Proc. Estonian Acad. Sci. Chem., 2001, **50**, 3, 156–166

SYNTHESIS AND CZE ANALYSIS OF PAMAM DENDRIMERS WITH AN ETHYLENEDIAMINE CORE

Janek PETERSON[a], Arkadi EBBER[a], Veiko ALLIKMAA[a], and Margus LOPP[b]

[a] Institute of Chemistry, Tallinn Technical University, Akadeemia tee 15, 12618 Tallinn, Estonia; janek@argus.chemnet.ee
[b] Department of Chemistry, Tallinn Technical University, Ehitajate tee 5, 19086 Tallinn, Estonia; lopp@chemnet.ee

Received 4 April 2001, in revised form 26 April 2001

Abstract. Generations 0 through 5 of ethylenediamine core poly(amidoamine) dendrimers were synthesized. The half-generations of the dendrimeric compounds were purified on silica gel and/or Sephadex LH-20. Capillary zone electrophoresis was applied to characterize the homogeneity of the individual generations.

Key words: PAMAM dendrimers, capillary electrophoresis, capillary zone electrophoresis.

INTRODUCTION

Dendritic polymers are unique “ball-shape” polymeric substances, whose molecular architecture consists of an initiator core and repeating units with branching and terminal groups. Each repeating unit bears a branching point to which two or several new repeating units are attached (Fig. 1).

In the case of poly(amidoamine) (PAMAM) dendrimers the initiator core is an ammonia or ethylenediamine (EDA) molecule. Ammonia has three and EDA four possible binding sites for amidoamine repeating units. The primary amino groups are on the surface of the molecule and two new branches may be attached to each of them (Fig. 2).

Owing to their unique properties such as solubility in water, well-defined molecular architecture, and spherical shape, dendrimers have found numerous applications in chemical, physical, and biological processes [1–7].

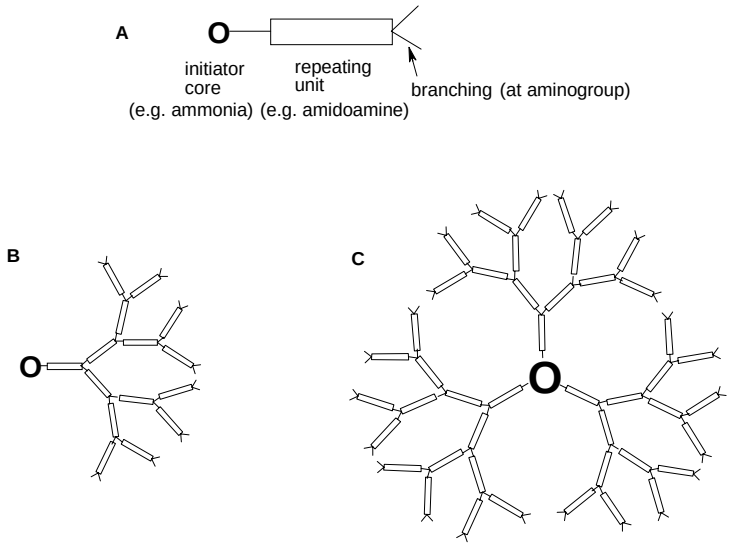

Fig. 1. Architecture of dendritic macromolecules: A – structural elements, B – dendron, C – dendrimer.

The construction of an EDA-core PAMAM dendrimer consists of two consecutive steps: Michael addition of primary amine (EDA in the very first step) to methyl acrylate followed by the amidation of the formed multiester (tetraester at the very beginning) with EDA (Fig. 2) [8, 9]. The tetraester is called PAMAM dendrimer of generation –0.5 (G–0.5) and the initial EDA itself may be considered as the generation G–1. The following amidation of the tetraester with EDA yields generation zero (G0) of the dendrimer with four terminal amino groups. The reiteration of this two-step procedure leads to higher dendrimer generations. Such a strategy of constructing dendrimers is known as the divergent synthesis method [10].

Most existing instrumental methods (e.g. IR spectroscopy, NMR, size exclusion chromatography, mass spectrometry with chemical ionization, electrospray ionization, matrix assisted laser desorption ionization, and so on) have been applied in investigations of dendrimers [8, 9, 11–15]. Capillary electrophoresis (CE) is one of the few separation techniques that can be used for separation of different generations [16], because in CE separation does not depend on the mass of the molecule, but its mass-to-charge ratio.

