THESIS ON NATURAL AND EXACT SCIENCES B17

SYNTHESIS, STRUCTURAL CHARACTERIZATION AND MODIFICATION OF PAMAM DENDRIMERS

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Dissertation in Organic Chemistry to be publicly examined in lecture hall VI-121,
Tallinn University of Technology, on December 18, at 10 a.m.,
for the Degree of Doctor of Philosophy

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ISSN
ISBN

Abstract

Starburst polyamidoamine (PAMAM) dendrimers, structurally defined spherical macromolecules, are composed of amidoamino subunits and are synthesized by simple repetitive steps. They are highly soluble in aqueous solutions and have a unique surface of primary amino groups. Taking advantage of these properties, PAMAM dendrimers have been employed as substrates to attach DNA, antibodies, contrast agents and radiopharmaceuticals. They have been found to be very efficient transfection systems for cells in culture.

In the present study, we attempted to characterize PAMAM dendrimers in the course of step-by-step synthesis and check the practical acceptance of the existing methodology. Purification methods were developed for separating intermediates and side-products and a combination of GC, CZE, MALDI-TOF MS and ^{1}H, ^{13}C NMR was used to characterize these products. Additionally, we describe a modification of the first generation of PAMAM dendrimers by attaching saccharides and oligopeptides.

We found that half generations –0.5, 0.5 and 1.5 of dendrimers could be purified by column chromatography on silica gel using methanol or its mixture with dichloromethane as an eluent. Generations G2.5–G4.5 of dendrimers could be purified by column chromatography on Sephadex LH-20 using methanol as an eluent. We found that full generations of dendrimers G1 and G5 could be separated from some defective products by help of HILIC and RP-HPLC.

We investigated decomposition of PAMAM dendrimers by using G-0.5 as a model compound and analysing products by GC. We found that the decomposition of PAMAM dendrimer G-0.5 generation into triester is caused by the *retro*-Michael reaction. Equilibrium between the Michael and *retro*-Michael reactions is shifted towards *retro*-Michael reaction at an elevated temperature. Similar decomposition is possible in the case of higher generations of PAMAM dendrimers.

CZE was used for the determination of the homogeneity of various full generations. CZE enabled us to discriminate between the dendrimer full generations up to fifth.

We found that MALDI-TOF MS gave a fast and accurate indication of the purity for all generations of EDA-core PAMAM dendrimers (G-0.5-G5) and allows identification of the defects and impurities present. The best matrix for the analysis was 2,5-dihydroxybenzoic acid mixed with β-D-fucose. For lower generations of dendrimers (G-0.5-G2), high-resolution mass spectra were obtained that allowed us also to determine the structures of impurities caused by major side-reactions. From generation G2.5 and higher, no more isotope resolution could be obtained.

We employed ^{1}H and ^{13}C NMR spectroscopy to determine the structural integrity and purity of each generation of Starburst PAMAM dendrimers. Analysis of the higher generations was complicated, mostly because of an increasing amount of defective dendrimers. In the ^{1}H spectra, the main bands were resolved and assigned up to G0.5, in the ^{13}C spectra, all signals from different positions were resolved and assigned up to G2.5. Up to ~2 ppm, long-range high field shifts were observed on core methylene carbons of the higher generations.

We modified the first generation of PAMAM dendrimers by attaching covalently β-D-glucopyranose, β-D-*N*-acetylglucosamine and disaccharide hyaluronic acid. The saccharides were linked by reductive amination of the aldehyde group of the sugar by amino groups of dendrimers at the presence of sodium cyanoboronhydride. To link the HA oligosaccharides (16-mer) to the dendrimer outer layer, carbodiimide chemistry was also used.

We used different linkers and carboxyl group activating reagents (SMCC, SPDP, BOP) to synthesize oligopeptide-dendrimer conjugates. The BOP reagent was found to be more efficient compared to SMCC and SPDP for attaching oligopeptide to the dendrimer.

PAMAM dendrimeeride süntees, struktuuri iseloomustamine ja modifitseerimine

Kokkuvõte

Starburst polüamidoamiin (PAMAM) dendrimeerid on korrapärase struktuuriga sfäärilised makromolekulid, mis koosnevad korduvatest amidoamiini ahelatest. Nende sünteesiks kasutatakse divergentset sünteesimeetodit. PAMAM dendrimeerid lahustuvad väga hästi vees, nende väliskihi moodustavad primaarsed aminorühmad, mis on füsioloogilise pH juures positiivselt laetud. Neil on olemas valkudele iseloomulik struktruurifragment - peptiidside - ning neid on võimalik sünteesida valkudele lähedase massiga. Lisaks on nad võimelised rakumembraani läbima. Nendest omadustest tingituna on PAMAM dendrimeerid leidnud laialdast kasutamist molekulaarbioloogias DNA, antikehade ja kontrastainete transpordil organismi.

Käesolevas töös sünteesiti PAMAM dendrimeeri põlvkonnad (G-0.5-G5) vastavalt teada olevale metoodikale, kusjuures igat pool- ja täispõlvkonda püüti maksimaalselt puhastada. Lähemalt uuriti nelja meetodi – GC, CZE ^{1}H, ^{13}C NMR ja MALDI-TOF MS - kompleksset rakendamist dendrimeeride pool- ja täispõlvkondade, samuti ka lähteainete ja kõrvalproduktide analüüsil. Pool- ja täispõlvkondadele töötati välja kromatograafilised puhastusmeetodid vaheühendite ja kõrvalproduktide eraldamiseks. Selles töös uuriti ka erinevate sahhariidide ja oligopeptiidide sidumse võimalikkust PAMAM dendrimeeri väliskihi külge.

Leiti, et PAMAM dendrimeeri madalamaid poolpõlvkondi (G-0.5, G0.5, G1.5) oli võimalik puhastada kolonnkromatograafiliselt, kus kolonni täidisena kasutati silikageeli ja eluendina metanooli või tema segu diklorometaaniga. Kõrgemaid poolpõlvkondi (G2.5-G4.5) puhastati samuti kolonnkromatograafiliselt, kus kolonni täidiseks oli Sephadex LH-20 ning eluendiks metanool. Täispõlvkondade puhastamiseks rakendati hüdrofiilse interaktsiooni ja pöördfaasi kõrgsurvevedelikkromatograafiat, mis võimaldasid eraldada esimesest ja viiendast põlvkonnast defektseid dendrimeere.

PAMAM dendrimeeride lagunemist uuriti lähemalt gaaskromatograafiliselt. Mudelühendina kasutati poolpõlvkonda G-0.5. Etüleendiamiini tuumaga PAMAM dendrimeeri põlvkonna G-0.5 (tetraester) lagunemine triestriks oli tingitud *retro*-Michaeli reaktsioonist. Tasakaal Michaeli ja *retro*-Michaeli reaktsiooni vahel nihkub temperatuuri tõustes

retro-Michaeli reaktsiooni suunas. Tõenäoliselt leiab analoogne lagunemine aset ka kõrgemate PAMAM dendrimeeride põlvkondade korral.

CZE kasutati erinevate täispõlvkondade homogeensuse uurimiseks. CZE võimaldas lahutada kõiki sünteesituid täispõlvkondi.

MALDI-TOF MS abil oli võimalik saada täpselt ja kiiresti informatsiooni PAMAM dendrimeeride defektide ja lisandite sisalduse kohta. Parimaks maatriksiks osutus 2,5-dihüdroksübensoehappe ja D-fukoosi segu. Dendrimeeri madalamate põlvkondade jaoks (G-0.5-G2) saadi kõrglahutuvusega spektrid, mis võimaldasid iseloomustada täpselt kõrvalreaktsioonidest tingitud lisandite struktuure. Alates põlvkonnast G2.5 ei õnnestunud saavutada isotooplahutuvust.

^{1}H ja ^{13}C NMR kasutati PAMAM dendrimeeride struktuurilise terviklikkuse ja puhtuse iseloomustamiseks. Komplitseerituks osutus kõrgemate põlvkondade struktuuri analüüs, mis oli põhiliselt tingitud defektsete dendrimeeride arvu kasvust. ^{1}H spektris lahutati põhipiigid ning määrati neile vastavad struktuurifragemenid kuni põlvkonnani G0.5. ^{13}C spektris õnnestus seda saavutada kuni põlvkonnani G2.5. Kõrgemate põlvkondade puhul täheldati nende tuuma metüleensetele süsinikele vastavate keemiliste nihete nihkumist ~2 ppm võrra kõrgemasse välja.

PAMAM dendrimeeri esimese põlvkonna modfitseerimiseks kinnitati kovalentselt tema väliskihi külge β-D-glükoosi, β-D-*N*-atsetüülglükoosamiini ja hüaluroonhappe disahhariidi. Sahhariidide aldehüüdrühma sidumuseks dendrimeeri aminorühmaga kasutati taandavat amineerimist naatriumtsüanoboorhüdriidiga. Hüaluroonhappe oligosahhariidi sidumiseks dendrimeeri väliskihi külge kasutati EDC-d.

Vastavate oligopeptiidide ja PAMAM dendrimeeri konjugaatide sünteesiks kasutati erinevaid linkereid ja karboksüülrühma aktiveerimisreagenti (SMCC, SPDP, BOP). BOP reagent osutus efektiivsemaks oligopeptiid-dendrimeer konjugaadi sünteesil võrreldes SMCC ja SPDP-ga.

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1. LIST OF PUBLICATIONS

This thesis is based on the following publications, which will be referred to by their Roman numerals as indicated below:

I Peterson, J., Ebber, A., Allikmaa, V., Lopp, M. Synthesis and CZE Analysis of PAMAM Dendrimers with an Ethylenediamine Core. Proc. Estonian Acad. Sci. Chem., 2001, 50, 3, 156-166.

II Peterson, J., Allikmaa, V., Pehk, T., Lopp, M. Fragmentation of PAMAM Dendrimers in Methanol. Proc. Estonian Acad. Sci. Chem., 2001, 50, 3, 167-172.

III Peterson, J., Allikmaa, V., Subbi, J., Pehk, T. and Lopp, M. Structural deviations in poly(amidoamine) dendrimers: a MALDI-TOF MS analysis. European Polymer Journal, 2003, 39(1), 33-42.

2. ABBREVIATIONS

BOP	benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate
CC	column chromatography
CI MS	chemical ionization mass spectrometry
Cys	cysteine
CZE	capillary-zone electrophoresis
DEDG	double exponential dendrimer growth
DIC	1,3-diisopropylcarbodiimide
DiHA	hyaluronic acid disaccharide
DMF	*N*,*N*-dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DIPEA	*N*,*N*-diisopropylethylamine
EDA	ethylenediamine
EDC	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
ESI MS	electrospray ionization mass spectrometry
ECM	extracellular matrix
FAB MS	fast atom bombardment mass spectrometry
GC	gas chromatography
Glc	D-glucopyranose
GPC	gel-permeation chromatography
GlcNAc	D-*N*-acetylglucosamine
HA	hyaluronic acid
HILIC	hydrophilic interaction chromatography
HPLC	high-pressure liquid chromatography
LALLS MS	low angle laser light scattering mass spectrometry
MALDI-TOF MS	matrix assisted laser desorption ionisation time of flight mass spectrometry
MW	molecular weight
NEM	*N*-ethylmaleimide
NHS	*N*-hydroxysuccinimide
NMR	nuclear magnetic resonance
PAMAM	polyamidoamine
PPI	polypropyleneimine
rt	room temperature
Sac	saccharide
SEC	size-exclusion chromatography
SMCC	succinimidyl-4-(*N*-malemidoethyl)-cyclohexane-1-carboxylate
SPDP	*N*-succinimidyl-3-(2-pyridyldithio)propionate
TCEP	tris-(2-carboxyethyl)phosphine hydrochloride
t_R	retention time
Z	benzyloxycarbonyl

3. INTRODUCTION

Dendrimers belong to a relatively new class of macromolecules. Their highly branched monodisperse structure leads to a number of interesting characteristics and features. Especially interesting are the properties that are connected with its globular void-containing shape. Geometrical peculiarities of the molecules give rise to somewhat unusual physical and chemical properties. The first publication in this topic appeared in 1978 [1], which dealt with the development and the realization of this fundamentally new molecular architecture. However, these first compounds were insufficiently characterized because of lack of appropriate analytical methods and instrumentation at that time. After the first publication a slow progress in the characterization of dendrimers followed, but soon afterwards it proceeded at a faster pace thanks to the development of new mass-spectroscopy methods at the beginning of 1990s [2,3]. Recent advances in the development of analytical instrumentation and methods (especially multidimensional NMR, MALDI MS) have supported the fast growth of a wide range of different methodologies of the syntheses of finely engineered well characterized dendritic structures, many of which incorporate functional moieties. This research is expected to lead to applications in many areas, such as chemical catalysis, materials science, particularly, in pharmacology and molecular biology.

For the last ten years polyamidoamine (PAMAM) dendrimers have been used in molecular biology as a carrier of different biologically active molecules [4-13]. As a carrier, a PAMAM dendrimer has a number of important advantages over other types of dendrimers. The most important property of a PAMAM dendrimer is its ability to pass eukaryotic cells membranes. PAMAM dendrimers are well soluble in water, and the amino groups in its outer shell are positively charged at the physiologic pH that enables good electrostatic interaction between a dendrimer and DNA (contains negatively charged phosphoric acid residues). To carry DNA into cells, higher generations (G5-G10) of PAMAM dendrimers are used [4,11,12]. PAMAM dendrimers also contain amide bonds that are similar to peptide bonds. The conjugates of PAMAM dendrimers with antibodies and DNA are stable and non-toxic [8,10,11,14-16]. The reasons of PAMAM dendrimer capability to penetrate cell membranes are not quite clear yet. It is known, however, that carrier abilities depend on the size, shape, and number of primary amino groups on the surface of the PAMAM polymer.

In the present study an attempt was made to characterize PAMAM dendrimers in the course of step-by-step synthesis of the polymer. A better characterization of the “real” geometry of the synthesized dendrimers is required in order to understand its interaction with other molecules. The other goal of the work was to search for new modified PAMAM dendrimers and methods of their modification. This work was planned as the first stage in a cell-specific DNA carriers project.

4. LITERATURE SURVEY

4.1. Dendrimers - a class of macromolecules

It is know that macromolecules may have many different structures. In its simplest form, the molecule has a linear long chain structure. The branched polymers forming cross-links that join the molecules into a network are also known. Branching without formation of the cross-links is also possible and in that case a polymer, where the chains start from one point, is formed. That type of polymer has a large number of branches (Figure 1) [17]. Examples of such biopolymers are carbohydrates amylopectin and glycogen.

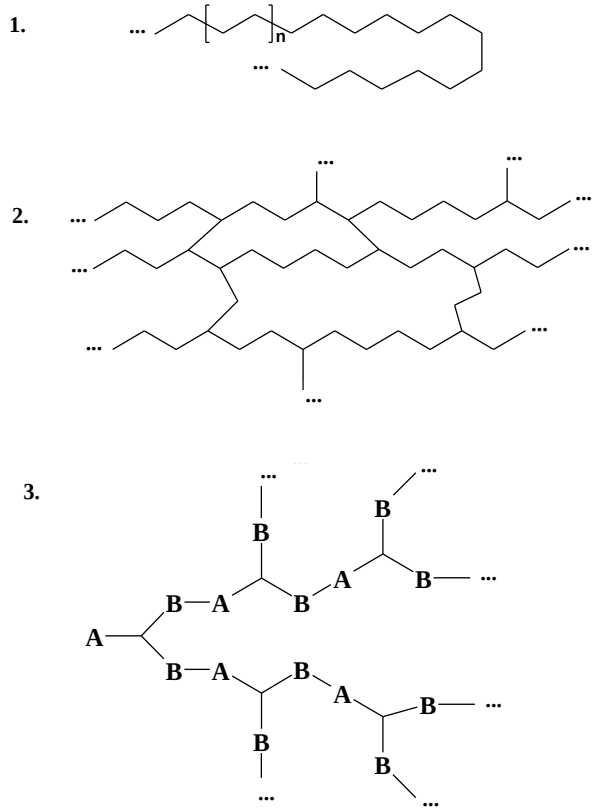
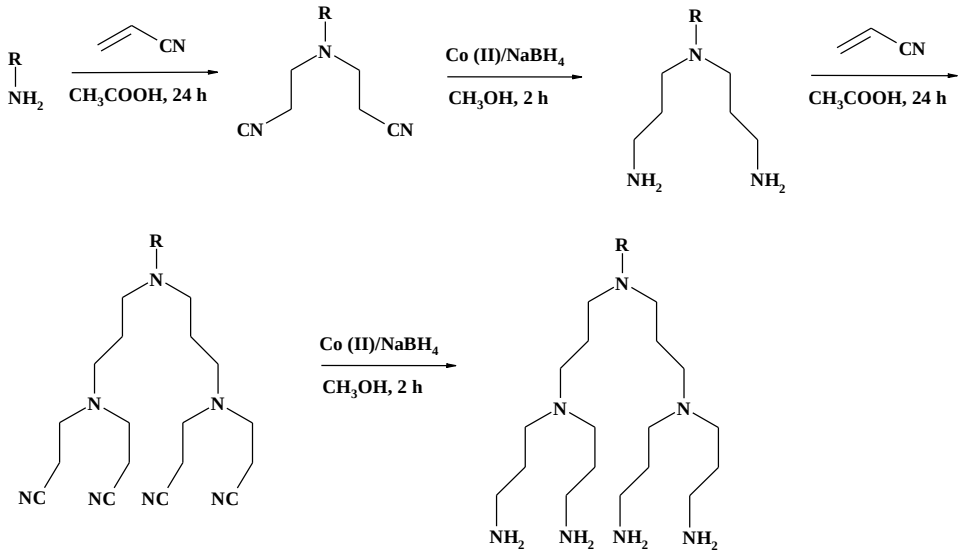


Figure 1. 1 - linear polymer, **2** – cross-linked branched polymer, **3** – branched polymer without cross-links (polymerization of the AB_2-type monomers where A- is reactive with B).

