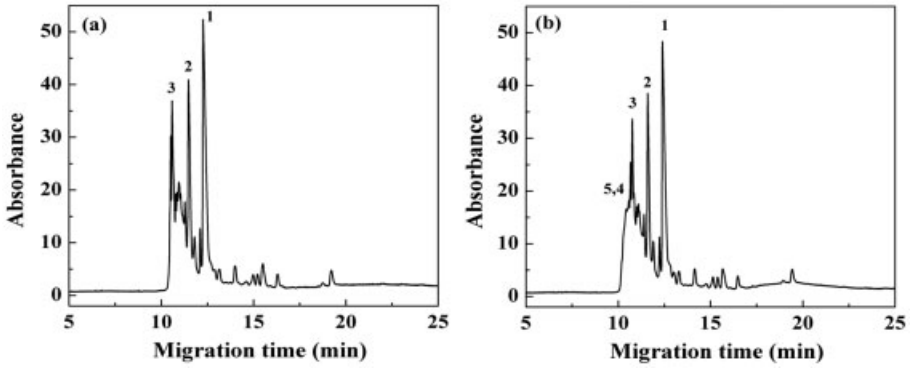

Figure 4. A typical capillary electropherogram of the mixture of PAMAM-SAH dendrimers of (a) generations 1–3 and (b) generations 1–5 using a PVA-coated capillary (id: 50 μm, total length: 64.5 cm, and effective length: 70 cm). Injection time: 4 s. The concentration of each generation is 1 mg/mL.

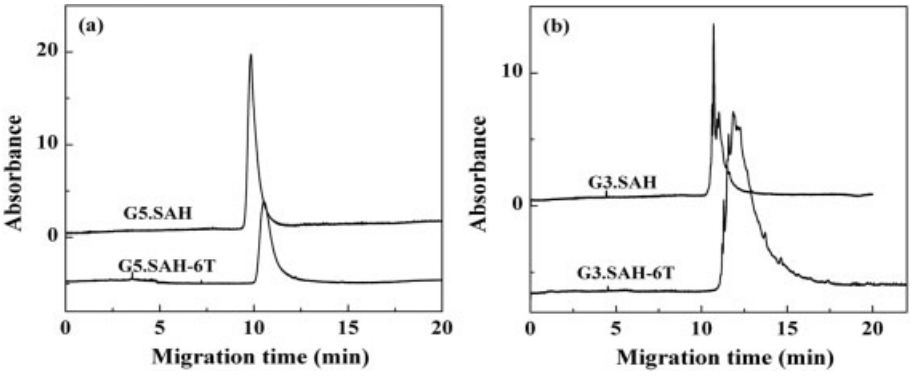

Figure 5. Capillary electropherograms of (a) G5.SAH and G5.SAH-6T and (b) G3.SAH and G3.SAH-6T analyzed using a PVA-coated capillary (id: 50 μm, total length: 64.5 cm, and effective length: 56 cm). Injection time: 4 s.

similar to the separation of higher generation PAMAM-SAH dendrimers without dye conjugation as mentioned above. The results obtained suggest that the synthetic procedures carried out are reproducible as evidenced from reproducible electropherograms.

4 Concluding remarks

In summary, we have demonstrated that a PVA-coated capillary can be utilized to characterize polyanionic PAMAM dendrimers with very high reproducibility. We show that under the conditions employed, counterion binding plays a major role in influencing the migration behavior of PAMAM-SAH dendrimers. The differences in the counterion binding for different generation dendrimers allow for the separation of lower generation dendrimers (G1–G3). This method can also be used for the analysis of dye-functionalized PAMAM-SAH dendrimers. Currently, we are analyzing various polycationic and polyanionic PAMAM dendrimer-based multifunctional nanodevices for various biological applications. The developed method should provide a truly reproducible approach for quality control of these nanodevices.

This work has been funded in whole or in part by the Michigan Economic Development Corporation-Life Sciences Corridor Fund, under award GR-472, as well as with Federal funds from the National Cancer Institute, National Institutes of Health, under award 1 R01 CA119409.

The authors have declared no conflict of interest.

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