The aim of the present study was to synthesize the first six generations (G0 through G5) of EDA-core PAMAM dendrimers and apply capillary zone electrophoresis (CZE) for separation of different generations and for the characterization of individual generations of synthesized dendrimers.

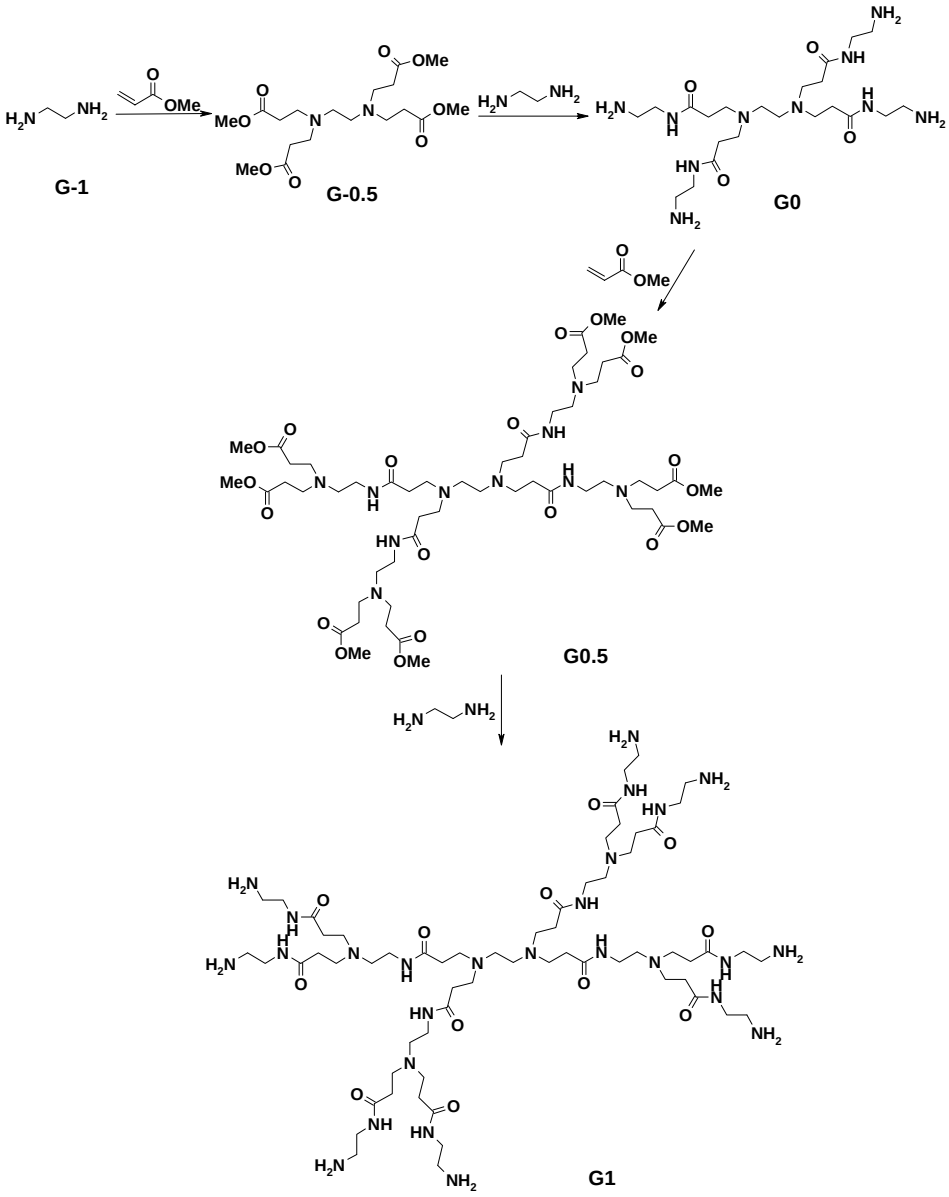

Fig. 2. Diagram of the synthesis of EDA-core PAMAM dendrimers.