In 1978, Vögtle developed a method that allowed the synthesis of branched polymers without cross-links (Scheme 1) [1]. That synthesis scheme ensured identical chains between branch points, because the subsequent reaction steps are chemically similar. Analogically branched, but not-cross-linked polymers have, since then, been obtained by many synthesis methods [18-27].



Scheme 1. Synthesis of "cascade molecules" by Vögtle et al. [1].

In the early 1980s, various names were proposed for such type of compounds, like silvanols [28], arborols [29], cascadols [30], cauliflower polymers [31], crowned arborols [32], molecular fractals [33], and polycules [34]. One of the proposed names was Starburst dendrimers that has now become a common name for such type of polymeric structures [18]. The word "dendrimer" is derived from two Greek words *dendron* (branch, tree) and *meros* (part). Regularity of the molecule is regarded as one of its most important properties. The requirement of regularity in dendrimeric structures excludes starch from the member of dendrimers, since the chains of starch between branching points differ in length [35]. At present the term "dendrimer" means a non-cross-linked regularly branched polymer.

4.2. The dendritic structure

Dendrimers differ from classical random coil macromolecules. They possess three distinctive architectural components, namely

(1) an initiator core,

(2) interior layers (generations), composed of repeating units and radially attached to the initiator core, and

(3) exterior (terminal functionality) attached to the outermost interior generation (Figure 2).

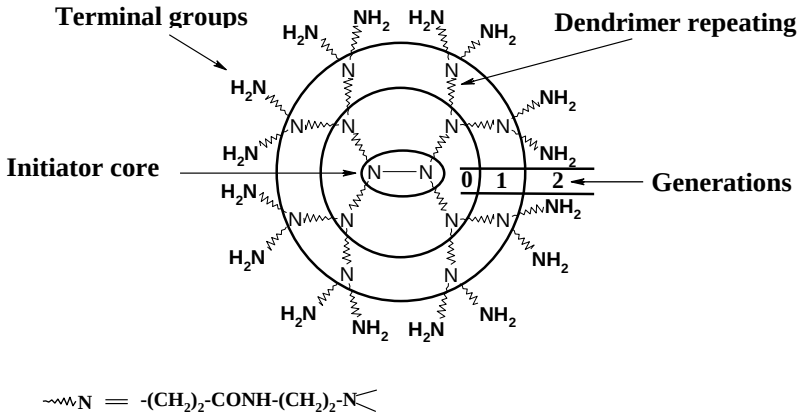


Figure 2. Structure of the polyamidoamine (PAMAM) dendrimer.

The dendrimer core is usually a multifunctional reactive compound. An important parameter for the dendrimer core is the so-called "core multiplicity" (N_c), which, for example, is $N_c = 3$ for ammonia and $N_c = 4$ for ethylenediamine (Figure 2). The selection of the initiator core is of great importance. It is the fundamental unit to which the dendrons are linked. Thus, its size, shape, multiplicity, and specialized functions will strictly influence the dendrimer throughout its construction [36,37].

The interior layer (or generation) is classically synthesized with two consecutive reactions. The first generation is composed of one or more repeating units directly connected to the core (Figure 2). Additional generations, each composed of a single layer of repeating units, are attached concentrically. The repeating units are typically composed of alternating structural units. For example, the repeating unit in the polyamidoamine dendrimer (PAMAM), $-CH_2CH_2CONHCH_2CH_2N=$, consists of two structural units, $-CH_2CH_2CO-$ and $-NHCH_2CH_2N=$ (Figure 2). Similarly, as the size and shape of the dendrimer depend on the core multiplicity, the same parameters also depend on the repeating unit multiplicity that is two for the PAMAM dendrimer.

The exterior shell of the dendrimer may contain reactive or passive functional groups. The chemically reactive surface groups may be used for further dendritic growth or for the modification of the dendritic surface. The chemically passive groups may be used to physically modify the dendritic surface (adjusting hydrophobic – hydrophilic ratios) [38]. The number of terminal groups can be computed from the following formula:

number of terminal units = $N_c(N_r)^G$,

in which G = number of generations

N_r = repeating unit multiplicity

N_c = core multiplicity

Dendrimer shapes have been investigated both experimentally and theoretically [17]. Namely, dendrimers have a denser structure and a shape closer to a sphere than to linear polymers. In that respect, dendrimers resemble many natural proteins acquire a relatively well-defined and comparatively compact conformation because of attractive forces between different parts of the chain.

4.3. Synthesis of dendrimers

In a classical polymer synthesis, the whole macromolecule is synthesized in one single step. As all the reactions occur simultaneously and competing with one another, the length of the polymer that finally forms is greatly different [17]. Every polymerization step requires two reactions that cannot occur simultaneously, at the end of every step, one specific compound is formed (Scheme 2).

I. **R**-Y + X-**R**-Z → **R**-**R**-Z

II. **R**-**R**-Z → **R**-**R**-Y

III. **R**-**R**-Y + X-**R**-Z → **R**-**R**-**R**-Z etc.

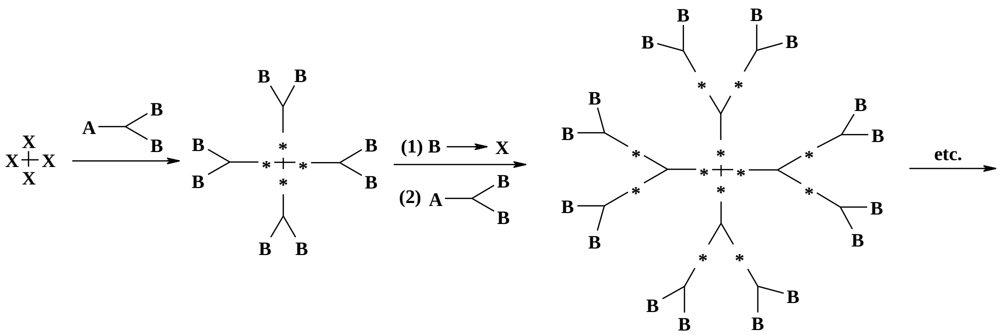
Scheme 2. Step-growth polymers (X, Y- reactive groups).

This is the way proteins and nucleic acids are synthesized by chemical methods [39,40]. But chemists find it hard to perform a large number of steps with a good yield. If every branch includes branching, the mass of the molecule will increase in geometric progression and fewer steps are required to obtain a large but well-defined molecule.

Dendrimers are constructed in a stepwise manner using repeating synthetic steps [41]. Each repetition cycle creates an additional layer of branches (generations) (Figure 2). Branching multiplicity is dependent on the building block valency, although, a new layer can be generated during the growth step from a non-branched building block as well [18,20]. The method is usually designed in a such way that all the terminal groups are fully derivatized before the next reaction is initiated. As the dendrimer is regular, its structure is well predefined by the selected structural units and the used synthetic method. This distinguishes dendrimers from other polymers and attracts interest. The methods of dendrimer synthesis are classified as the divergent [19-22], convergent [23,24,42-45], the double-stage convergent [26,46], double exponential dendrimer growth (DEDG) [27,46], comb-burst [47], and hyper branching strategies [48,49].

4.3.1. Divergent dendrimer growth

In the divergent strategy, pioneered by Tomalia [18] and Newkome [22], dendrimers are built from the central core out to the periphery. In the repeating cycle, some number (n) of reactive groups on the dendrimer periphery reacts with the same number of monomer units to add a new layer or generation to the dendrimer. In the next repeat cycle, 2n or 3n reactive sites will be available depending on the monomer unit's branching multiplicity (2 or 3) [50]. Thus, the number of coupling reactions increases with each successive generation (Scheme 3).



Scheme 3. Dendritic growth via divergent approach with AB_2-type chain extenders. Protection/deprotection steps (B→X) are not necessary if selective chemistry can be adapted. Dots represent the bonds formed between A and X groups [17].

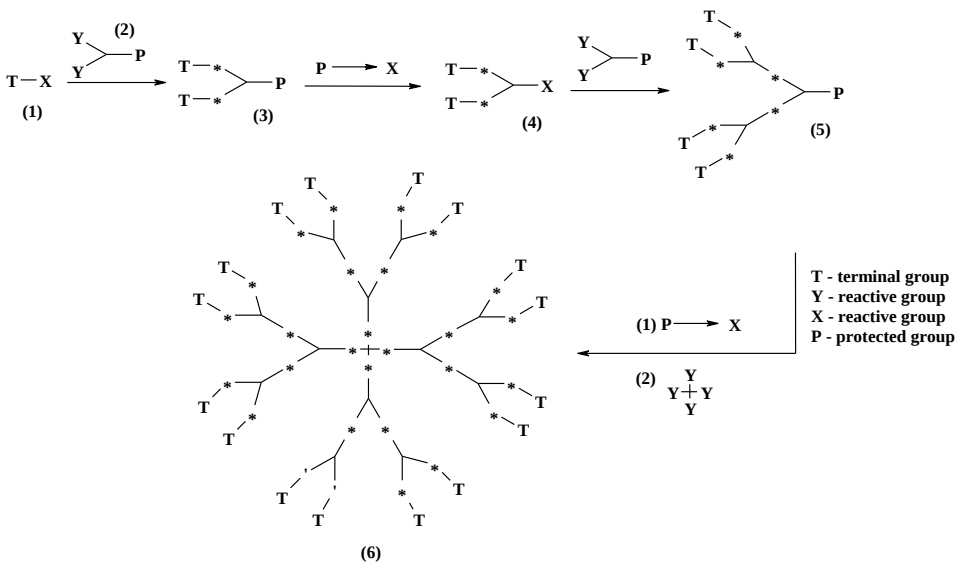
In divergent synthesis, every generation (x) requires the addition of 4×2^x monomers (x - being the number of generation). If the polymerization reaction is not complete, then $4\times2^x+1$ polymer molecules of different molecular mass can form. These defected polymers differ very little from each other and are hard to separate. In practice, the defective dendrimers are not separated. As a result, defects will accumulate in the synthesis of higher generations. The divergent approach is successful for the production of large quantities of dendrimers since in each generation-adding step, the molecular mass of the dendrimer is almost doubled (in the case if the branching multiplicity is two). A wide variety of the dendrimer families have been synthesized by these divergent methods: poly(amidoamine) [18,51], poly(ether) [52], poly(siloxane) [53], poly(thioether) [54], poly(amidoalcohol) [22], poly(amine) [36], poly(phosphonium) [55], poly(alkane) [56], poly(nucleic acid) [57], and poly(organometallic) [58] types. Both the commercially available PAMAM (polyamidoamine) and PPI (poly(propyleneimine)) dendrimers are made by the divergent method.

4.3.2. Convergent dendrimer growth

Classical examples of the convergent approach to the dendrimer synthesis can be found in the studies of Fréchet [23], Miller [24], and Moore [42,43]. In contrast to the divergent strategy, in this approach, the dendrimer is built from the periphery toward the central core (Scheme 4).

Dendron growth is designed to occur *via* a limited number of reaction sites. The synthesis starts first with a monomer unit **1**, possessing a functional group that will eventually constitute the dendron surface functionality (T) and a reactive functionality (X). The monomer unit is now coupled to a branch cell reagent **2**, containing at least two coupling sites and a protected functionality (P) to give **3** as illustrated in Scheme 4. After coupling, the functional group (P) is transformed to (X) to give the reactive branched cell reagent **4**, which is then allowed to react with **2** to produce the new cascade fragment **5**. This dendron

has a single reactive group (X), which may be used to couple to a multivalent core to produce dendrimers **6**.



Scheme 4. Dendritic growth *via* convergent approach. Dots represent bonds formed between two reactive groups Y and X [37].

In the convergent synthesis, only n chains are added in each reaction step, the n+2 compounds formed are very different and easy to separate, but the yields of individual compounds are much less than in the divergent synthesis. The convergent strategy is often limited to the dendrimers of lower generation number. Dendritic wedges (= branches or "dendrons") (Scheme 4, **5**) of higher generations encounter serious steric problems in the final reaction - attachment to the core. A wide variety of the dendrimer types have been synthesized by these convergent methods, like poly(arylalkyl ether) [23], poly(arylester) [59], poly(arylene) [60], and poly(arylacetylenic) [61]. The limits of both approaches have yet to be firmly established. At present we can say that by using the convergent approach less than eight generations dendrimers can be obtained, whereas using the divergent syntheses of dendrimers, as many as 10 generations may be synthesized.

4.3.3. Other accelerated growth techniques

Since both divergent and convergent synthesis methods are time-consuming and tedious, a synthesis method was needed that would allow relatively easily as many functional groups in the outer layer as possible. To solve the problem, the so-called double-stage convergent strategy, double exponential dendrimer growth (DEDG), comb-burst, and hyper branching strategies were developed.

Double-stage convergent strategy

The double-stage convergent growth approach consists of two parts. Part I covers the preparation of essentially monodisperse dendritic macromolecules (hyper core) with a large number of functional groups evenly distributed at the periphery. Part II discusses coupling of performed dendritic fragments (branched monomer) to the highly functionalized macromolecules [26]. It was found that the use of this technique not only reduces the number of convergent growth steps but also simplifies the construction of large structures. An additional advantage of the method is its great versatility that may be used for the preparation of totally new functional globular structures or molecular devices. For example, the hyper core and the outer dendritic layer may be built by help of very different functionalities that provide molecules with unusual chemical and physical properties [26]. This method is used, for example, to construct large polyether dendrimers [26].

Double exponential dendrimer growth

Double exponential dendrimer growth (DEDG) is an accelerated convergent scheme for the preparation of monodendrons *via* a bi-directional synthesis. Double exponential growth begins with a diprotected monomer of the type A_pB_p and involves a repetitive use of a set three reactions (selective deprotection of $A_p \rightarrow A$, selective deprotection of $B_p \rightarrow B$, and coupling of A + B $\rightarrow$ A-B). The result of this method is a discrete straight-chain sequence of the type A_p-$(BA)_{(2^n-1)}$-Bp (n – the generation number). This process can be used for monomers of higher functionality leading to the double exponential dendrimer growth. The disadvantages of DEDG are that a pair of orthogonal protecting groups (rather than one) is required and the number of generations that can be realized before steric crowding occurs is apparently very limited. The yields of DEDG strategy are worse than for either divergent or convergent synthesis. The double exponential growth scheme has been used for the synthesis of a third-generation (255-mer) phenylacetylene monodendron [27] and α-sialodendrimers [62].

Comb-burst strategy

A linear polymer with a large number of reactive groups could be used as a dendrimer core. In this case, the molecule is a macromolecule to start with, but the synthesis method itself can be classified in one of the above mentioned groups [47].

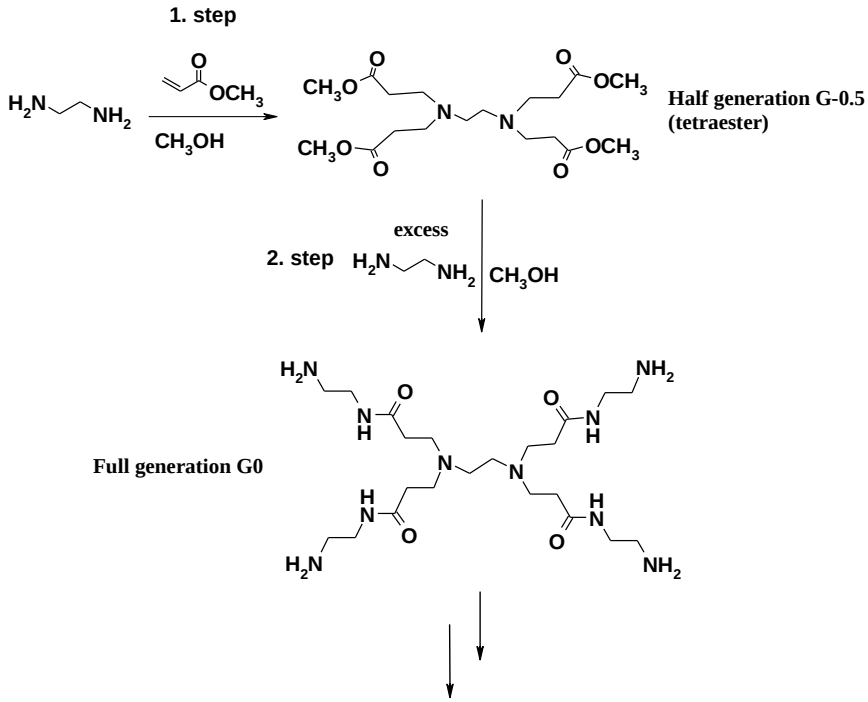
Hyper branching strategies

Recent advances in the synthesis of low-polydispersity hyper branched polymers have promoted interest in their dendrimer-like properties [48]. In the synthesis of a hyper branched polymer, AB_n ($n \geq 2$) type monomers are polymerized, but systematic protecting and deprotecting is not used. This allows synthesis of branched polymers in a single step, but the molecule is inevitably far less regular than in the case of other methods [63].

