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Separation of poly(amidoamine) (PAMAM) dendrimer generations by dynamic coating capillary electrophoresis ☆

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Abstract

The separation of compounds possessing amino groups (peptides, proteins, polyamino compounds) by capillary zone electrophoresis suffers from the interaction (sticking) of these solutes with the capillary wall. This sticking can result in the absence or incomplete separation of compounds or even in their retention in the capillary. Polyamidoamine (PAMAM) dendrimers are a class of spherical polymers with primary amino groups at the surface. These compounds can be separated reasonably well at acidic pH but not at neutral pH. A new method based on the dynamic coating of the capillary was developed for the separation of these compounds at pH 7.4. The method comprises separation in a fused-silica capillary (57 cm total length, 50 cm to the detector, ID 75 μm) and a background electrolyte consisting of a Tris-phosphate buffer (50 mmol/L, pH 7.4) and 0.05% (w/v) polyethyleneimine. This system is suitable for the separation of 7 generations of dendrimers (generations 0–6). The dynamic coating agent (polyethyleneimine) also improves the separation at acid pH.

Keywords: PAMAM dendrimers; Dynamic coating; Capillary wall; Capillary electrophoresis

1. Introduction

The separation of amino-containing compounds by capillary zone electrophoresis, such as peptides and proteins, is complicated due to their sticking to the capillary wall. This sticking is caused by adsorption of the charged sites of the proteins to fixed, negatively-charged sites (silanol groups) on the capillary wall, by electrostatic interaction of the proteins with the wall and by hydrophobic forces. There are three main methods to minimize this sticking: (i) the use of acidic or basic pH (high or low pH); (ii) a permanent capillary coating; and (iii) a dynamic (and non-covalent) coating [1,2]. Generally speaking it is possible to say that any compound possessing amino groups (or another positively-charged compound) can stick to the silica capillary.

Dendrimers are synthetic, highly branched, nearly spherical and symmetrical macromolecules with well-defined sizes and compositions. The name dendrimer derives from "δενδρον" ("dendron"), which in the Greek language means tree. These polymers are unique, "spherical macromolecules", whose molecular architecture consists of a core and repeating units with branching and terminal groups. Each repeating unit contains a branching point to which two or several new repeating units are attached. Dendrimers most often used in analytical chemistry in the separation science are PAMAM (polyamidoamine macromolecules). These dendrimers have amine surfaces and EDA (ethylenediamine) cores. I. There are also another kinds of dendrimers with other functional groups on the surface like carboxyls, hydroxyls and acetamides [3].

Regarding the construction of PAMAM dendrimers we can briefly summarize (for details see some specialized article, e.g. [3–6]) that it starts with the Michael addition of methyl acrylate to EDA. The result of this reaction is a tetraester molecule – the so-called generation −0.5 (G-0.5). After reaction with an excess of EDA (amidation), the dendrimers belong to generation

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0 (G0) with a nucleophilic surface. Repetition of these reactions leads to the higher dendrimer generations. The molecular mass and number of terminal groups of dendrimers rapidly increase with the number of generations. The number of terminal groups for the full generation N (GN) of EDA-core PAMAM dendrimers is given by the formula 4×2^{GN} (4 = number of repeating units linked to the core, 2 = multiplicity of the branching). Half-generations refer to dendrimers with ester functional groups on their surface [7,8].

An interesting application of dendrimers is their use as vectors for gene transfer into eukaryotic cells [9,10]. Dendrimers can also be used as a pseudostationary phase for micellar electrokinetic chromatography [8,11]. Stathakis et al. [12] demonstrated the usability of various generations, G0 (cationic), 0.5, 1.5, 2.5 and 3.5 (anionic), for the separation of proteins. Regrettably, the separation efficiencies were quite low.

There are a few papers describing the separation of the various generations of PAMAM dendrimers with an EDA core by capillary electrophoresis (for a review, see [8,13]).