EXPERIMENTAL

Synthesis of PAMAM dendrimers

Materials

EDA was purified by distillation over CaH_2. Methyl acrylate was Aldrich Chemical 99% pure material and was used as received. Solvents were Aldrich

Chemical HPLC grade. For column chromatography 5×50 cm columns packed with Sephadex LH-20 and silica gel (0.063–0.200 mm; Merck) were used.

General procedure for the synthesis of ester-terminated (half-generation) PAMAM dendrimers

Dendrimers were prepared by a divergent synthesis scheme using the reagent excess method starting from EDA by consecutive Michael addition and ester amidation reaction [7]. The full-generation dendrimer (EDA in the very first step) was dissolved in methanol, cooling the mixture in an ice bath. Then methyl acrylate was added dropwise at stirring to the dendrimer solution (under argon). A slight excess of methyl acrylate (~10%) was used in the Michael addition step. The mixture was kept at room temperature several days and at 45 °C a few days. The excess of methyl acrylate and solvent were removed under vacuum at the temperature below 50 °C resulting in a nearly colourless viscous syrup. Details of the performed syntheses are presented in Table 1.

Table 1. Details of the syntheses of half-generation PAMAM dendrimer

Generation	Amidoamine solution				Methyl acrylate, mmol	Reaction time at room temperature and at 45 °C, h	Yield, g
	Starting poly(amidoamine)			MeOH, mL			
	G	g	mmol				
– 0.5	EDA	20	333.3	1000	1360	168	130.7
0.5	G0	13.7	25.4	200	232	96 + 24	26.7
1.5	G1	38.9	27.0	200	870	96 + 24	59.5
2.5	G2	11.5	3.52	80	231	48 + 72	15.9
3.5	G3	15.5	2.25	170	288	96 + 72	24.7
4.5	G4	3.9	0.28	190	75	24 + 120	5.9

The synthesized half-generations were purified chromatographically: the generations –0.5, 0.5, and 1.5 were purified by column chromatography on silica gel and G2.5, G3.5, and G4.5 by column chromatography on Sephadex LH-20. Methanol or its mixture with dichloromethane was used as the chromatographic eluent (Table 2).

Table 2. Parameters of purification of the synthesized half-generation PAMAM dendrimers

Initial sample		Column	Eluent	Yield, g
Generation	Amount, g			
G–0.5	0.11	Silica gel	CH_2Cl_2(10) : MeOH(1)	0.10
G0.5	1.0	Silica gel	CH_2Cl_2(10) : MeOH(1)	0.7
G1.5	19	Silica gel	MeOH	10.5
G2.5	0.24	Sephadex LH-20	MeOH	0.17
G3.5	9.68	Sephadex LH-20	MeOH	4.8
G4.5	6.0	Sephadex LH-20	MeOH	4.5

General procedure for the synthesis of amino-terminated (full-generation) PAMAM dendrimers

EDA was dissolved in methanol and the solution was cooled in dry ice down to −30 °C. In another reaction vessel multiester was dissolved in methanol and the flask was also cooled down to −30 °C. The cold multiester solution was gradually added to the EDA solution at a rate keeping the temperature below −25 °C under argon. After the addition was completed the mixture was allowed to warm to room temperature and the reaction continued for several days (the details of the syntheses are presented in Table 3). The excess of EDA was distilled off as an azeotrope with *n*-butanol. As a result a pale amber-coloured syrup was obtained. The structure of synthesized PAMAM dendrimers is described in detail in [17].

Table 3. Preparation of full-generation PAMAM dendrimers

Genera-tion	Multiester solution				EDA solution		Reaction time at room tempe-rature, h	*n*-Butanol, L	Yield, g
	Multiester			MeOH, mL	EDA, mol	MeOH, mL			
	G	g	mmol						
0	– 0.5	10	25	25	2.97	100	168	1.0	14.4
1	0.5	27.2	23.5	74	9.03	345	144	1.5	41.4
2	1.5	7.5	2.7	20	2.40	80	144	1.5	12.2
3	2.5	12.8	2.1	45	3.41	130	144	1.5	17.2
4	3.5	4.8	0.4	80	3.74	120	168	1.5	6.1
5	4.5	3.04	0.12	90	2.3	266	192	1.5	3.9