Other co-polymers of dendrimers and linear polymers also exist, some of which are obtained *via* growing of dendrimers onto sites on the polymer, others *via* attachment of dendrimers by bonds or chains [17,46].

4.4. PAMAM dendrimer

The starburst polyamidoamine (PAMAM) type dendrimer was first synthesized by Tomalia et al. in 1985 [18]. This dendrimer has interesting chemical and physical properties (e.g. it is a polyelectrolyte-base). A relatively simple divergent synthetic method for that dendrimer also exists (Scheme 5). The dendrimer core may be ammonia and primary amines like ethylenediamine (EDA) and tris(2-aminoethyl)amine. The first step of the synthesis of the PAMAM dendrimer is a Michael addition reaction of the amine to the double bond of an acrylic ester (usually methyl or ethyl acrylate). The reaction proceeds fast with a good yield. This reaction results in a tetraester (Scheme 5). The resulting tetraester represents the so-called half generation (G–0.5) of the PAMAM dendrimer. The second stage of the synthesis is an amidation reaction. The ester group of methyl acrylate reacts with an amino group of ethylenediamine. As a result, the full generation (generation 0; G0) dendrimer is formed. Now the outer layer of the dendrimer consists of free amino groups. In the amidation reaction, the amine is taken in a large excess to suppress the formation of intramolecular cycles and clusters [18,64,65]. Both steps are carried out in methanol. By repeating these two steps, the following higher generations up to the G10 have been obtained [36].



Scheme 5. Diagram of the synthesis of EDA-core PAMAM dendrimers.

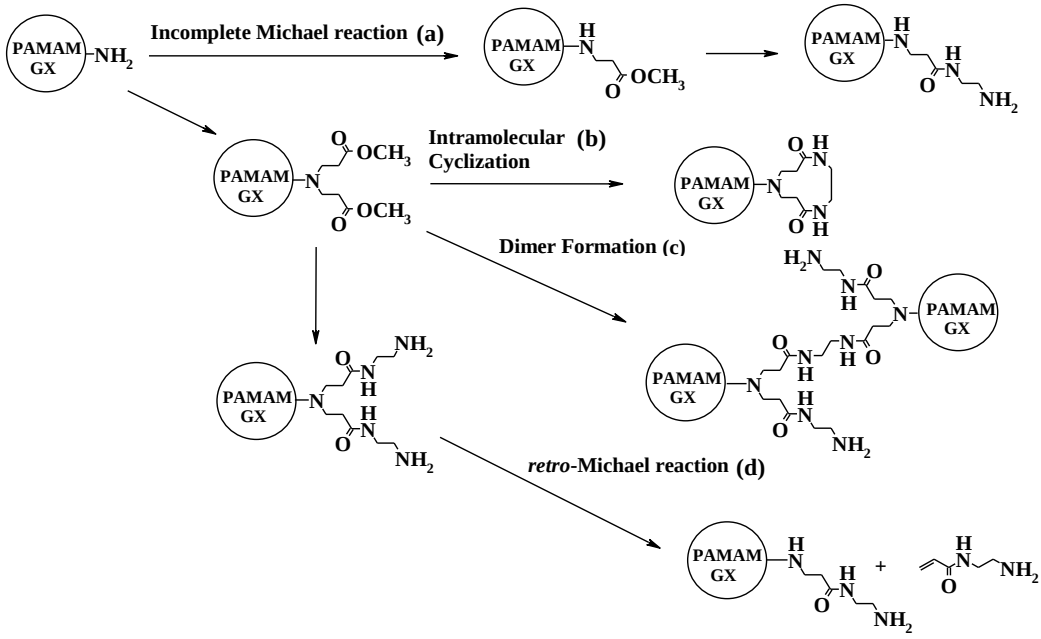
Several analytical techniques, including size exclusion chromatography (SEC) [36,64], low angle laser light scattering (LALLS) [36,66], infrared spectroscopy (IR) [67], capillary electrophoresis (CE) [68, I,

II], mass spectrometry (MS) (chemical ionisation (CI) [18], fast atom bombardment (FAB) [18], electrospray ionization (ESI) [69] and matrix assisted laser desorption ionization time of flight (MALDI-TOF) [70, IV]), and ^{1}H, ^{2}H, ^{13}C NMR [18,64,68,71] have been used for the characterization of PAMAM dendrimers.

4.4.1. Defects of the PAMAM dendrimer

Digressions from non-ideal dendrimer growth occur because of a variety of reasons and include the following (Scheme 6):

(1) incomplete Michael addition reactions
(2) intramolecular cyclization
(3) fragmentation due to *retro*-Michael reaction
(4) solvolysis of terminal functionality



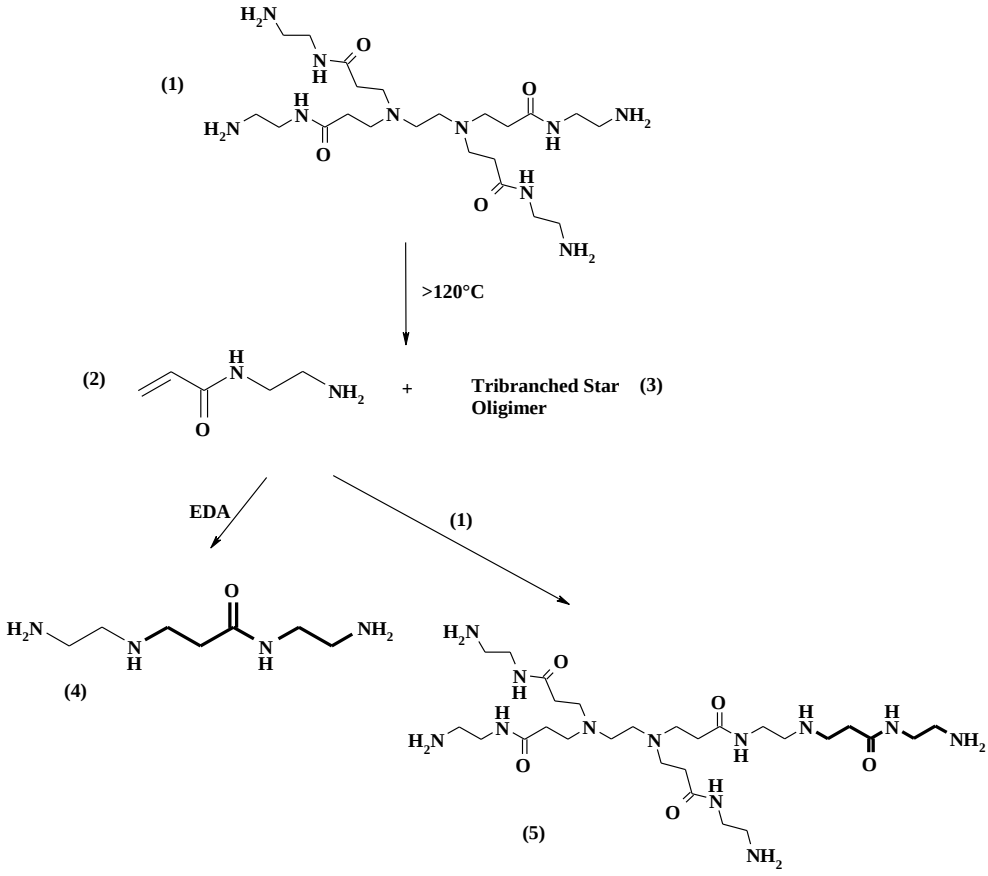
Scheme 6. Possible side-reactions in the PAMAM dendrimer synthesis.

The most important side-reaction is the incomplete Michael reaction, which gives a dendrimer where one or several branches are missing (Scheme 6, **a**). Another possible side-reaction is intramolecular cyclization (Scheme 6, **b**). This type of reaction occurs only during the formation of a full generation of the dendrimer. It is important to maintain a large excess of amine to avoid this side-reaction; nonetheless, such intramolecular cycles tend to form at higher temperatures [18]. As a result, that branch of the dendrimer is terminated. Since

the PAMAM dendrimer is synthesized by the divergent method, every step includes a large number of separate reactions. Because of that, defects are highly likely to form.

Retro-Michael reaction (Scheme 6, **d**) occurs at higher temperatures (≥ 60 °C) and at a very small extent also below 60 °C [64]. This reaction gives a dendrimer with missing (deleted) branches (Scheme 6, **d**). It was found that the zero generation dendrimer (core = EDA) could be reversed at a high temperature (i.e., > 100-120 °C) either in the presence or the absence of the EDA [18]. As a result of that reaction, the N-(2-aminoethyl) acryl amide chain is cleaved and a defective dendrimer with one missing branch is formed (Scheme 7). As can be seen from Scheme 7, the *retro*-Michael reaction results in the removal of N-(2-aminoethyl) acryl amide chain (**2**) from the ideally branched molecule and the new structure is again able to give the Michael addition both with ethylenediamine and an ideally branched dendrimer.

Besides these three main side-reactions, there is a possibility for a so-called dimer formation (Scheme 6, **c**), where two dendrimer molecules connect to each other in the amidation stage [64].



Scheme 7. Side-reaction due to the *retro*-Michael reaction [18].

One reason for the dendrimer fragmentation can be solvolysis of the amide bond. The modelling studies of the sixth-generation ammonia core dendrimer show that the internal surface area is 124% more available for solvent interaction than the external area [36]. It is assumed that all the amide bonds in a dendrimer, both internal and on the surface, are equivalent in relation to solvolysis. If the solvolysis occurs in the outermost amide bonds of PAMAM dendrimers, the number of amino groups in the dendrimer outer layer is decreased. As a result, charge density is decreased and that is unfavourable with regard to the transport efficiency of the dendrimer as a carrier in biological systems [12]. Formation of defective dendrimers also occurs if the unreacted EDA is incompletely removed from the reaction mixture. In that case, lower generation dendrimers are formed parallel to the main dendrimer in the course of the synthesis.

The bridged PAMAM dendrimers (dimers) can be identified and their influence can be minimized by the process optimization and/or purification techniques. It would be difficult to prepare dendrimers beyond generation 4 and 5 so that bridging is avoided [64]. It has been shown that generations 0-10 can be obtained in a highly monodispersed form with symmetrical branches of 95 mol% or greater per generation [72]. The properties of the defective dendrimers only slightly differ from the perfect ones and, therefore, they are hard to separate by physical methods.

4.4.2. Applications of the PAMAM dendrimer

There is an urgent need in molecular biology in various carriers of molecules like DNA, medicines, antibodies, contrast substances, radiopharmaceuticals, etc. into a cell. Up no now the most widely used carriers are based on viruses, lipids, polylysine, polyethylene imine, etc. For the last ten years, PAMAM-type dendrimers have been of special attention from that aspect. A variety of structural features, such as their size, internal cavities, and surface channels make PAMAM dendrimers ideal synthetic analogues of proteins, enzymes and viruses. The advantages of PAMAM dendrimers are

(1) their very good solubility in water

(2) the unique outer layer consisting of amino groups that are positively charged at the physiological pH.

The complexes of PAMAM dendrimers with DNA and antibodies are stable and non-toxic [8,10,11,14-16]. To carry out transfection, the higher generations G5-G10 of PAMAM dendrimers have been used [4,11,12]. The transfection capability of these generations is probably due to the high number of surface amino groups and the spherical shape of G5-G10 dendrimers. These characteristics allow simultaneous interaction with both negatively charged phospholipides on cell membranes and negatively charged phosphoric acid residues of DNA.

PAMAM dendrimers also give complexes with metal ions. Because of its relative stiffness (the chains are not too long and freely folding), it does not surround the metal ions from all directions. Such metals, bound to PAMAM,

but freely accessible by a reagent (in a reduced state) have been used for catalysis [73,74].

Due to its large charge and well defined (monodisperse) state, PAMAM dendrimers have been used in electrokinetic analysis, enabling good reproducibility [75,76]. These polymers can be used as calibration standards in size-exclusion chromatography [77] and as the delivery agent for X-ray contrast compounds [78,79].

Easily accessible PAMAM dendrimers have been used as the dendrimeric starting substances for the covalent addition of various substituents [80-86].

4.5. Characterization of dendrimers

The characterization of the structure of polymers, including dendrimers, is a complicated task. For the determination of PAMAM dendrimers, NMR [87] and IR spectroscopy [88] has been used. Both of these methods, as well as some other methods of chemical analysis (C, H, N analyses, CZE, LALLS, CI and FAB mass spectrometry, SEC), reveal the chemical nature of polymer repeating units. But neither of these methods allows us to describe the general architecture of the molecule. The molecule consists of similar repeating units, and to distinguish the signals of the units from different location of the molecule is quite impossible.

Also, the mass analysis of macromolecules is a difficult task. The standard methods for mass determination, like osmometry that gives an absolute value of the mass of the molecule, prove much less accurate in the case of macromolecules, giving only a certain average value of the molecular mass. The mass distribution can be determined (if possible) by the methods that separate polymers according to their size, such as chromatographic techniques (like GPC and SEC) [89].

All of these methods depend on the comparison of the analyte with a reference polymer sample of the known molar mass, composition, structure, and conformation. Even then these methods are not sufficiently precise: dispersity indices (defined as the ratio of number averaged and mass averaged molar mass) below some per cent cannot be determined. Since chromatography is a non-destructive method, the outlet from the column may be analysed in order to determine the absolute molecular mass. However, the capability of chromatographic methods in separating very complex mixtures has its limit. Also, it is possible to analyse such small quantities online: electrospray mass spectrometry has been done successfully by laser light scattering, both for SEC output [23] and GPC output [90].

Mass spectroscopy gives the absolute mass with a good accuracy: the errors are in tenths of per cent in the case of most inaccurate techniques, but usually smaller by one or two orders of magnitude [3]. That method can be applied if the molecule gives an easily identifiable molecular ion, which is simple in the case of smallest molecules. However, that is not so simple for large molecules. Because of that the so-called soft ionization techniques have been developed. The most suitable methods for macromolecule mass

determination are electrospray (ES) [69,91] and MALDI [90,92-94] MS. The other methods, like fast-atom bombardment, desorption-chemical ionisation, electrohydrodynamic, and secondary ion mass spectroscopy, cause excessive fragmentation of the molecule if its mass exceeds 10000. Additionally, the field desorption mass spectrometry is useful for molecular masses somewhat above 10000, but sample preparation in that case is difficult. Also, plasma desorption mass spectrometry like ES is useful up to molecular mass of 50000. However, ES is more favourable because it uses a flow of liquid sample. Thus its input could be obtained from the chromatography output. The limitations of molar mass determination arise from the possibility of multiple ionization: multiply charged ions form a large number of lines for the same molecule [94].

4.6. Modification of dendrimers

4.6.1. Modification of dendrimers by carbohydrates

Glycoconjugates like glycoproteins [85,96], glycopeptides [97], and glycopolymers [80] are quite common compounds in biochemistry. Already for the last ten years, such conjugates where various saccharides have been covalently bound to the functional groups of the outer layer of the dendrimer (glycodendrimers) have aroused great interest (Figure 3). Glycodendrimers are a new type of glycoconjugates, many of which have biological activity. As a result, they have been used widely, mostly in mutual interaction studies of saccharides and proteins on the molecular level.
Glycodendrimers can be divided into two classes:

- **(a)** Glycodendrimers, where saccharides (mostly mono- or disaccharides) are bonded to the outer layer of a dendrimer. This requires the existence of reactive functional groups on the outer layer of the dendrimer (e. g. $-NH_2$, -COOH).
- **(b)** Glycodendrimers where the saccharide itself (as a so-called “building block”) is used in the dendrimer synthesis.

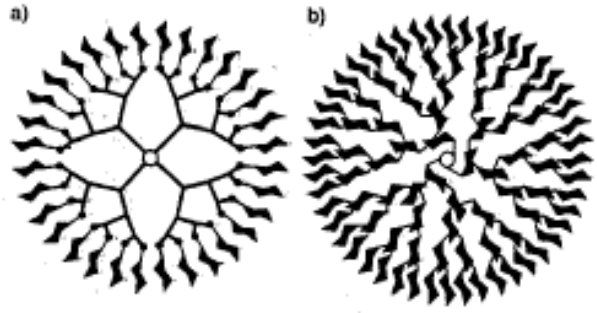
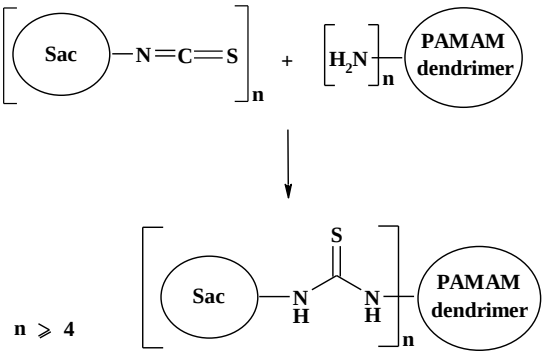


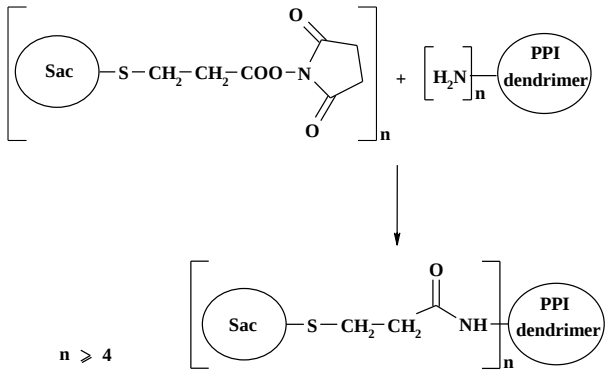
Figure 3. a – A dendrimer covered with carbohydrates, where the saccharides are located bound to the dendrimer outer layer, **b** – A dendrimer synthesized from carbohydrates [82].