Brothers II et al. [14] separated dendrimers in an uncoated fused-silica capillary. Full generations were separated at pH 2.7 (100 mmol/L phosphate buffer) and half-generations were separated at pH 8.2 (borate buffer). These methods allow the separation of ammonia-core PAMAM dendrimers (generations 0–5) and show the instability of EDA-core PAMAM dendrimer half-generations. A similar method was used by Ebber et al. [7] for the separation of PAMAM dendrimers with a EDA core in an uncoated fused-silica capillary. Full generations were separated at pH 2.7 (100 mmol/L phosphate buffer) and half-generations at pH 7.8 (20 mmol/L phosphate buffer). This method allows the separation of generations 0 through 5 and again showed the hydrolysis of half-generations.

Shi et al. [15] demonstrated the possibility of reproducibly separating EDA-core PAMAM dendrimers in uncoated and silanized silica capillaries. The reproducibility was poor with uncoated capillaries. The separation was performed at pH 2.5 (50 mmol/L) and enables the separation of generations 2 through 5.

The same group [16] demonstrated the separation of EDA-core PAMAM succinamic acid dendrimers (generation 2 to 7) and revealed that the electrophoretic mobility of the individual generations of PAMAM polyanions was similar. This indicates that the separation mainly depends on their approximately identical charge/mass ratio. The separation was made in an uncoated fused-silica capillary at pH 8.3 (20 mmol/L borate buffer).

Slab-gel electrophoretic methods for the characterization of PAMAM dendrimers have also been described [14,17,18].

The principal objective of the work presented here was to develop a method for the separation of different generations of PAMAM dendrimers (from generation 0 to 6) at their physiological pH (7.4) by dynamic coating capillary electrophoresis. Indeed, most of the potential applications of these compounds are biological and their analysis at conditions that simulate the body's environment may be required. Furthermore, it was also our aim to evaluate the effect of dynamic coating when an acidic pH (2.5) was used in the separation.

2. Materials and methods

2.1. Capillary electrophoresis

All runs were performed on a Beckman P/ACE system 5500 (Beckman, Fullerton, CA, USA). A bare fused-silica capillary of 57 cm total length (50 cm to the detector) with an ID of 75 μm was used. Detection was performed by UV absorbance recording at 214 nm. The sample was injected electrokinetically (2 s, 10 kV). The concentration of dendrimers was approx. 1 mg/mL (except for G0 – 2 mg/mL, and G4 and G6 – 0.5 mg/mL) after dilution of the original aqueous solution delivered by the manufacturer (ca. 160-fold with water). The separations were run at 20 °C with an applied voltage of 20 kV. Immediately before the analysis (every day) the capillary was washed step-wise with water (5 min), 1 mol/L HCl (5 min), water (5 min) and the background electrolyte (5 min).

Phosphate buffers (0.05 mol/L or 0.1 mol/L) at pH 2.5 or pH 7.4 (pH was adjusted with 3 mol/L HCl or 1 mol/L NaOH) and Tris-phosphate buffers (0.05 mol/L or 0.1 mol/L) at pH 2.5 or pH 7.4 (pH was adjusted with H_3PO_4) were used. Before analysis, the background electrolyte was filtered using a 45-μm Millex-HV filter (Millipore, Bedford, MA, USA). A number of modifiers were tested (percentages are expressed in w/v percentages): hexadecyltrimethylammonium bromide (0.01%, 0.025%, 0.05%, 0.1%, 0.2%), ethylenediamine (0.025%, 0.05%, 0.1%) and polyethylenimine (0.025%, 0.05%, 0.1%). The phosphate buffer and Tris-phosphate buffer were finally modified by 0.05% PEI (polyethyleneimine) (selected as the best modifier).

All generations of dendrimers were identified using the spiking method but individual generations were also analysed separately.

2.2. Chemicals

PAMAM dendrimers (EDA cores) of different generations (G0–G6) were purchased from Dendritech (Midland, MI, USA). Sodium dihydrogen phosphate, hydrochloric acid and phosphoric acid were produced by Lachema (Brno, Czech Republic) and were of p.a. quality. Tris-phosphate and the modifiers (hexadecyltrimethylammonium bromide, ethylendiamine and polyethylenimine) were produced by Sigma (St. Louis, MO, USA). All solutions were prepared in Milli-Q water (Millipore) and filtered through a 45-μm Millex-HV filter (Millipore). Mesityloxide (Sigma) was used as the neutral marker of electroosmotic flow.