Capillary zone electrophoresis

Capillary zone electrophoretic separations of synthesized dendrimers were performed on a home-made CE setup consisting of a high voltage power supply unit (International High Voltage Electronics, Inc.), a UV detector ISCO CV^4, and an uncoated fused silica capillary (Polymicro Technology, Phoenix) with an ID of 75 μm, total length of 75 cm, and length to the detection window of 50 cm. UV absorption was measured at 212 nm and the detector signal was recorded through ADC (Keithley) at a frequency of 4.2 Hz. The half-generations of dendrimers were analysed in a 0.02 M phosphate buffer (pH = 7.8). For full-generations a 0.1 M phosphate buffer (pH = 2.7) was used. The procedure of analysis was similar to that of Brothers et al. [16] used for separation of ammonia-core PAMAM dendrimers. At the beginning of each day, the capillary was rinsed with 0.1 M NaOH for 5 min and then with the run buffer. Before each injection the capillary was rinsed with Milli-Q water and buffer. For full-generations the voltage of 18 kV was applied while in the case of half-generations the voltage was 16 kV. The samples were introduced electrokinetically at the anodic end of the capillary and the direction of the electroosmotic flow was towards the grounded cathode. The

analysed samples were prepared by dissolving 1–2 mg of dendrimer in 1 mL of buffer and were run immediately after dissolving.

RESULTS AND DISCUSSION

Analysis of the complex polymeric (dendrimeric) mixtures is a complicated problem not only because of the large number of different regular structural units, but also because of numerous possible "structural errors" present in the polymeric mixture. There are at least three main types of primary side-reactions in PAMAM dendrimer synthesis. The most important of them is the incomplete Michael addition causing the appearance of unsymmetrical dendrimeric structures (e.g. structure A in Fig. 3). Another possible side-reaction is intra-molecular cyclization. This type of reaction can only occur during the formation of a full-generation of the dendrimer. The presence of many functional groups in the outer shell provides different cyclization possibilities. As a result, a wide variety of the cyclic products may be formed (structure C in Fig. 3). Beginning from the first generation, a third side-reaction, the *retro*-Michael reaction, is also possible (giving rise to the structures A, Fig. 3). In order to suppress the side reactions in the amidation step, significant excess of EDA is used. The possibility of the formation of dendrimer dimers (or oligomers) has to be considered as well (structure B in Fig. 3).

CZE is the most straightforward and simple method for separation of different generations of dendrimers and for analysing the homogeneity of the single generations. It may give also valuable information about "structural errors" in several first dendrimer generations.

In CZE separation occurs when the analyte molecules are charged and have different apparent electrophoretic mobilities. A straightforward approach assumes that the resistance experienced by the ion when flowing through the liquid medium is approximately proportional to the ion's mass (or size) and mutual separation of ions takes place according to the charge-to-mass ratio. Therefore, half- and full-generations of PAMAM dendrimers exhibit different electrophoretic behaviour depending on the pH of the running buffer. Dendrimer's full-generations have terminal amino groups that become protonated in an acidic medium and the electrophoretic mobility of positively charged molecules is in the direction of the electroosmotic flow (i.e. towards the cathode). When the number of generation increases the calculated charge-to-mass ratio remains essentially constant (assuming the protonation of all amino groups) and electrophoretic velocities of different generations should be close. Nevertheless, our experiments showed that in an acidic phosphate buffer, where the electroosmotic flow is practically suppressed, first six generations of EDA-core PAMAM dendrimers with molecular masses ranging from 516 to 28 788 Da could be separated (Fig. 4). Similar results were obtained earlier for ammonia-core PAMAM dendrimers [16]. However, the profile of possible "structural errors" for EDA-core dendrimers is more complicated.