In the synthesis of the glycodendrimers of the first type, various full generation PAMAM and PPI type dendrimers have mostly been used. Lindhorst and Kieburg [98-101] have synthesized glycodendrimers, where the acylated isotiocyanate derivatives of β-D-mannose, β-D-glucose, β-D-galactose, β-D-cellobiose and β-D-lactose have been covalently attached to the outer layer of the PAMAM dendrimer (G0, G1, G2) (Scheme 8).



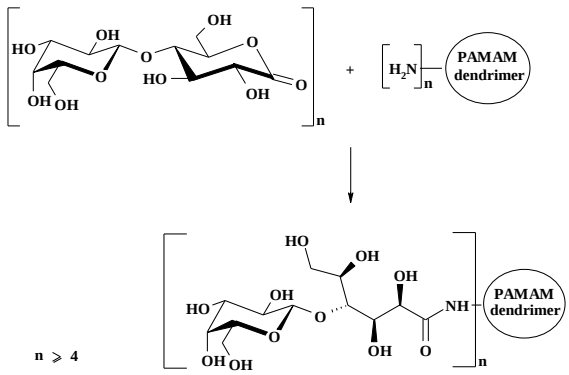
Scheme 8. Attaching carbohydrates to the PAMAM dendrimer *via* thiourea linkage.

The standard Zémplen method was used to remove acyl groups [102]. An analogous method for binding saccharides to dendrimers has also been used by Roy et al. who synthesized a dendrimer with the outer layer consisting of β-D-mannose residues [103]. Stoddart et al. modified PPI dendrimers with 4, 8, 16, 32 and 64 amino groups with thiogalactoside and thiolactoside derivatives, employing the *N*-hydroxysuccinimide method for linking (Scheme 9) [104-106].



Scheme 9. Attaching carbohydrates to PPI dendrimers using *N*-hydroxysuccinimide.

Okada et al. [107-109] synthesized the corresponding glycoconjugates from the second, third and fourth generation PAMAM dendrimers, using β-D-lactonolactone and β-D-maltonolactone. The resulting glycodendrimers are also known as “sugar balls” (Scheme 10).



Scheme 10. Attaching saccharides to a PAMAM dendrimer by amidation of its terminal amino groups with aldonolactones [107].

Roy and co-workers have published a series of the synthesis of dendritic glycosides based on polylysine and evaluated their biological activities [81]. The first compound in this class (e.g. the octamer illustrated in figure 4) incorporated *N*-acetylneuraminic acid (sialic acid) and *N*-acetylglucosamine as terminal residue (Figure 4) [110].

A series of similar polylysine-based dendritic glycosides, bearing nonreducing β-glucosaminyl, β-actosyl, β-lactosaminyl, and α-mannosyl residues, have also been prepared [111,112]. The construction of the above mentioned polylysine scaffoldings and the key couplings of sugar thiols to the terminal *N*-chloroacetyl groups were performed on a solid support (Figure 4 and Scheme 11) [81,113].

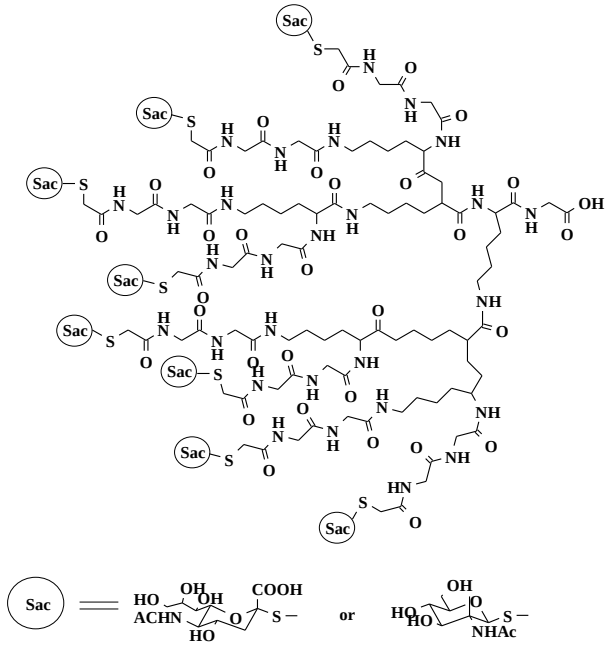
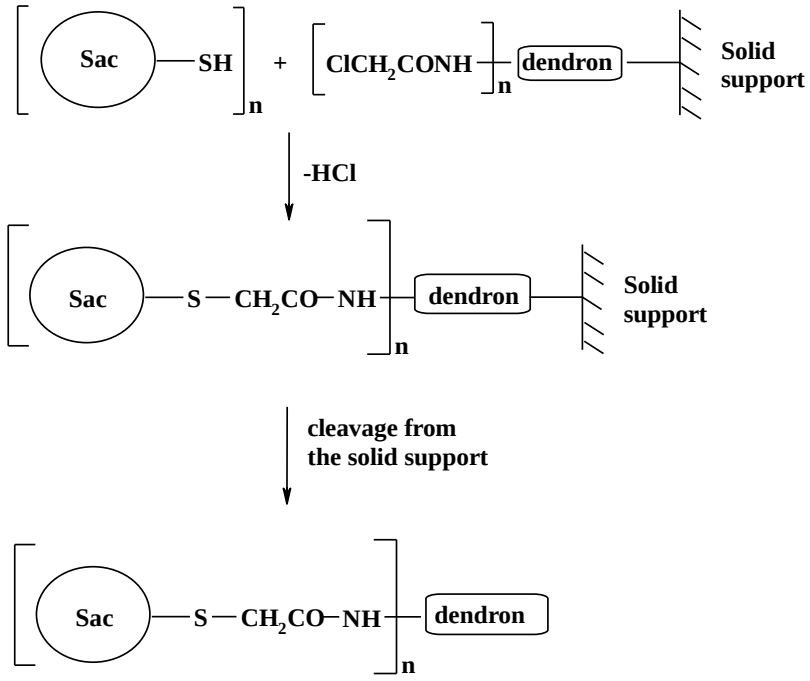


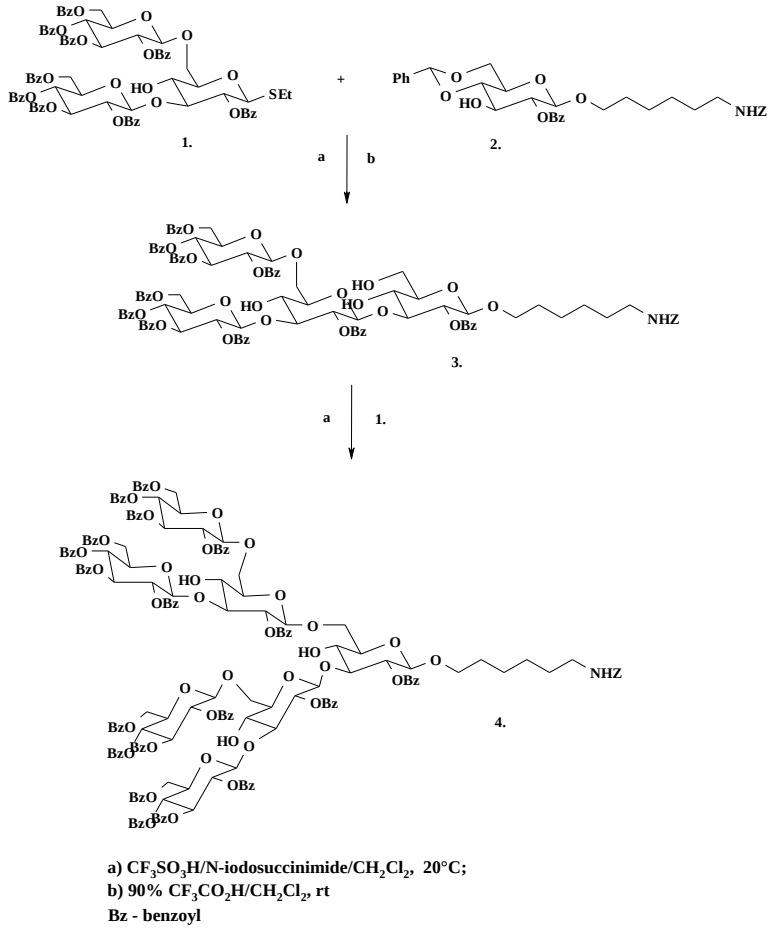
Figure 4. Dendritic polysialoside based on the highly branched oligipeptide core [81].



Scheme 11. Attaching thioylated saccarides to *N*-chloroacertyl termini of a dendritic wedge.

Using this method, the hydroxyl groups of the corresponding saccharides were acylated and they were deacylated only after binding the saccharide to dendron. Instead of polylysine dendritic wedge, Roy et al. have prepared phosphotriester group [81], gallic acid [114] and 3,3'-iminobis(propylamine) [115] based dendrons. The same dendrons were also modified with glucosamine, lactose and sialic acid residues, according to the similar methodologies described above [116].

Synthetic carbohydrate glycodendrimers are polysaccharide analogues that could obviously mimic a broad spectrum of polysaccharide structures and properties. Similarly to cyclodextrines they contain internal voids and are able to complex with different molecules. Glycodendrimers, e.g. glycoheptaoside (**4**), can be used as dendritic wedges [117,118]. The focal amino group can be generated by the removal of the Z (benzyloxycarbonyl) protecting group and, subsequently being available to react with a central core component (Scheme 12) [119].

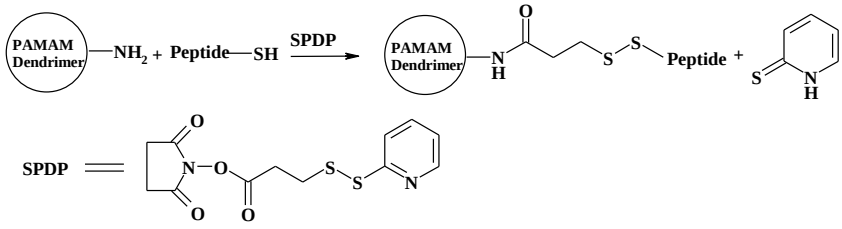


Scheme 12. Synthesis of the branched glycoheptaoside [119].

4.6.2. Modification of dendrimers by oligopeptides

Articles on the modification of dendrimers with oligopeptidies are relatively rare. Most attention has been focused on the PAMAM dendrimer as a carrier of different molecules in a biological system. Through the reactive amino groups present on the outer layer of PAMAM dendrimer it is possible (under mild reaction conditions) to link various peptide compounds to it. From literature it is known that for the synthesis of protein-protein, protein-peptide and enzyme-antibody conjugates, various linkers are used [120]. Szoka et al. have used the linker *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) to bind oligopeptide GALAcys covalently to the outer layer of the fifth generation of the PAMAM dendrimer (Scheme 13) [13]. SPDP is a heterobifunctional linker that carries a *N*-hydroxysuccinimide (NHS) and 2-pyridyldisulfide groups. When the NHS ester reacts with a primary amino group, a stable amide

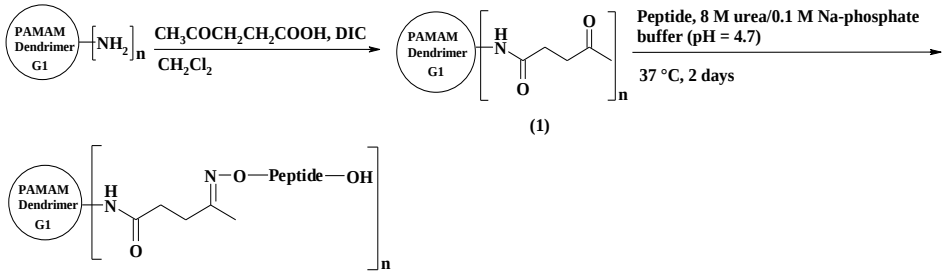
bond is formed and the 2-pyridyldisulfide reacting with the thiol group of the oligopeptide gives a disulfide bond (Scheme 13). On average, ten oligopeptides were bound to a single fifth generation PAMAM dedrimer. This type of binding has certain disadvantages: a cell may contain reducers that are capable of reducing the disulfide bond. An attempt was made to bind DNA to the resulting conjugate, but it was found that the interaction between DNA and the corresponding conjugate was weak. Also, the corresponding transfection effect was weaker than with an unmodified dendrimer.



GALAcys = WEAALAEALAEALAEHLAEALAEALEACAA

Scheme 13. Modification of the PAMAM dendrimer by oligopeptide using the SPDP reagent.

Lambert et al. linked oligopeptide to the first generation PAMAM dendrimer using a ketone as the linker [121]. To obtain peptide modified dendrimers, they introduced a ketone functionality to the surface of a first generation of the PAMAM dendrimer to obtain the keto-dendrimer (**1**). That compound was then condensed with aminoxy peptides to produce a peptide dendrimer (**2**) (Scheme 14). On average, 5 oligopeptides were linked to a single dendrimer by help of this method.



DIC = 1,3-diisopropylecarbodiimide

Peptide sequence = H_2NOCH_2CO-EYLNKIQNSLSTEWSPASVT-OH

Scheme 14. Synthesis of peptide dendrimers by Lambert et al [121].

Mihara et al. have synthesized the peptide dendrimers in which amphiphlic α-helix peptides containing cysteine (Cys) at their N-terminus were attached to the PAMAM dendrimer through the thioether linkage [122].

5. AIMS OF THE STUDY

So far the PAMAM dendrimer is the only one that has found a wide practical use, especially in molecular biology. As it is an efficient transfection agent in the biological systems, it is important to develop new modifications to make the dendrimer macromolecule more cell-specific.

Hitherto, the analysis and purification of the higher generations of PAMAM dendrimers and their conjugates has been problematic.

As our (and our co-operation partners') scientific interests were connected with the use of dendrimer conjugates in molecular biology, it was important to study the synthesis, analysis and the purification methods of dendrimers, and to elaborate their modification methods.

The main goals of the present investigation were:

- to study a step-by-step synthesis of EDA-core PAMAM dendrimers with an amidoamine-repeating unit in order to check the practical acceptance of the existing methodology
    - synthesize and purify chromatographically the first six generations (G0 through G5) of PAMAM dedrimers
    - separate the reaction side-products for the structure elucidation
- to characterize individual generations of the synthesized dendrimers
- to study the formation of defective dendrimers
    - the decay of PAMAM dendrimers in *retro*-Michael reaction
    - structural deviations in PAMAM dendrimers by stepwise synthesis/purification of the PAMAM dendrimer with simultaneous analysis
- to investigate the possibilities of modification of PAMAM dendrimers with saccharides
- to investigate the possibilities of modification of PAMAM dendrimers with oligopeptides

6. RESULTS AND DISCUSSION

6.1. Synthesis and purification of various generations of PAMAM dendrimer (I, III)

The main goal of the present study was to elaborate the synthesis of the EDA-core PAMAM dendrimers with amidoamine-repeating six generations (generations 0 through 5) in order to check the practical acceptance of the existing methodology and to prepare necessary quantities of dendrimers, and also their side-products for the structure elucidation (Figure 5).

The first six generations of EDA-core PAMAM dendrimers were synthesized in a stepwise process [I]. Preparation of every full generation consists of two subsequent steps: Michael addition of amino groups of the dendrimer (or in the very first case of EDA) to methyl acrylate, resulting in a multiester intermediate (which is a half generation dendrimer). The synthesis is followed by the amidation of the multiester intermediate with a large excess of EDA, resulting in a full generation dendrimer (Scheme 5). Capillary zone electrophoresis [I], ^{1}H and ^{13}C NMR spectroscopy [III] and MALDI-TOF MS [III] were used to determine the homogeneity of the synthesized dendrimers. According to the classical divergent method (PAMAM dendrimers are synthesized only by a divergent method), no purification is used. After the reaction is completed, only the excess of reagents are removed to prevent formation of side-products or formation of lower generation of dendrimers.

As we were interested in the side-reactions of the dendrimer synthesis, we also made an attempt to remove defective dendrimers (Scheme 6). Such side-products would otherwise accumulate in the course of the synthesis, complicating the analysis of the higher generation of dendrimers. Also, we tried to fractionate the major side-products and characterize them to understand better what side-reactions occur during the dendrimer synthesis. Because of those reasons, we introduced chromatographic purification steps and found that these methods were suitable in both synthesis steps: for the purification of full and half generations of dendrimers.