2.3. Calculations of resolution, number of theoretical plates and electrophoretic mobility

The resolution R was calculated using the equation $R = 2\,\Delta t\,(W_1 + W_2)^{-1}$, where Δt is the difference in migration times of the two solutes involved and W_1 and W_2 are the peak widths at the baseline.

The number of theoretical plates N was calculated according equation $N = 5.54\,(t_m/W_h)^2$, where t_m is the migration time and W_h is the peak width at the half height of the peak.

The electrophoretic mobility μ_{ep} was calculated according equation $\mu_{ep} = \mu_{app} - \mu_{eof}$. Where μ_{app} is apparent mobility of the analyte and μ_{eof} is electroosmotic flow mobility. Mobilities were calculated as: $\mu = (L_d/t_m)/(V/L_t)$, where L_d is length of the capillary from the inlet to the detector, t_m is migration time, V is applied voltage, and L_t is total length of the capillary.

3. Results and discussion

The dendrimers studied (PAMAM dendrimers with an EDA core) are compounds with a high number of charged amino groups at the surface. An increase in generation corresponds to an increase in molecular mass and in the number of primary amino groups. Generation 0 has a molecular mass of only 517 and 4 primary amino groups while generation 6 has a molecular mass of 58,048 and 256 primary amino groups (these numbers are theoretical only) So it is not surprising that the positively-charged PAMAM dendrimers strongly bind to the silica surface of the capillary.

The separation of highly positively-charged compounds (with a high number of amino groups on the surface) at approximately neutral pH is highly problematic. As was mentioned earlier, separation can be prevented at this pH due to adsorption to the capillary wall, but this effect can be suppressed by dynamic coating. We examined the activity of some amine-derived compounds selected on the basis of our previous studies [2] (percentages are expressed as w/v percentages): hexadecyltrimethylammonium bromide (0.01%, 0.025%, 0.05%, 0.1%, 0.2%), ethylenediamine (0.025%, 0.05%, 0.1%) and polyethylenimine (0.025%, 0.05%, 0.1%). A good example of the influence of the modifiers tested on the separation of the different generations of PAMAM dendrimers is presented in Fig. 1. In this case, it can be underlined that the influence of ethylenediamine, which is a core compound of dendrimers, was also studied. It was observable that the lowest generations (0, 1) can be successfully separated with a good efficiency but the resolution of higher generations is unsatisfactory.

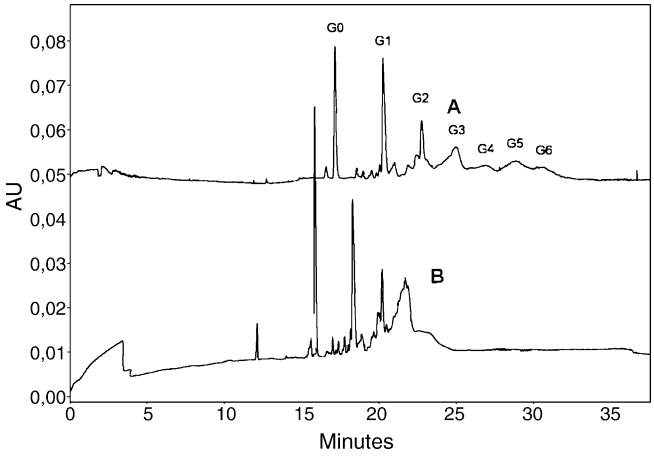

Fig. 1. Influence of background modifier on electrophoretic separation of dendrimers (G0–G6) at pH 7.4, 50 mmol/L Tris-phosphate buffer. (A) 0.05% (w/v) PEI (polyethylenimine); (B) 0.025% ethylenediamine (EDA).

Table 1
Resolution of individual dendrimer generations (in all cases, resolution values listed is with respect to previous generation)

Generation	pH 7.4 with PEI	pH 2.5 with PEI	pH 2.5 without PEI
1	6.7	6.4	9.0
2	5.3	2.9	2.0
3	2.3	1.6	1.0
4	0.8	1.1	0.8
5	0.6	0.9	0.5
6	0.3	–	–

Separations were performed with a Tris-phosphate buffer (for details see Section 2).