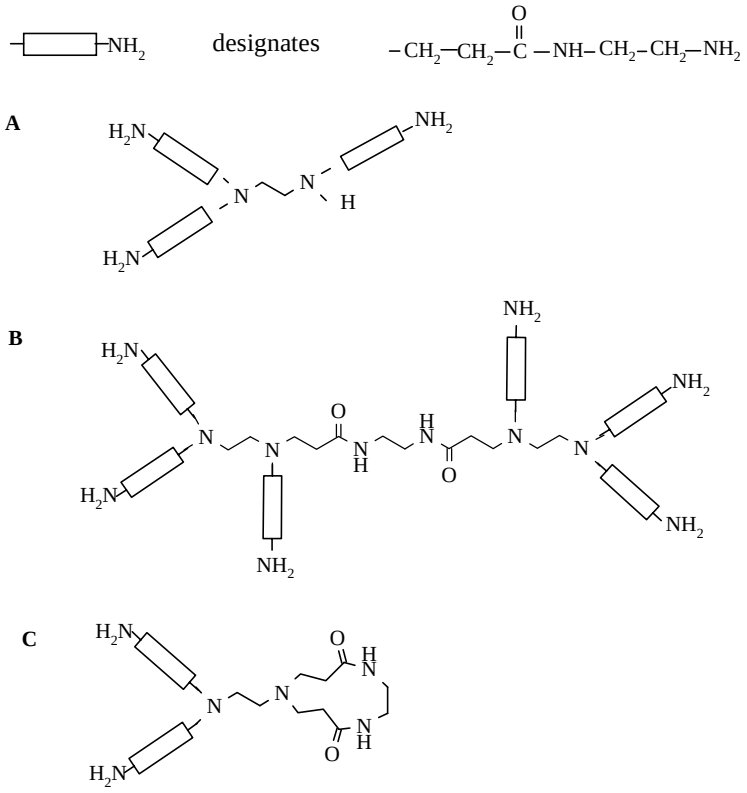

Fig. 3. Defective structures of the G0 dendrimer: A – missing arm, B – dimer, C – intramolecular cyclization.

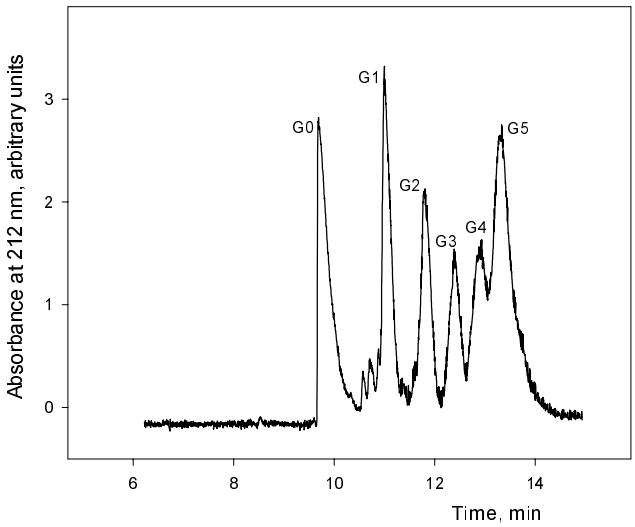

Fig. 4. Electropherogram of first six full-generations of EDA-core PAMAM dendrimers.

Electropherograms of single generations allow to assess the presence of side products in synthesized dendrimers (Fig. 5). Starting from the G0 all full-generations contained the previous generation and side products of formation reactions as impurities (the same was observed in the commercial Starburst dendrimer purchased from Aldrich, Fig. 5D). Also, all generations contain some impurities that are migrating slower than the main component. Those impurities may be attributed to the "structural errors" (see Fig. 3). At each cyclization the molecule loses 60 Da of mass and two terminal amino groups. In a perfect dendrimer structure each $–NH_2$ group "carries" roughly 200 Da of mass and it is obvious that because of increased mass-to-charge ratio the cyclic product should have lower electrophoretic mobilities. These slowly migrating products can be seen especially clearly in the electropherogram of G0, where they form several peaks corresponding to various looping possibilities (Fig. 5A).

Half-generation dendrimers (amidoesters) are not ionized at conventional for CZE pH values and we did not succeed in separating the mixture of two different half-generation dendrimers. In a moderately basic phosphate buffer with