MALDI-TOF MS mass spectrometry was found to be the best method to characterize the purified products and separated side-products. In fact, for lower generation of dendrimers (up to G2), the resolution is within one mass unit that allowed us not only to estimate the purity of the separated products but also to define the structures of the side-products. Additionally, silica gel TLC was used to analyse the purified half generation fractions to find the presence of any side-products. Unfortunately, the NMR analysis appeared not very suitable for the characterization of the side-products and higher generation of dendrimers. The method requires a relatively large amount of pure material and the interpretation of the spectra of larger molecules is complicated due to a multitude of overlapping signals.

The half generation dendrimers (G-0.5-G4.5, multiester intermediates) were purified using column chromatography on silica gel and/or size-exclusion chromatography. The column chromatography on silica gel of G-0.5-G1.5 of PAMAM dendrimers was performed by using the methanol/dichloromethane mixture. In the case of G–0.5 and G0.5 generation, the method was effective

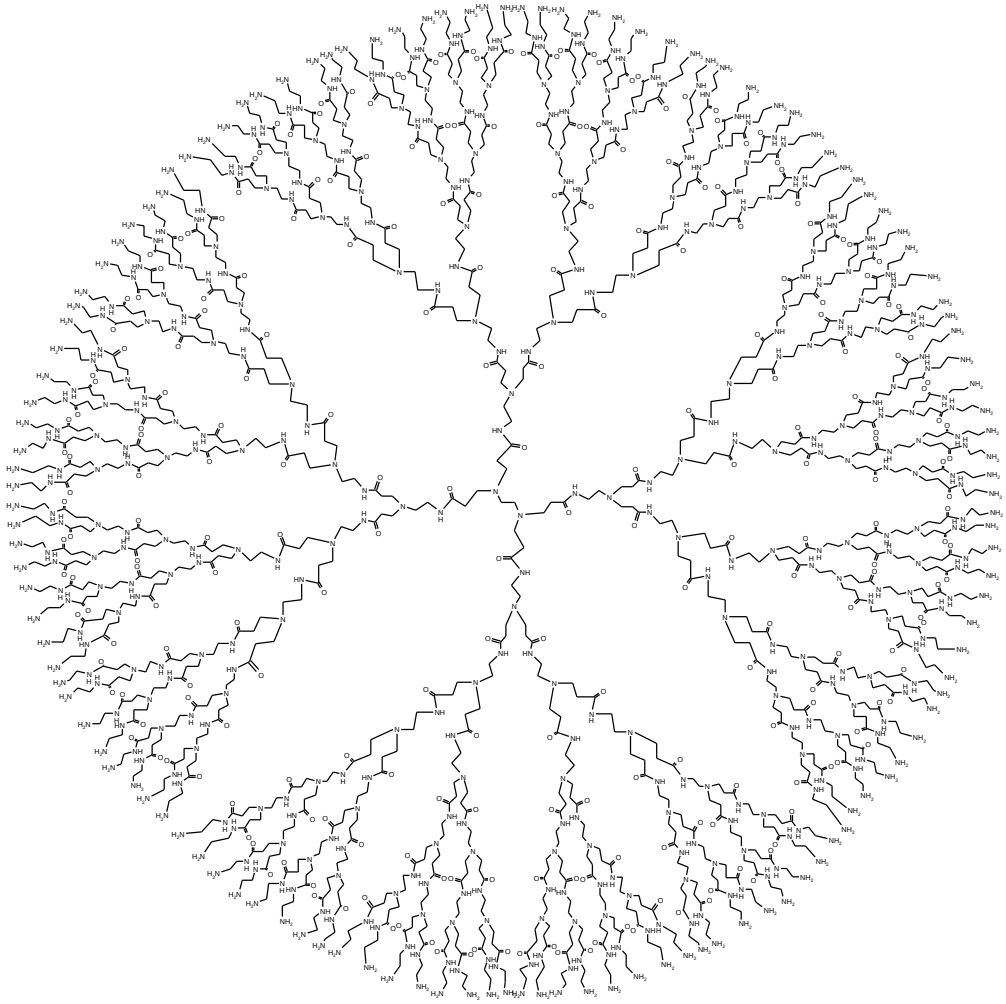


Figure 5. Fifth generation of the Starburst PAMAM type dendrimer.

and defective intermediates were fully removed. For G1.5, the method was less efficient, but we were able to remove some defective intermediates and lower generation impurities (G-0.5 dendrimers).

For half generations G2.5-G4.5, the silica gel chromatography was not suitable and these compounds were purified on Sephadex LH-20 gel, using methanol as an eluent. The method allowed us to separate efficiently lower molecular weight side-products.

Purification of full generations dendrimers is complicated because of the presence of a large number of amino groups in the outer shell (there are no literature references about that purification possibility). In the present work, we tested different chromatographic methods in order to find the best purification procedure.

We found that it is possible to separate selectively some defective compounds from G1, using HILIC and RP-HPLC chromatography.

The HILIC method was more suitable for selective separation of defective dendrimers that contained intramolecularly cyclized branches (-60 Da) and RP-HPLC for separating dendrimers with missed branches (-114 Da). The exact conditions are described in [III] (see Appendix, No. 1).

HILIC separation that depends on the number of amino groups in the outer layer of the dendrimer allowed a good separation of the defects caused by intramolecular cyclization from the crude first generation of the dendrimer (Figure 7, compounds **2** and **5**) [III].

The RP-HPLC method was especially suitable for the separation of dimerized dendrimers and dendrimers with deleted branches. The chromatograms of the first generation dendrimers are presented in the Figure 6A. The lowest molecular mass defect in the first generation is the compound **5** (Figure 7, t_R = 6.00-7.60 min., Figure 6A), which could be completely separated from the mixture. By using the RP-HPLC, the content of defective dendrimers (Figure 7 compounds **3** and **4,** t_R = 7.60-8.30 min., Figure 6A) in the purified product also decreased markedly. Still, the defective dendrimer **2** (Figure 7, t_R = 8.30-8.90 min., Figure 6A), having mass very close to that of G1 itself, could not be separated by this chromatographic method. The HPLC method also allowed the separation of the dendrimer "dimers" (Figure 7, compounds **6** and **7**, t_R=8.90-10.00 min., Figure 6A). Dimer **6** is known to be formed during the full generation synthesis stage, Scheme 6 **c** [69]. Dimer **7** could be formed during the half generation formation, as a result of the opening of intramolecularly cyclisized dendrimer branch, followed by the acylation of the resulting amino groups by the methyl ester of another dendrimer [III]. Usually the part of this kind of defective compounds in a crude product is so small that it could not be clearly detected by MALDI MS before chromatographic purification. The spectra in [III, Figure 6] prove that the defective dendrimers observed in the mass spectra of various generations of PAMAM dendrimers were formed in the course of the side-reactions during the synthesis rather than during the fragmentation of an ideally branched dendrimer during the analysis.

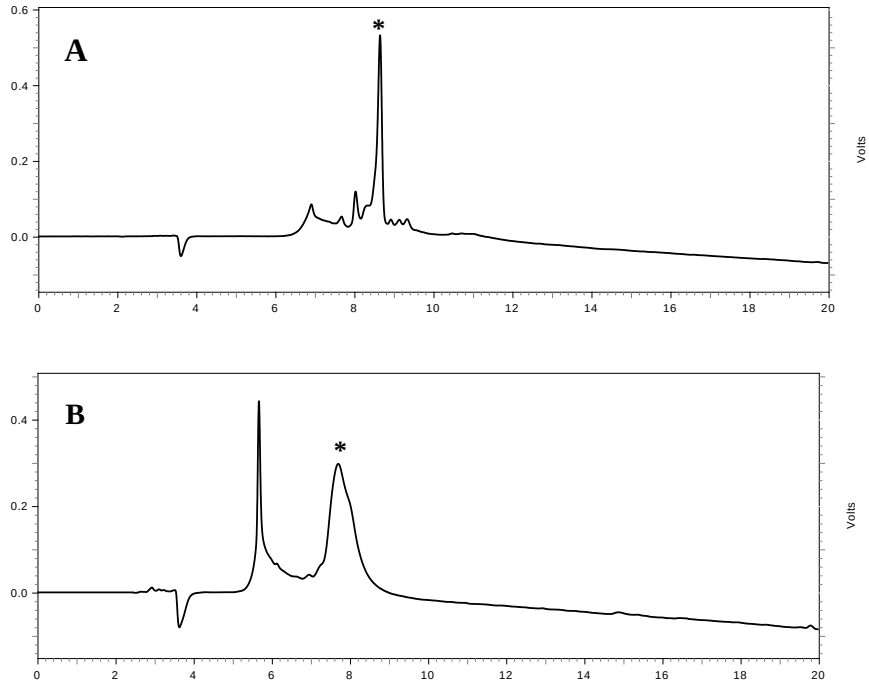


Figure 6. **A** - RP-HPLC chromatogram of the first generation G1 dendrimer. **B** - RP-HPLC chromatogram of the fifth generation G5 dendrimer. The eluent system was: **A** - 0.1% trifluoroacetic acid in water, **B** - acetonitrile. The linear gradient 0-50% of **B** in 20 min was used (1 ml/min).

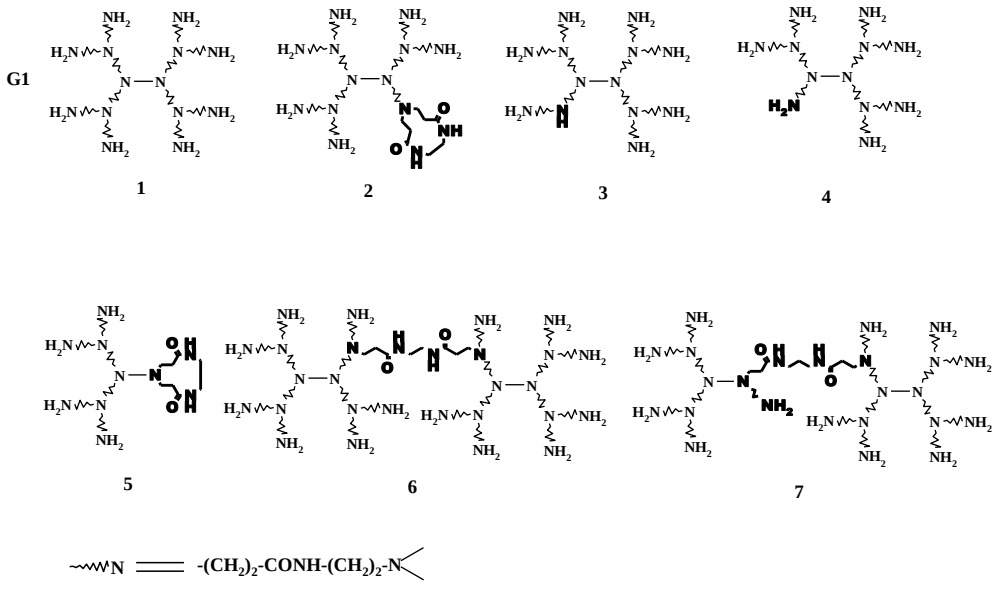


Figure 7. Defective structures of G1 dendrimer.

It is possible to analyse and purify the fifth generation of the PAMAM dendrimer (G5) chromatographically by RP-HPLC using the same conditions as for the first generation. The G5 is purified from the defective dendrimers which have a mass in the range 5000-15000 Da (according to the MALDI-TOF MS). The corresponding chromatogram is shown in the Figure 6B (t_R = 7.40-8.60 min., G5) The RP-HPLC was also used to separate G5 dendrimer from its conjugates with lower molecular weight side-products.

6.2. Complex investigation of structural deviation in PAMAM dendrimers using GC, CZE, ^{1}H and ^{13}C NMR and MALDI-TOF MS

6.2.1. *Retro*-Michael degradation of PAMAM dendrimers. GC analysis (II)

It is known that the dendrimers (especially its half generations) partially degrade in time [123]. Also, it is known that higher full generations PAMAM dendrimers (G5-G10), which are used for DNA transfection into cells, may lose their ability to mediate transfection after prolonged storage [12,124]. An assumption was made that the loss of activity is connected with the degradation of the dendrimer.

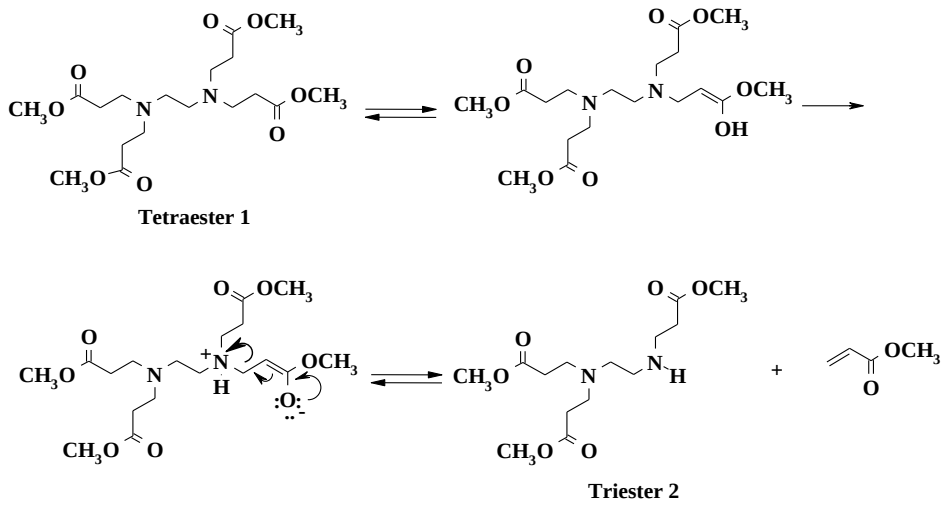
The key reactions of the PAMAM dendrimer synthesis (the Michael reaction) is known to be reversible [125]. The reverse reaction- the *retro*-Michael reaction is considered as one of the reasons for the observed changes in dendrimer properties. Also, the content of defective PAMAM dendrimers may be connected with the *retro*-Michael reaction. Therefore, we investigated the *retro*-Michael reaction in order to elucidate conditions of the reaction and its extent experimentally [II].

We followed the stability of the PAMAM dendrimer (G-0.5) in the methanol solution at different temperatures (-15 °C; 4 °C; ~20 °C; 50 °C). The G-0.5 generation of a dendrimer is the only generation having sufficiently low boiling point to be analysed by GC. That enables us to quantify exactly the amounts of the formed degradation products. To help to identify the products, the corresponding tetraester and triester of G-0.5 dendrimer (Scheme 15) were separately prepared and characterized by NMR analysis [II]. Attempts to analyse the higher generations of dendrimers by GC were not successful (probably due to the decomposition of dendrimers) at high temperature.

We observed the decomposing caused by *retro*-Michael reaction (Scheme 15) occurs already at 4 °C. In the course of the degradation, a corresponding triester product is formed. Only at -15 °C, the rate of *retro*-Michael reaction was low and no considerable decomposition was observed. In all cases, the equilibrium between the triester and tetraester products (Michael and *retro*-Michel reaction) was achieved (tetraester/triester ratio after 20 days: 50 °C 29/33 (achieved already after 165 h); ~20 °C 83/15; 4 °C 90/9; –15 °C 98/2).

It is obvious that similar *retro*-Michael decomposition occurs also in the case of higher generation dendrimers. Therefore, these results could be similarly applied when considering the stability of the higher generations of

PAMAM dendrimers (e.g. during storage of dendrimers). The obtained result may also be interpretated as one of the reasons for changes of the properties of PAMAM dendrimers during the storage over a long period (especially at room temperature). This might also be the reason why we did not succeed in obtaining a pure sample of the tetraester when using silica gel column chromatography purification, but obtained always a mixture of tetraester/triester 98.9/0.5. The practical conclusion from the result gained is straightforward: the possibility of the *retro*-Michel reaction should always be considered in preparation and storage of regular PAMAM dendrimers.



Scheme 15. The decomposition of tetraester to triester (*retro*-Michael reaction).

6.2.2. Separation of PAMAM dendrimer generations. CZE analysis (I)

Analysis of dendrimers is a complicated problem not only because of the large number of different regular structural units, but also because of numerous possible defective dendrimers present in a mixture. CZE method is the most straightforward and simple analytical method that may be used in the case of complicated separation/analysis problems. We investigated the possibilities of separation of different generations of dendrimers (homogeneity of the single generation) by using this method. Also, we wondered whether we can obtain valuable information about defective products in several low dendrimer generations by using this technique.

CZE separation of substances occurs when the analyte molecules are charged and have different apparent electrophoretic mobilities. It is assumed that the resistance experienced by the ion when flowing through the liquid medium is approximately proportional to the ion's mass (or size) and the mutual separation of ions takes place according to the charge-to-mass ratio [126]. This is the reason why half and full generations of PAMAM dendrimers exhibit

different electrophoretic behaviours depending on the pH value of the running buffer.

Dendrimer full generations have terminal amino groups that become protonated in an acidic medium and the positively charged molecules move during the electrophoretic process in the direction of an electroosmotic flow (i.e. towards a cathode). The calculated charge-to-mass ratio remains essentially constant for all PAMAM generations (assuming that all amino groups are protonated) and electrophoretic velocities of different generations should be close to each other. Nevertheless, our experiments showed that in the acidic phosphate buffer (pH = 2.7), where the electroosmotic flow is practically suppressed, the first six generations of EDA-core PAMAM dendrimers with molecular masses ranging from 516 to 28788 Da could be separated (Figure 8).