The best separation was obtained with the system containing polyethylenimine at a concentration of 0.05% (w/v). The resolution is excellent for the lower generations (0–2) and it was even possible to see some peaks that are probably due to by-products and incompletely formed generations, so-called "structural errors" [7] (Table 1). The numbers of theoretical plates (see Table 2) demonstrate the efficiency of the separation for low generations. The higher generations (3–6) do not give sharp peaks, a feature that reflects the structure of a large ball (with a molecular mass of approx. 7000 and higher) with a high number of primary amino groups on the surface. This high concentration of amino groups on the surface means a higher possibility for interaction with the negatively-charged capillary wall. For this reason, the requirements set on the dynamic coating are enormous. In the system we developed, it was still possible to separate up to generation 6 (molecular mass 58,048; 256 free amino groups) – but the numbers of theoretical plates were low (Table 2).

All previously published methods used acidic pH for the separation of different generations of dendrimers by capillary electrophoresis, since this prevents/minimizes the dendrimer's adsorption on the wall [14,15]. Silanization of the wall was only used to obtain a higher reproducibility for the separation [15]. Neutral or basic pH (7.8 or 8.2) were only used for studying the separation of the half-generations (neutral ester or negatively-charged carboxylate on the surface) [7,14].

For separations at acidic pH, a phosphate buffer is routinely used – for example, in the traditional separation of proteins. However, it is often not the best buffer and so we tried comparing two buffers – phosphate and Tris-phosphate (Fig. 2) – in the separation of different generations of PAMAM dendrimers at acidic pH. In the figure, the separation is visibly similar for

Table 2
Number of theoretical plates

Generation	pH 7.4 with PEI	pH 2.5 with PEI	pH 2.5 without PEI
0	106750	182665	338843
1	148684	54392	296100
2	61467	72812	38674
3	6843	48402	31629
4	3442	45014	33326
5	2095	26745	31270
6	2807		

Separations were performed with a Tris-phosphate buffer (for details see Section 2).

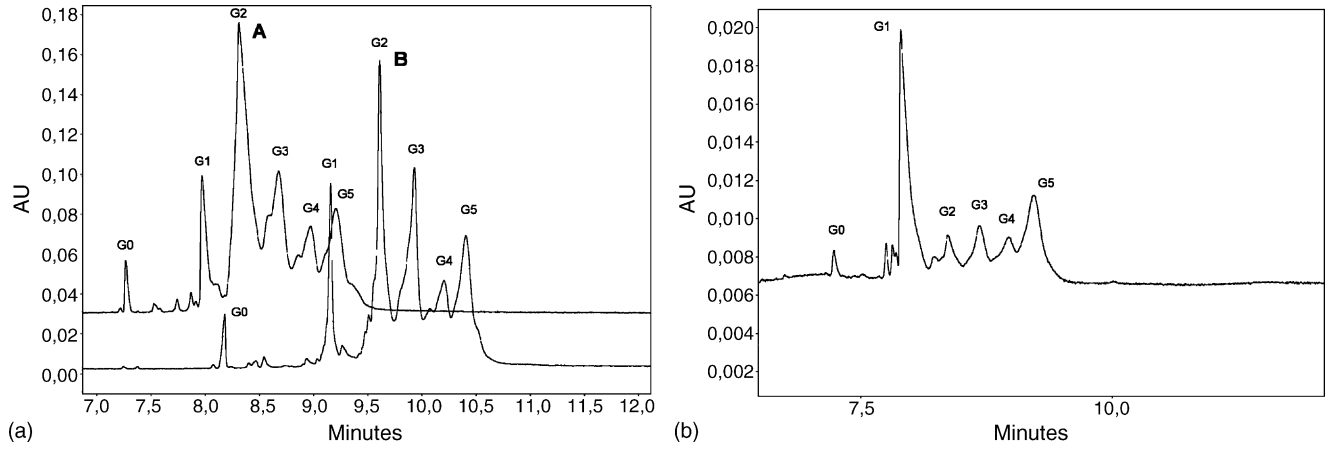

Fig. 2. Electropherogram of various generations (0–5) of dendrimers at acidic pH (2.5). Separation conditions: 20 kV, *a*: (A) 100 mmol/L phosphate buffer; (B) 100 mmol/L Tris-phosphate buffer, *b*: 100 mmol/L Tris-phosphate buffer with 0.05% (w/v) PEI (polyethylenimine).