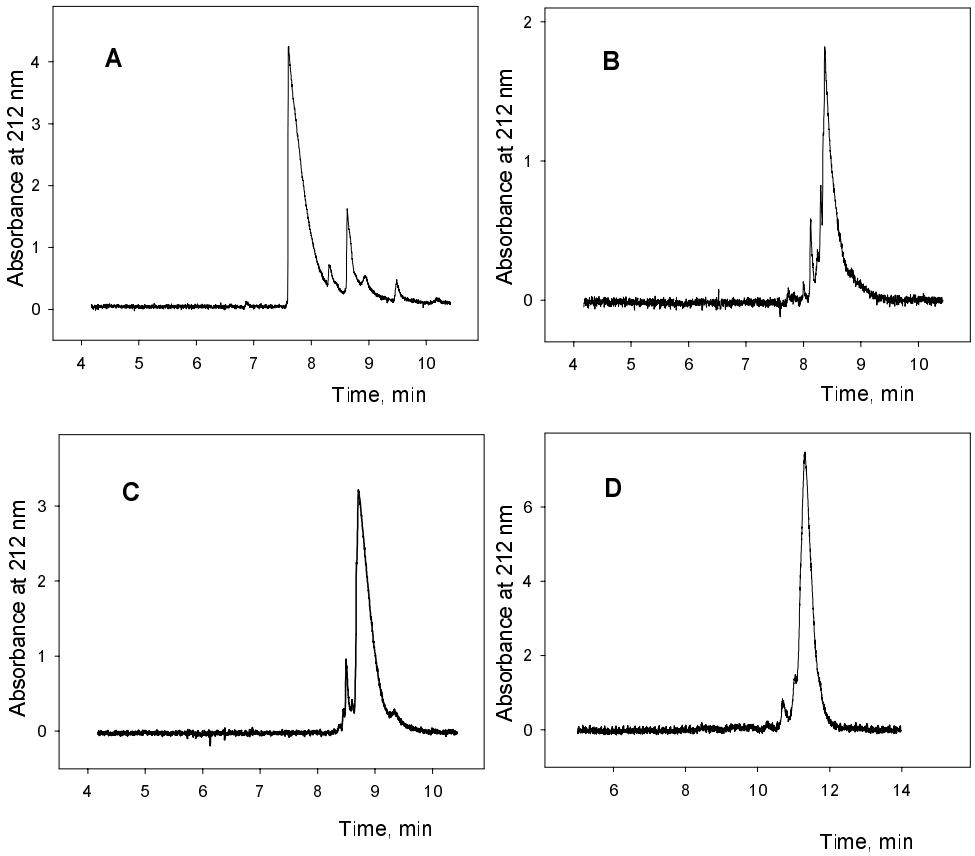

Fig. 5. Electropherograms of individual full-generations of EDA-core PAMAM dendrimers. A, B, C – synthesized generations G0, G1, and G2; D – Starburst G4 dendrimer from Aldrich.

pH = 7.8, however, half-generation dendrimers are markedly labile and hydrolyze readily. So we could observe various carboxylate anions and kinetics of hydrolysis (Fig. 6).

Compared to the separation of full-generation dendrimers, the cathode directed electroosmotic flow in this case is relatively fast and the electrophoretic migration of carboxylate anions is against the electroosmotic flow. The electropherograms in Figs. 6 and 7 show that for G–0.5 and G0.5 the hydrolysis products can be well separated. Starting from G1.5 the hydrolysis products could be only partly separated and G2.5 revealed only one unresolved peak. For lower generations the hydrolysis products may be used for elucidation of errors in the dendrimer's structure and for the characterization of individual half-generations.

CONCLUSIONS

Synthesis of PAMAM dendrimers always generates "structural errors". Capillary zone electrophoresis may serve as a suitable tool for quantitative estimation of the amount of these "errors" in first generations and for the analysis of generational homogeneity of PAMAM dendrimers. Also, CZE may serve as a complementary method for the characterization of single generations of dendrimers.