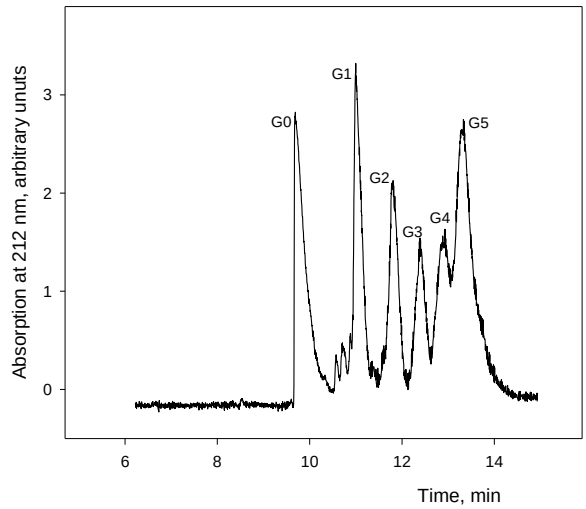


Figure 8. Electropherogram of the first six generations of EDA-core PAMAM dendrimers.

Half generation dendrimers (amidoesters) are not ionized at the pH range commonly used for CZE and we did not succeed separating the mixture of different half generation dendrimers. In a moderately basic phosphate buffer with pH = 7.8, however, half generation dendrimers are remarkably labile and hydrolyze readily. In these conditions, we observed fast hydrolysis of the ester groups of dendrimers (clearly seen by CZE).

6.2.3. Identity of dendrimer generations. ^{1}H and ^{13}C NMR spectroscopy (III)

High field (11.7T) ^{1}H and ^{13}C NMR spectroscopy was used to follow the addition of new generations to the EDA core of PAMAM dendrimeric molecules. The addition of identical branches to the initial core and the fast increase of the molecular weight of the dendrimer results in the overlapping of signals of intensities strongly different from non-equivalent positions. Thus, in the ^{1}H spectra already in generation 0.5 out of 9 different bands, only 7 are clearly resolved. In ^{13}C NMR spectra all signals from different positions are resolved up to generation 2.5 (Figure 9).

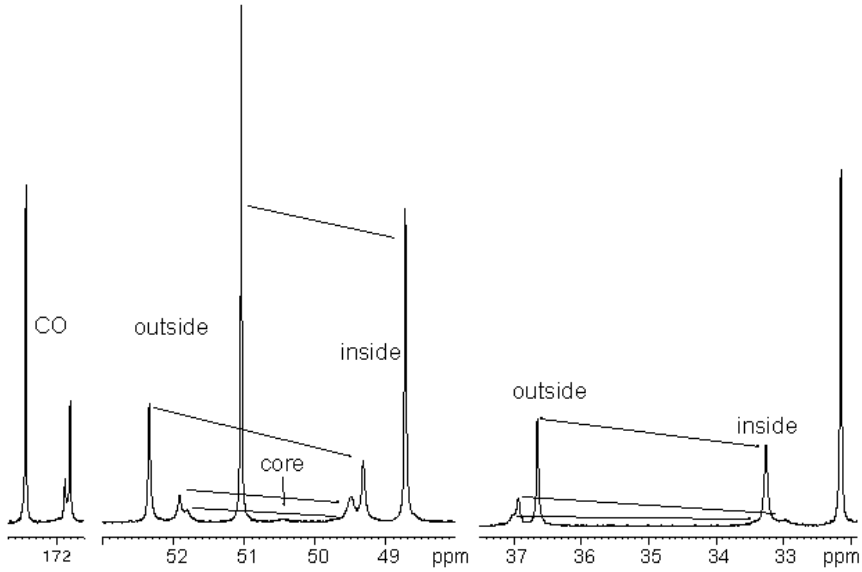


Figure 9. 125.77 MHz ^{13}C NMR spectrum of EDA core based PAMAM G2.5 dendrimer in $CDCl_3$. Indicated by arrows are pairs of connected methylene carbons. Inside the carbons there are broad lines. The assignment of lines is given in [III, Table 1].

The chemical shift data for ^{1}H and ^{13}C nuclei ($CDCl_3$ solution) for the first generations of PAMAM dendrimers are given in [III, Table 1]. The solubility of higher-generation PAMAM dendrimers in chloroform is too low for NMR studies and, therefore, some CD_3OD was added. Comparison of the spectra obtained with the published NMR data on EDA core PAMAM dendrimers [18,36,65] reveals some obvious assignment errors and misprints in earlier publications. Usually, in raw synthetic products, several irregular branching products were always observed in low concentrations. In different layers of dendrimer molecule, the same nearest environment forms for the same atomic groups and this is a good starting point for the assignment of different signals within individual layers. Despite the possible solvent, concentration and impurity effects, some general trends from long-range effects could be

observed. The most remarkable of them is a nearly 2 ppm change of the core methylene carbon shift to a higher field. Within the pairs of two connected methylene groups between the CO group and N atom, or between two N atoms, the inner methylene carbon in this pair has always a broader signal (Figure 9). This can be attributed to the lower mobility of this methylene group in the movement towards the core of the dendrimer molecule. This phenomenon has been observed even on the outer layer of the dendrimer molecule and was also referred to in the published spectrum of the terminal, the $-NHCH_2CH_2NH_2$ group of the PAMAM dendrimer [36].

6.2.4. Structural deviations of PAMAM dendrimers. MALDI TOF MS analysis (III)

In this work we investigated the potential of MALDI-TOF MS as an analytical tool for the analysis and control of the synthesis of Starburst PAMAM dendrimers. Using the spectra of the lower generation dendrimers, we also intended to provide a closer view on the fragmentation mechanism during the MALDI analysis.

Different standard matrixes, such as 2,5-dihydroxybenzoic acid (dhb), 4-hydroxy-3-methoxycinnamic acid (ferulic acid), α-cyano-4-hydroxycinnamic acid, dithranol, and dhb/fucose 1:1 mixture, were tried for dendrimers. We found dhb/fucose is the matrix of choice for full generation dendrimers in both, providing the best signal and causing least fragmentation. We also proved α-cyano-4-hydroxycinnamic acid to be a "hot" matrix, causing most fragmentation just as in the case of peptides and proteins. We used both linear and reflectron modes to measure mass spectra and obtained isotope resolution up to mass 4000 Da. This provided us with precise mass numbers, facilitating precise conclusions about both ideally branched and defective molecular structures, and also allowed us to identify a novel dissociation channel.

MALDI mass spectra were measured for all half and full generations up to G5 in the linear mode and up to G2.5 in the reflectron mode. Most of the analyses were done in the linear mode. Using this mode, the molecular dissociation products are, in general, visible and the discrimination against high mass is smaller. The full isotopic resolution and superior accuracy of the reflectron mode was used to confirm our assignments of the defective dendrimer structures and prompt decay products. The limitation of the reflectron mode is that we see only the prompt decay products that are formed within the initial delay between the laser pulse and acceleration pulse, but we lose all information about the ions that decompose in the flight time, as these are not focused on the detector by our gridless ion reflector.

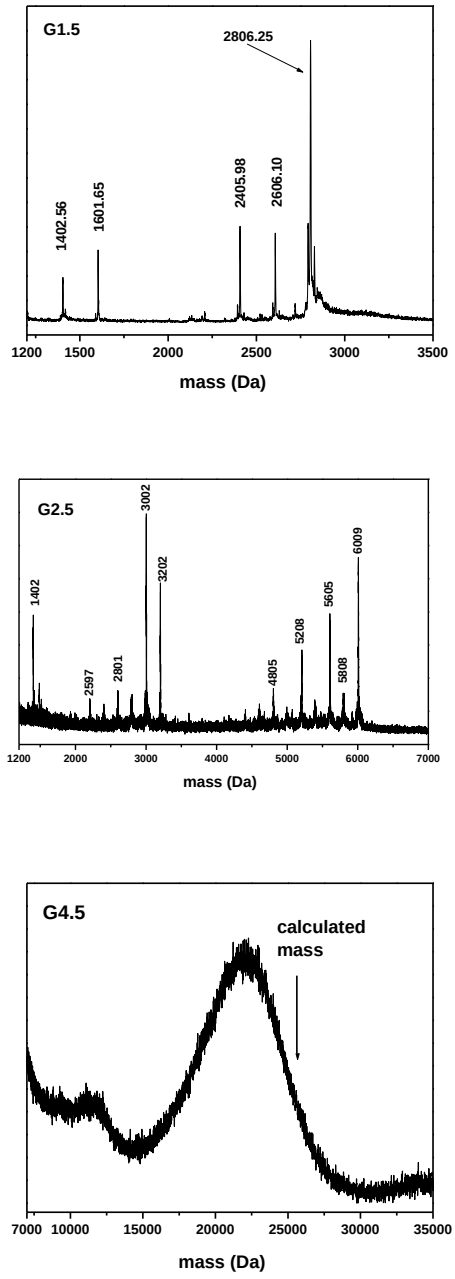


Figure 10. MALDI-TOF MS spectra of the G1.5, G2.5 and G4.5 PAMAM generations.

We obtained spectra for half generations with all matrixes tried. Up to G1.5 (Figure 10), the spectra near MALDI threshold consist of sharp lines corresponding to the ideally branched and the defective protonated dendrimers, their fragmentation products and weak lines corresponding to the Na and K

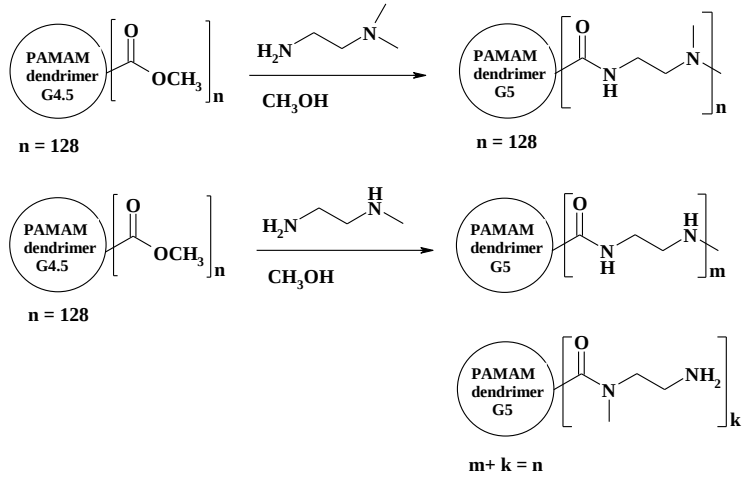
adducts. We have matched all lines in the spectra with the corresponding structures of defective dendrimers ([III], Figure 4). Beginning with G2.5 (Figure 10), unresolved background emerges and for G4.5 (Figure 10), no line structure is observable any more. Still, molecular weight distributions are readily obtained.

For dendrimer full generations, good spectra could be obtained with standard matrixes only for G0 and G1 ([III], Figure 3). For higher generations, the spectra lines became weak, excessive laser power was needed, and for G4 and G5, strong enhancement of the low mass range was observed due to strong mass discrimination or extensive prompt decay of molecules. As there was no reason to expect that full generations are harder to ionize than half generations, we assumed that the reason is the matrix incompatibility. We continued with a mixed matrix dhb/fucose 1:1 that provided us with equally good spectra for both half and full generations. It is a well known "cold" matrix that is used to suppress fragmentation in peptide research and does it in the case of dendrimers as well. As in the case of the half generations, the higher generations (G4 and G5) do not give the line spectra ([III], Figure 3). Still, general molecular weight distribution is well observed.

6.3 Modification of PAMAM dendrimers

6.3.1. Modification of PAMAM dendrimers by *N*-substituted and *N,N*-disubstituted ethylenediamine

The outer layer of the ordinary PAMAM dendrimer is covered with primary amino groups. In this work, the modified fifth generation PAMAM dendrimers with *N,N*-dimethylamino or *N*-methylamino groups in the outer layer were synthesized according to Scheme 16:



Scheme 16. Modification of the end groups of the PAMAM dendrimer with *N,N*-dimethylethylenediamine or *N*-methylethylenediamine.

In the first case, during the synthesis of G5, instead of ethylenediamine, *N*,*N*-dimethylethylenediamine was added to G4.5. As a result, the outer layer of the dendrimer consists of *N*,*N*-dimethylamino groups (Appendix, No. 2a).

In the second case (reaction of *N*-methylethylenediamine with G4.5), there is also a possibility of the reaction of G4.5 ester group with *N*-substituted amino group. Thus, the outer layer of this dendrimer consists of methylamino and amino groups (Appendix, No. 2b).

These types of dendrimers were used in the investigation of the transfection of DNA. It was established that their transfection effect was very weak in comparison with the ordinary G5 PAMAM dendrimer (see Part 6.4).

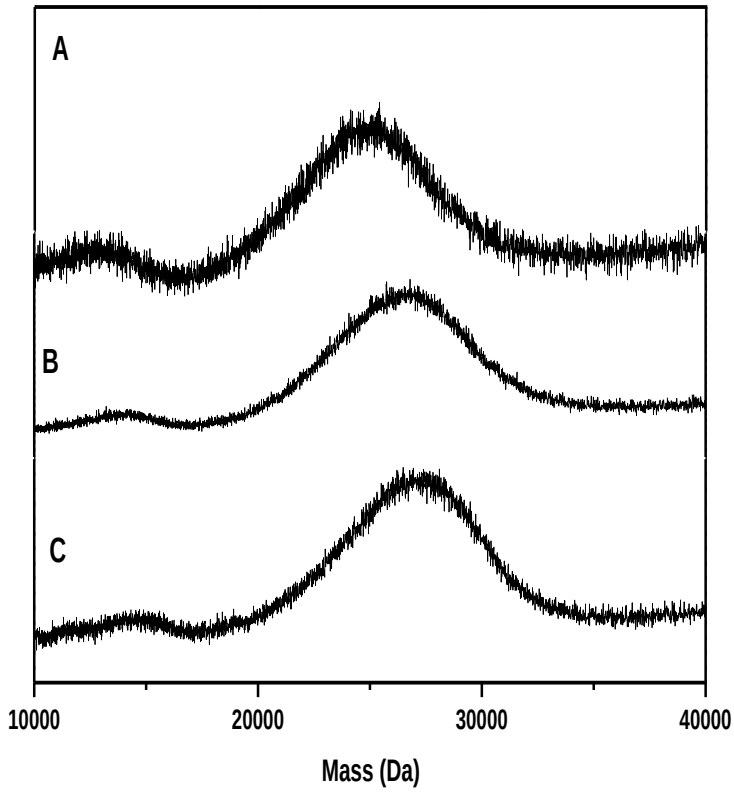


Figure 11. MALDI-TOF MS spectra of the fifth generation of PAMAM dendrimers (**A**), dendrimers modified with N-methylethylenediamine (**B**) and dendrimers modified with *N*,*N*-dimethylethylenediamine (**C**).

6.3.2. Modification of PAMAM dendrimers with carbohydrates

In order to investigate the possibility of making dendrimers more cell-specific in various biological processes (for example, in transportation of DNA into cell), we have investigated the binding of oligosaccharides covalently to the surface of a dendrimer.

It is known from literature that several cell membrane receptors like CD44, RHAMM, ICAM-1 are sensitive to hyaluronic acid (HA) [127,128]. Based on that information, we made an attempt to synthesize a glycoconjugate of HA oligosaccharide and the PAMAM dendrimer.

Hyaluronic acid is a linear polysaccharide consisting of alternating 1,4-linked units of 1,3-linked glycuronic acid and *N*-acetylglucosamine (Figure 12) and is one of several glycosaminoglycan components of the extracellular matrix (ECM) of the connective tissue. HA differs from the other major glycosaminoglycans in that it does not contain sulfate groups.

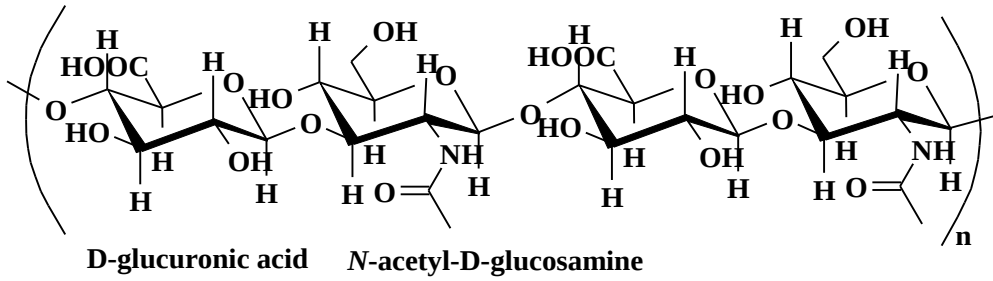


Figure 12. Tetrasaccharide fragment of HA (disaccharide repeat units are shown).

HA has been chemically modified *via* carboxyl group, *via* hydroxyl groups, *via* the reducing end, and in some cases partially *via* *N*-deacetylated materials [129]. Our final goal was to develop a method that allows us to attach hyaluronic acid oligosaccharide molecules to the outer layer of the fifth generation PAMAM dendrimer.