both buffers and only a slightly better resolution was obtained with the Tris-phosphate buffer. Surprisingly, the separation is slower when the Tris-phosphate buffer is used, but is shown to be influenced by the conductivity of the buffer used and not by adsorption to the capillary wall (the current was 88 μA for the Tris-phosphate buffer and 156 μA for the phosphate buffer).

To prevent sorption to the silanol groups of the capillary wall we used a dynamic coating of polyethylenimine (PEI) that was previously selected as the best additive at a higher pH (Fig. 2). The strong influence of PEI as a dynamic capillary wall modifier was confirmed as it had a great impact on the separation/resolution of individual generations. This finding is in agreement with our previous results concerning the dynamic coating of the capillary for the separation of low-molecular mass peptides at acidic pH [2].

The resolutions of the described systems can be compared in Table 1. It is obvious that systems incorporating dynamic coating with PEI are more efficient (the only exceptions being for generation 0 and 1, which are still satisfactory). If we compare the electropherograms, it is noticeable that a better separation of the "structural errors" for generations 0–2 was obtained for the system with PEI at pH 7.4.

The electrophoretic mobilities of dendrimers are influenced by dynamic coating (as is shown at Table 3). At the acid pH the electrophoretic mobilities are higher in the system with PEI in compare with system without PEI. We suppose that dynamic coating covers capillary wall and protects it against interactions with amine analytes. In this case these changes in the electrophoretic mobilities due to the lack of interactions with silanol groups. Of course, it could be mention that elimination could not be completed and some interactions can still exist, as was mentioned by Shi et al. [15]. Because these interactions are limited, the electrophoretic mobilities are higher. At the neutral pH the separation of PAMAM dendrimers in the plain buffer only is impossible. The dynamic coating of the capillary enables to separate 7 generations of PAMAM dendrimer. From these findings we can speculate that the separation of PAMAM dendrimers in the system with dynamic modification is not based only on the interactions with silanol groups on the capillary wall and can depend on the charge density of the dendrimers' structures. As it was calculated by Shi et al. [15] these separations reflect the theoretical charge density of dendrimers, but they did not reflect their density measured by titration technique. From these published results it is possible to presume that also in the system of dynamic coating the interactions with capillary wall can influence the separation. This speculation is supported by the broading of peaks of higher dendrimer's generations.

Table 3
Electrophoretic mobilities of various dendrimers generations ($m^2\ s^{-1}\ V^{-1}$)

Generation	pH 7.4 with PEI	pH 2.5 with PEI	pH 2.5 without PEI
0	1.42×10^{-7}	3.41×10^{-7}	2.89×10^{-7}
1	1.21×10^{-7}	3.13×10^{-7}	2.58×10^{-7}
2	1.08×10^{-7}	2.96×10^{-7}	2.45×10^{-7}
3	9.89×10^{-8}	2.86×10^{-7}	2.37×10^{-7}
4	9.20×10^{-8}	2.77×10^{-7}	2.31×10^{-7}
5	8.62×10^{-8}	2.70×10^{-7}	2.27×10^{-7}
6	8.15×10^{-8}		

Presented method enables to separate compounds with high content of the amino groups using relatively simple method based on the dynamic coating of the capillary wall.

4. Conclusions

A suitable method was developed for the analysis of highly positively-charged amino compounds with high molecular mass (dendrimers). The interaction between amino groups and silanol groups was prevented by the dynamic coating of a fused-silica capillary with an amino compound (polyethylenimine) present in the background electrolyte, and so without requiring an extremely acidic pH for the separation of these compounds.

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