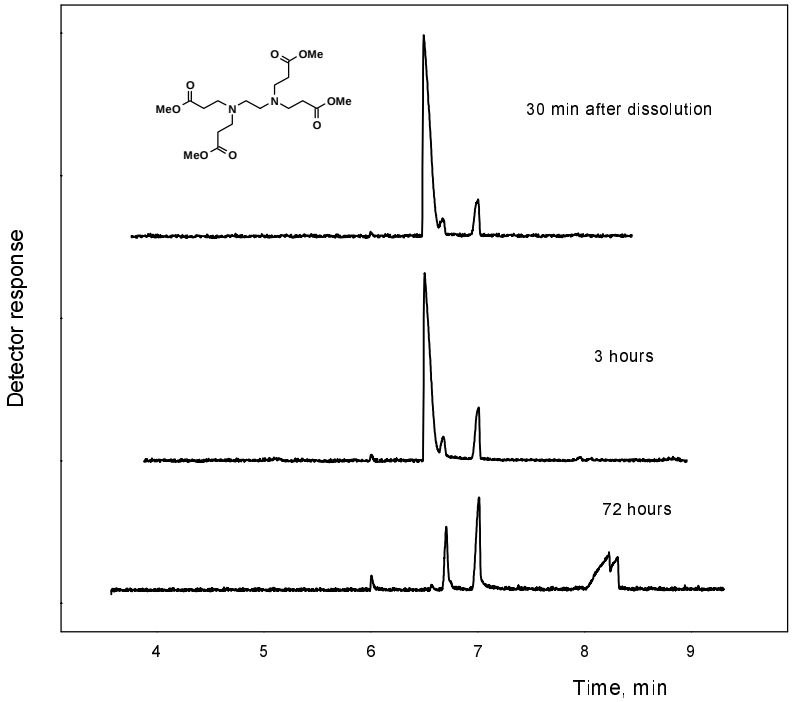

Fig. 6. Electropherograms of the generation of G–0.5 PAMAM dendrimer demonstrating the hydrolysis of multiester in phosphate buffer at pH = 7.8.

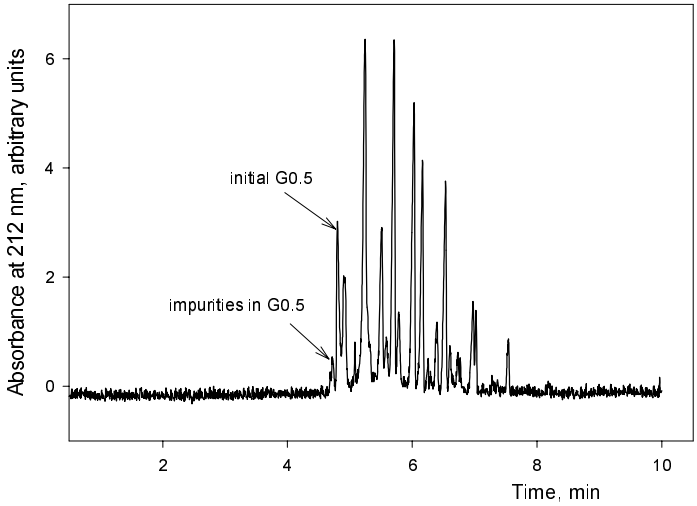

Fig. 7. Electropherogram of G0.5 hydrolysis products. Sample stayed in phosphate buffer (pH = 7.8) 24 h.

ACKNOWLEDGEMENT

The authors would like to thank the Estonian Science Foundation (grants Nos. 3834, 3781, 3164) for the financial support.

REFERENCES

1. Jansen, J. F. G. A. & Meijer, E. W. The dendritic box: Shape-selective liberation of encapsulated guests. *J. Am. Chem. Soc*., 1995, **117**, 4417–4418.
2. Wooly, K. L., Hawker, C. J. & Fréchet, M. J. Unsymmetrical three-dimensional macromolecules: Preparation and characterization of strongly dipolar dendritic macromolecules. *J. Am. Chem. Soc*., 1993, **115**, 11496–11505.
3. Percec, V., Johansson, G., Ungar, G. & Zhou, J. Fluorophobic effect induces the self-assembly of semifluorinated tapered monodendrons containing crown ethers into supramolecular columnar dendrimers which exhibit a homeotrobic hexagonal columnar liquid crystalline phase. *J. Am. Chem. Soc*., 1996, **118**, 9855–9866.
4. Dubin, P. L., Edwards, S. L., Kaplan, J. I., Metha, M. S. & Tomalia, D. A. Carboxylated Starburst dendrimers as calibration standards for aqueous size exclusion chromatography. *Anal. Chem*., 1992, **64**, 2344–2347.
5. Dubin, P. L., Shun, L., Metha, E. & Metha, M. S. Quantitation of non-ideal behavior in protein size-exclusion chromatography. *J. Chromatogr*., 1993, **635**, 51–60.
6. Kukowska-Latallo, J. F., Bielinska, A. U., Johnson, J., Spindler, R., Tomalia, D. A. & Baker, J. R. Efficient transfer of genetic material into mammalian cells using Starburst polyamidoamine dendrimers. *Proc. Natl. Acad. Sci. USA*, 1996, **93**, 4897–4902.
7. Tang, M. X., Redemann, C. T. & Szoka, F. C., Jr. *In vitro* gene delivery by degraded polyamidoamine dendrimers. *Bioconjugate Chem*., 1996, **7**, 703–714.