As we had a very small quantity of ready-made oligosaccharide (16 monomeric units) available (1 mg) for the synthetic experiments, it was necessary to develop the reaction conditions on the model compounds. Based on the knowledge that the MALDI-TOF MS spectra of the early generation PAMAM dendrimer are well resolved and that these dendrimers can be obtained relatively free from defects, we have selected purified G1 as a model compound ([III], Figure 3). The saccharides selected as model compounds for the synthesis were D-glucose (Glc), D-*N*-acetylglucosamine (GlcNAc), and disaccharide unit of hyaluronic acid (DiHA) (Figure 13).

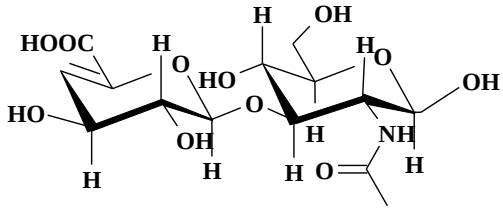
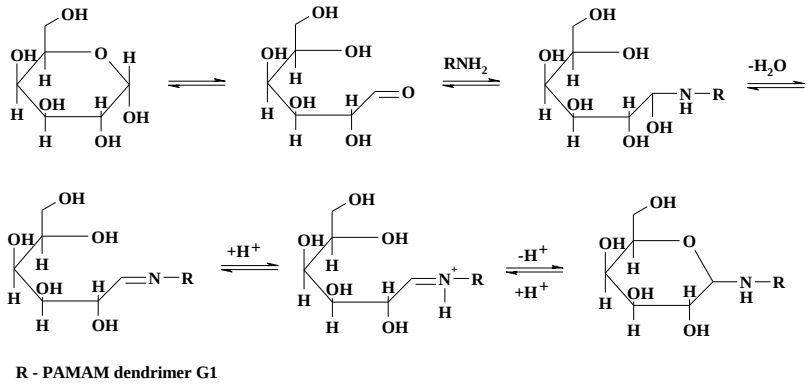


Figure 13. Hyaluronic acid disaccharide.

The aldehyde form of the saccharide reacts with the amino group of the dendrimer resulting Schiff base (imine) (Scheme 17).



Scheme 17. Intermediates in the attaching of saccharide to the first generation PAMAM dendrimer.

6.3.2.1. Reactions between G1 dendrimer and saccharides

The reaction between dendrimer G1 and D-glucose or D-*N*-acetylglucosamine or disaccharide of hyaluronic acid was conducted under the same conditions (Appendix, No. 3). In all cases, Schiff base-type compounds were formed, but the reactivity of saccharides toward dendrimer was different.

Using Glc as a saccharide, the following products were identified from the mass spectra: G1+1×Glc (1592.48 Da), G1+2×Glc (1754.10 Da), G1+3×Glc (1916.06 Da), G1+4×Glc (2077.54 Da) and G1+5×Glc (2239.10 Da) (Figure 14A). A similar addition of Glc to the defective dendrimers was also observed. An analogous situation occurs in the case of GlcNAc (Figure 14B). The only principal difference between those two cases was that in the last case, the mass spectra did not reveal the compounds G1+4×GlcNAc and G1+5×GlcNAc and the peaks corresponding to the products G1+1×GlcNAc (1633.27 Da), G1+2×GlcNAc (1836.67 Da), G1+3×GlcNAc (2039.02 Da) had a lower intensity. When hyaluronic acid disaccharide was used, only the compound G1+1×HA disaccharide was detected in the mass-spectra.

It was found that under given conditions, the equilibrium between the corresponding saccharide and G1 dendrimer was established in about one day (Appendix, No. 3, Figure 15). As the formation of Schiff's base in aqueous environment is reversible, a certain amount of the starting compounds is always present. These compounds may again result in numerous side-products. It was clearly observed on standing of saccharide-dendrimer conjugates.

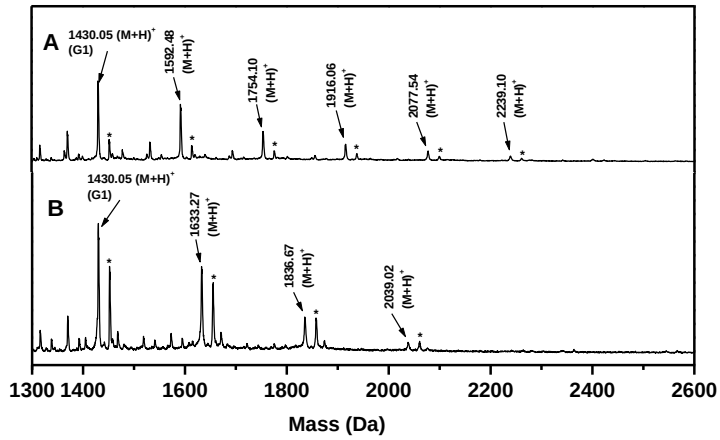


Figure 14. MALDI-TOF MS spectra. **A** – the reaction mixture of the first generation of PAMAM dendrimer and Glc in water solution, **B** - the reaction mixture of the first generation of PAMAM dendrimer and GlcNAc in water solution. ***** - corresponding sodium ion.

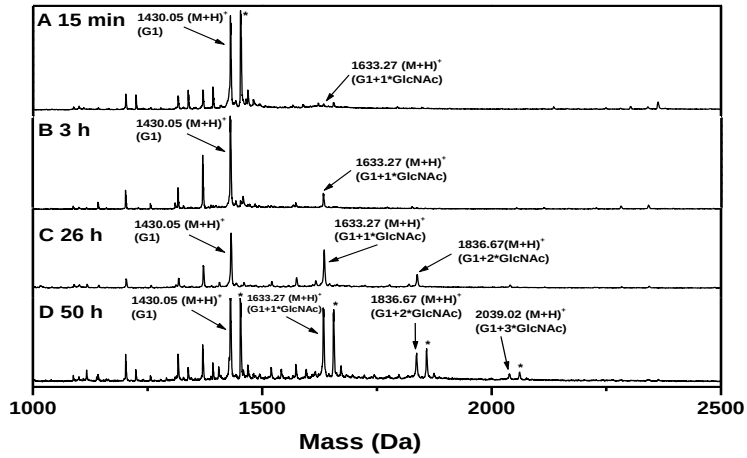
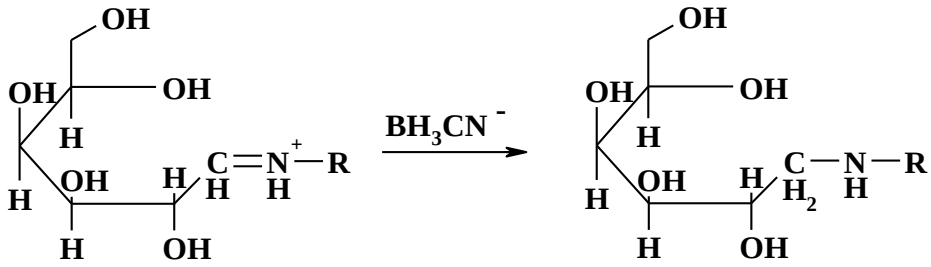


Figure 15. MALDI-TOF MS spectra of samples taken at different times during the reaction that show the establishment of the equilibrium between the first generation PAMAM dendrimer and GlcNAc. ***** - corresponding sodium ion.

6.3.2.2. Reaction between G1 dendrimer and saccharides in the presence of $NaCNBH_3$

The influence of the equlibrium may be eliminated by reducing the imine group into a secondary amine. The Schiff's base can be reduced into a secondary amine with sodium cyanoborohydride (frequently used reagent for such purposes in biochemistry [130-133]). The elimination of the imine from the reaction mixture makes the reaction unidirectional (Scheme 18).



R - PAMAM dendrimer G1

Scheme 18. Reduction of the Schiff base to the secondary amine by $NaCNBH_3$.

When $NaCNBH_3$ was added to the reaction mixture, containing G1 and saccharide, in the mass-spectra, the lines exceeding mass by two units were observed (Appendix, No. 3). That is exactly what was expected: after reduction of the Schiff's base, the corresponding mass numbers should increase by two mass units (Scheme 18). When the reaction mixture was analysed 28 h later, low-intensity lines, with mass numbers exceeding those of G1 and Glc addition products by +25 Da appeared in the mass spectra. Since the reaction mixture contains CN^- (originating from $NaCNBH_3$), there is reason to think that the compound is formed by adding CN^- to the corresponding Schiff base (which forms from the unreacted saccharide and dendrimer). When the reaction mixture was analysed two and seven days later, a new peak corresponding to the unidentified compound with mass +26 Da appeared and the intensities of the peaks corresponding to the target products decreased. The intensities of the spectra lines corresponding to the +25 Da adducts did not change during this period. A similar phenomenon also occurred when GlcNAc was used instead of Glc (Figure 16).

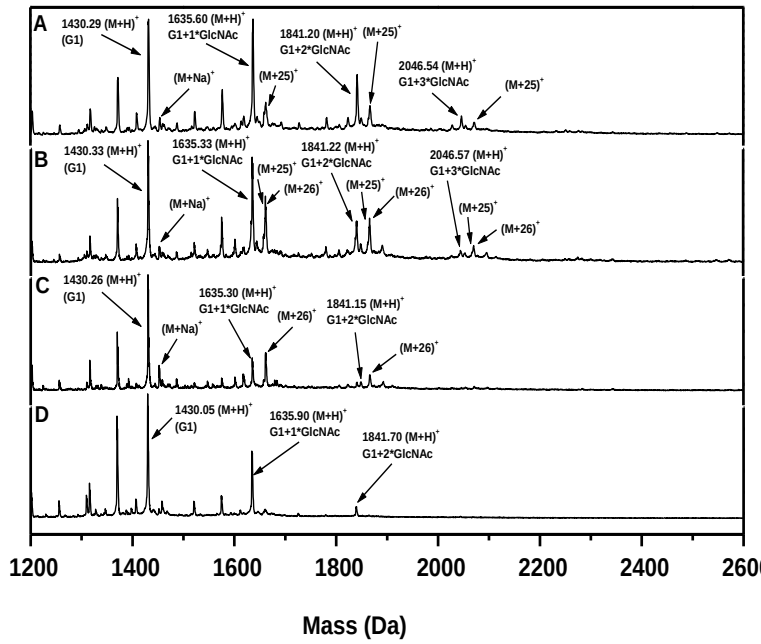


Figure 16. MALDI-TOF MS spectra characterizing the ratio between reaction products and impurities in the reaction mixture during the reduction of Schiff base between G1 and GlcNAc with $NaCNBH_3$.
A - 28 h after the beginning of the reaction
B - 2 days after the beginning of the reaction
C - 7 days after the beginning of the reaction
D - reaction products purified of impurities with RP-HPLC

In the case of GlcNAc, the reaction was slower compared to Glc, because of that the products G1+4×GlcNAc and G1+5×GlcNA were missing and the peaks corresponding to compounds G1+1×GlcNAc (1635.90 Da), G1+2×GlcNAc (1841.70 Da), G1+3×GlcNAc (2046.58 Da) had a lower intensity than in the case of Glc (Figure 17). The reaction seemed to be even slower with hyaluronic acid disaccharide only, and so a weak signal corresponding to a single saccharide molecule added to G1 dendrimer (1793.39 Da) was detected (Figure 17).

To avoid the side-reactions between the dendrimer conjugate and CN^- anion, the reaction mixture was purified by RP-HPLC, which allowed the separation of inorganic salts (NaCN and Na_3BO_3), unreacted saccharide and impurities with mass < 900 Da (Figure 18). According to the MS analysis, the conjugate did not contain any side-products after the purification (Figure 16D). The purified conjugate was stable on prolonged standing. Thus, the optimum conditions for preparation of G1 dendrimer-saccharide conjugates require a

simultaneous reduction of the formed Schiff's base by sodium cyanoborohydride and chromatogrphic purification by RP-HPLC. It was also found that too large excess of $NaCNBH_3$ increased the formation of side-products.

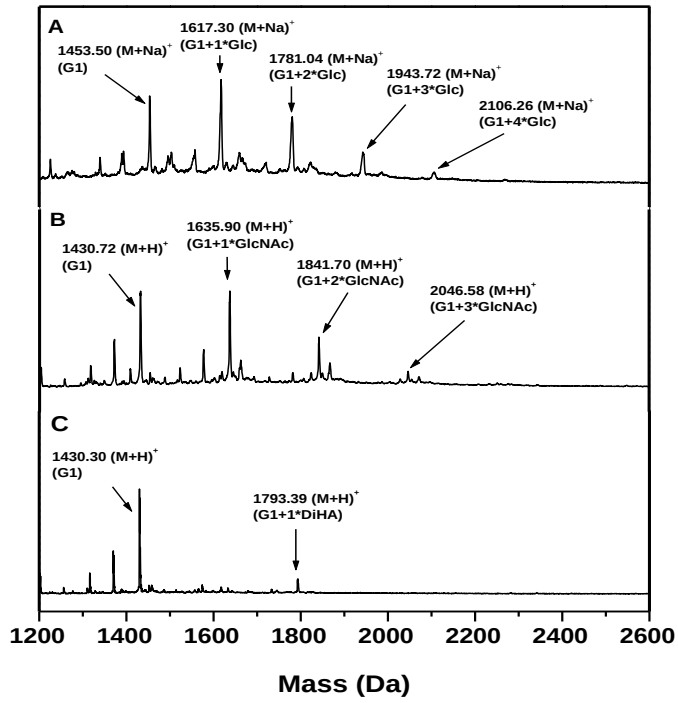


Figure 17. MALDI-TOF MS spectra of the crude products after the reduction of Schiff base between G1 dendrimer and various glycosides (**A** – Glc, **B** – GlcNAc, **C** – DiHA) with $NaCNBH_3$.

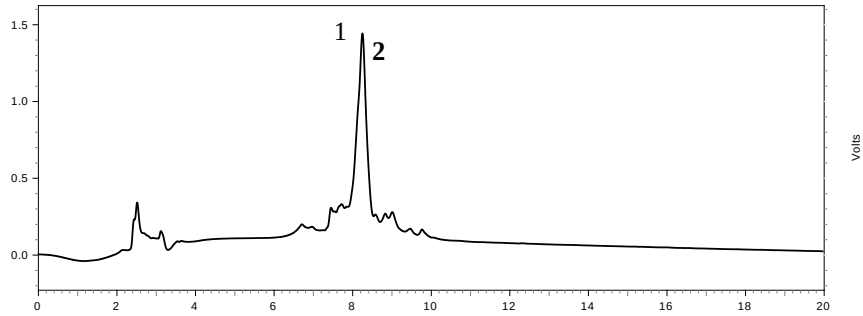
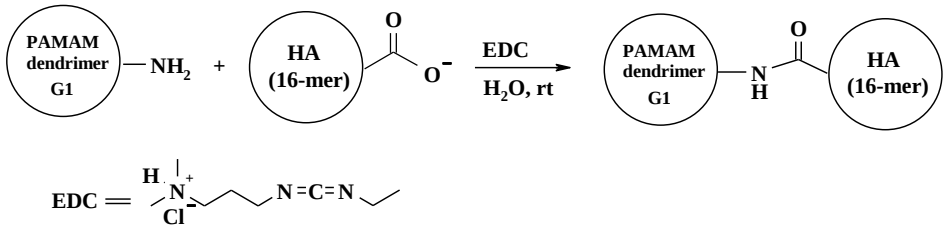


Figure 18. RP-HPLC chromatogram of the reaction mixture containing G1 dendrimer (including defective molecules) (**1**) and the corresponding conjugates of G1 dendrimer and GlcNAc (**2**). The eluent system was: **A** - 0.1% trifluoroacetic acid in water, **B** - acetonitrile. The linear gradient 0-50% of **B** in 20 min was used (1 ml/min).

6.3.2.3. Binding the hyaluronic acid oligosaccharide to G1 dendrimer by EDC carbodiimide

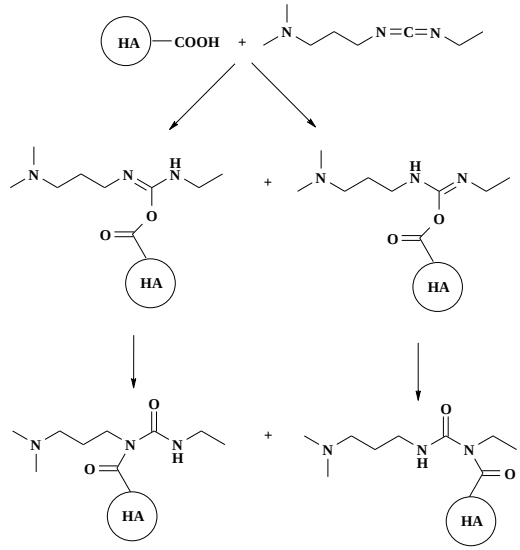
In order to find a method that might be used also on a micro scale, we tried to attach HA oligosaccharide 16-mer (contained also small amounts of 14-mer and 18-mer as impurities) to the first generation dendrimer, using carbodiimide chemistry (Scheme 19, Appendix, No. 4).



1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride

Scheme 19. Attaching hyaluronic acid oligosaccharide to G1 using EDC.