8. Tomalia, D. A., Naylor, A. M. & Goddard, W. A., III. Starburst dendrimers: Molecular-level control of size, shape, surface chemistry, topology, and flexibility from atoms to macroscopic matter. *Angew. Chem., Int. Ed. Engl*., 1990, **29**, 138–175.
9. Tomalia, D. A., Baker, H., Dewald, J. R., Hall, M., Kallos, G., Martin, S., Roeck, J., Ryder, J. & Smith, P. A new class of polymers: Starburst-dendritic macromolecules. *Polym. J*., 1985, **17**, 117–132.
10. Tomalia, D. A. & Durst, H. D. Genealogically directed synthesis: Starburst/cascade dendrimers and hyperbranched structures. *Top. Curr. Chem*., 1993, **165**, 193–313.
11. Meltzer, A. D., Tirrell, D. A., Jones, A. A. & Inglefield, P. T. Chain dynamics in poly(amidoamine) dendrimers. A study of ^{13}C NMR relaxation parameters. *Macromolecules*, 1992, **25**, 4541–4548.
12. Meltzer, A. D., Tirrell, D. A., Jones, A. A. & Inglefield, P. T. Chain dynamics in poly(amidoamine) dendrimers. A study of ^{2}H NMR relaxation parameters. *Macromolecules*, 1992, **25**, 4549–4552.
13. Smith, P. B., Martin, S. J., Hall, M. J. & Tomalia, D. A. A characterization of the structure and synthetic reactions of polyamidoamine "Starburst" polymers. In *Applied Polymer Analysis and Characterization* (Mitchell, J., Jr., ed.). Hanser, München/New York, 1987, 357–385.
14. Kallos, G. J., Tomalia, D. A., Hedstrand, D. M., Lewis, S. & Zhou, J. Molecular weight determination of a polyamidoamine Starburst polymer by electrospray ionization mass spectrometry. *Rapid Commun. Mass Spectrom*., 1991, **5**, 383–386.
15. Xu, Z., Kahr, M., Walker, K. L., Wilkins, C. L. & Moore, J. S. Phenylacetylene dendrimers by the divergent, convergent, and double-stage convergent methods. *J. Am. Chem. Soc*., 1994, **116**, 4537–4550.
16. Brothers, H. M., II, Piehler, L. T. & Tomalia, D. A. Slab-gel and capillary electrophoretic characterization of polyamidoamine dendrimers. *J. Chromatogr. A*, 1998, **814**, 233–246.
17. Peterson, J., Allikmaa, V., Subbi, J., Pehk, T. & Lopp, M. Structure deviations in PAMAM dendrimers. A complex investigation using ^{1}H and ^{13}C NMR and MALDI-TOF MS. Unpublished results.

ETÜLEENDIAMIINTUUMAGA POLÜ(AMIDOAMIIN)DENDRIMEERIDE ERINEVATE PÕLVKONDADE SÜNTEES JA ANALÜÜS KAPILLAARTSOONELEKTROFOREESIGA

Janek PETERSON, Arkadi EBBER, Veiko ALLIKMAA ja Margus LOPP

On sünteesitud mitmeid etüleendiamiintuumaga erineva põlvkonna (G0–G5) polü(amidoamiin)dendrimeere. Neist poolpõlvkonnad G–0,5, G0,5 ja G1,5 puhastati kromatograafiliselt silikageelil, G2,5–G4,5 aga Sephadex LH-20-l. Kapillaartsoonelektroforeesi kasutati erinevate põlvkondade homogeensuse (eelnevate põlvkondade sisaldus ning tekkivate "struktuurivigade" sisaldus) iseloomustamiseks ning nende lahutamiseks.