RP-HPLC allowed the purification of the reaction mixture from compounds with mass < 900 Da. The unreacted oligosaccharide was also collected as a separate fraction. In the MALDI-MS spectrum of the main fraction (t_R – 8.10-8.60 min, Figure 19), the desired product (4465.08 Da) was observed, however, in quite low intensity (Figure 20). The spectrum revealed also a peak that corresponds to a conjugate (3208.62 Da, Scheme 20) that is likely a hyaluronic acid *N*-acylisourea. The presence of the conjugates of 14-mer (2828.88 Da) and 18-mer (3587.45 Da) was also observed. The formation of *N*-acylisourea is a reaction that has been described earlier in the literature (Scheme 20) [134].



Scheme 20. Possible reaction pathways for coupling of EDC with HA [134].

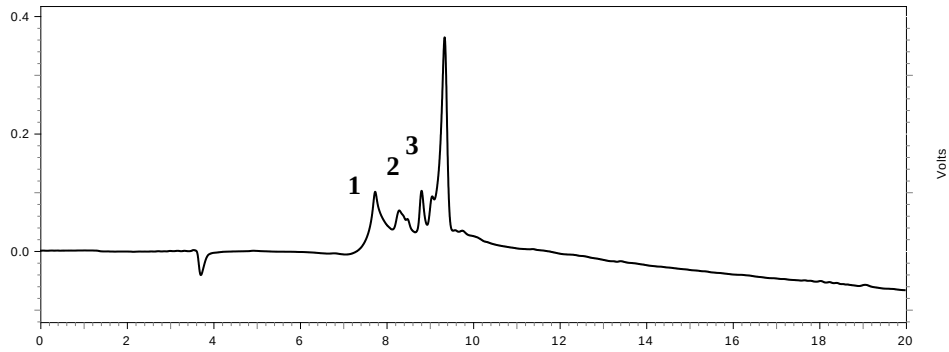


Figure 19. RP-HPLC chromatogram of the reaction mixture containing 16-mer oligosaccharide of HA (**1**), G1 dendrimer (including defective molecules) (**2**), corresponding conjugate of G1 dendrimer and oligosaccharide (**2**) and modifications of oligosaccharide (**3**) (Scheme 20).

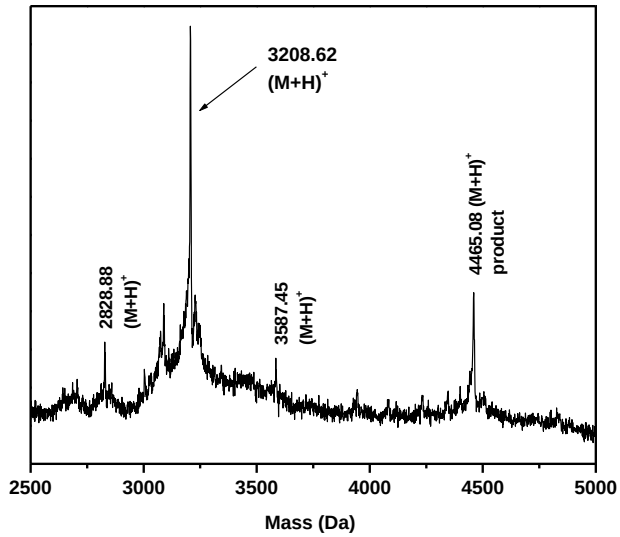
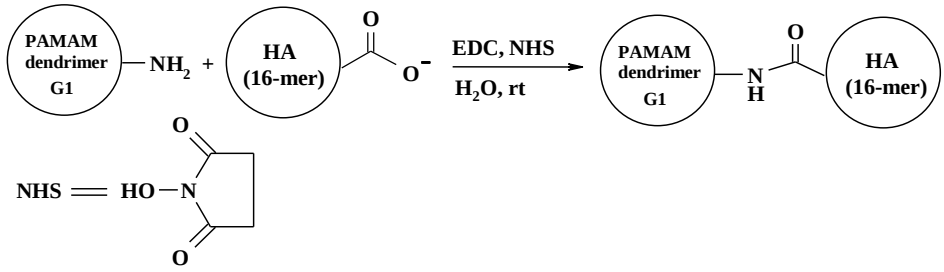


Figure 20. MALDI-TOF MS spectrum of the purified conjugate of G1 dendrimer and 16-mer oligosaccharide. The fraction still contains some modifications of 16-mer oligosaccharide as impurities (Scheme 20).

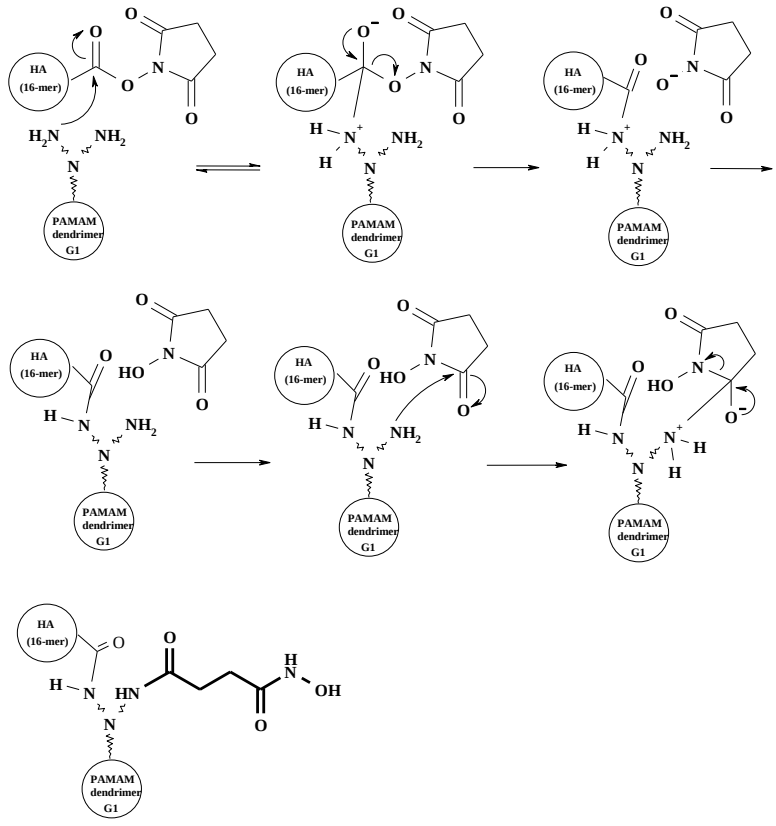
In an attempt to prepare an active intermediate that would have a larger half-life in a water environment compared to *O*-acylisourea, we prepared a *N*-hydroxysuccinimide ester of the hyaluronic acid (Scheme 21, Appendix, No. 5).



N-hydroxysuccinimide

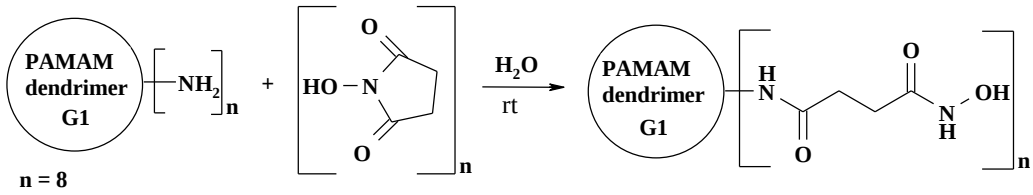
Scheme 21. Strategy for attaching HA to dendrimer through EDC and NHS.

RP-HPLC allowed the removal of the impurities with mass < 900 Da from the reaction mixture. The unreacted oligosaccharide was collected into a separate fraction. The main fraction (t_R – 8.00-8.60 min contained a certain quantity of the modified product (4580.91 Da) that had a MW 115 Da higher than the desired product (Figure 21 and 22). The formation of that product may be explained by Scheme 22.



Scheme 22. The synthesis scheme for glycodendrimer modified with *N*-hydroxysuccinimide.

In the mass spectrum, the peak that corresponds to a modified oligosaccharide (3208.02 Da) dominated again. The main fraction after purification also contained some modifications of G1 dendrimer. One of them, a compound with mass 1545.74 Da, might have been formed as a result of the re-amidation reaction between the amino group of dendrimer and *N*-hydroxysuccinimide. That reaction is presented in Scheme 23.



Scheme 23. Addition of *N*-hydroxysuccinimide to dendrimer.

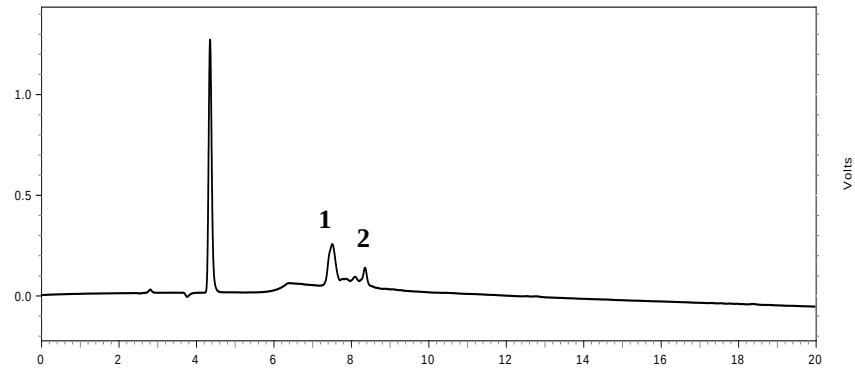


Figure 21. RP-HPLC chromatogram of a reaction mixture containing 16-mer oligosaccharide of HA (**1**), G1 dendrimer (including defective molecules) (**2**), corresponding modification of the conjugate of G1 dendrimer and oligosaccharide (**2**), modifications of G1 dendrimer and modifications of oligosaccharide (**2**) (Scheme 20 and 23). The eluent system was: **A** - 0.1% trifluoroacetic acid in water, **B** - acetonitrile. The linear gradient 0-50% of **B** in 20 min was used (1 ml/min).

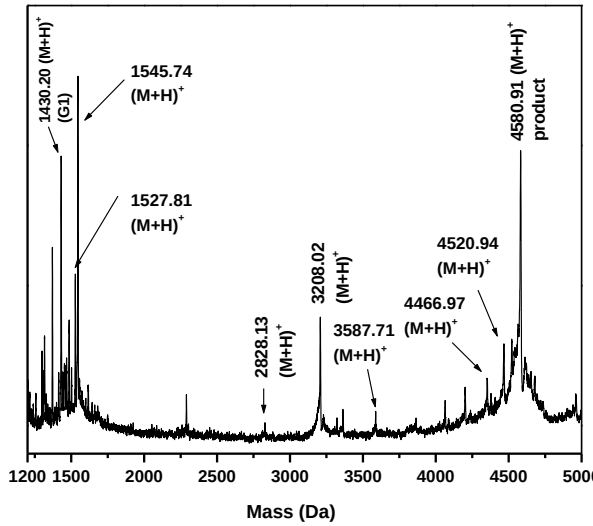


Figure 22. MALDI-TOF MS spectrum of the purified conjugate of G1 dendrimer and 16-mer oligosaccharide. The fraction contains various modifications of 16-mer oligosaccharide and G1 dendrimer as impurities (Scheme 20, 23).

Despite a low yield, it was demonstrated that the HA oligosaccharide (16-mer) could be linked to the G1 dendrimer. The reason of the low yield in EDC-supported coupling reaction could be that the reaction was performed in an aqueous medium, since the HA was in the sodium salt form. Probably in organic solvents (DMSO, DMF) hyaluronic acid oligosaccharide ammonium salt may give a better reaction.

An attempt to link the HA oligosaccharide (16-mer) to the G1 dendrimer by reductive amination was not successful. Theoretically, the method should work and that was also demonstrated in the case of HA disaccharide. Due to the lack of HA oligosaccharide, we were limited to a single experiment and the reaction had to be conducted on a very small scale (1 μl) that made precise monitoring and controlling of the reaction conditions impossible.

We demonstrated that saccharides could be linked to the PAMAM dendrimer by using carbodiimide chemistry and by reductive amination. To produce the conjugates on the preparative scale and to optimize substantially bigger amounts of hyaluronic acid, oligosaccharide is needed.

6.3.3. Modification of PAMAM dendrimers by oligopeptide

The other type of cell-specific dendrimers may be dendrimer-peptide conjugates. Therefore we investigated a possibility to bind oligopeptides covalently to the outer layer of the fifth generation PAMAM dendrimer. In order to check and compare different synthesis possibilities, we investigated the

binding of oligopeptides to model dendrimer compound. Again, for the purposes of simplicity and a possibility of price analysis of the products by MALDI TOF MS, G1 dendrimer was chosen as the model compound.
In the present work, we used two oligopeptides (oligopeptide I and II) with different amino acid sequences (Figures 23 and 28).

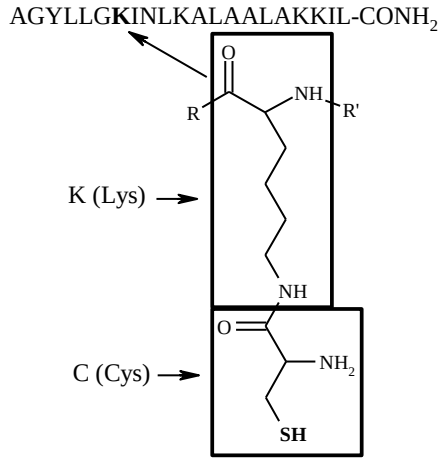
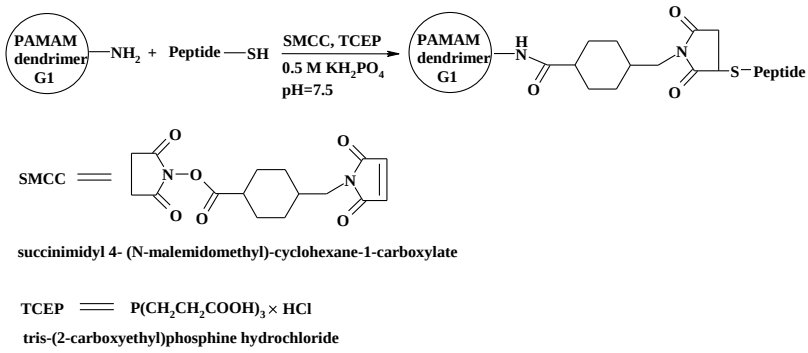


Figure 23. The amino acid sequence of the oligopeptide I. The amino group at the side chain of Lys at position of the peptide was acylated with Cys residue. The thiol group of the Cys side chain attempted to attach to the linker.

First, we made an attempt to bind the oligopeptide I to the outer layer of the PAMAM dendrimer *via* a SMCC linker, capable of reacting with thiol groups (Appendix, No. 6). SMCC linker is known and widely used in binding different proteins to each other [135]. It is a heterobifunctional linker which has NHS ester on the one end and maleimide function on the other end of the molecule (Scheme 24). When the NHS ester reacts with a primary amino group, a stable amide bond is formed. The maleimide, on the other hand, can react with thiol groups of oligopeptide (Scheme 24). Since the thiol group can easily be oxidized into disulphide, a reducing agent like TCEP is added to the reaction mixture in order to avoid that problem (Scheme 24).



Scheme 24. Attaching oligopeptide to the outer layer of the dendrimer by SMCC linker.

We performed the experiment with the oligopeptide I. When the reaction mixture was analysed by MALDI TOF MS, we found that the G1 dendrimer had reacted with 1 to 4 molecules of linkers (G1+1×SMCC (1688.53 Da $(M+K)^+$), G1+2×SMCC (1907.65 Da $(M+K)^+$), G1+3×SMCC (2126.58 Da $(M+K)^+$), and G1+4×SMCC (2345.80 Da $(M+K)^+$)), but not with peptide. Masses that correspond to the conjugates of defective dendrimers with linkers were also found. In addition to the unreacted oligopeptide (2323.42 Da $(M+K)^+$), a dimer peak corresponding to the mass of peptide dimer (4604.84 Da $(M+K)^+$), oxidized *via* the disulfide bridge, was observed in the spectrum.

A double bound of the maleimide is also able to react with the amino group and it is very likely that after acylation of the dendrimer amino group with NHS ester, an intramolecular reaction followed. That would explain why the masses that correspond to the conjugates of dendrimers and oligopeptides were not found. This hypothesis is further supported by the fact that only dendrimers reacted with 1-4 linkers were found in the spectra. In this case, four attached linkers correspond to a case where all eight amino groups of the dendrimer have reacted (Figure 24).

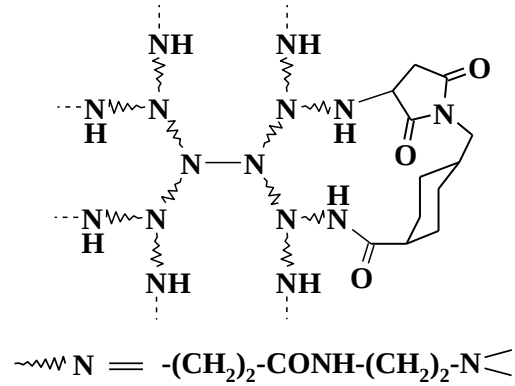


Figure 24. Possible side-reaction occuring during the derivatization of the dendrimer with SMCC linker.

If we change the order of the addition of the reagents by adding the linker to oligopeptide first, then low yields will also be expected due to the large number of side-reactions: the corresponding oligopeptide contains four amino groups and one thiol groups, which can all react with the linker.

Indeed, when we tried to bind cystein to the dendrimer the results similar to those described above were obtained. The only differences observed were: cystein dimer was not observed in the spectrum; a compound with the mass number (1809.04 Da $(M+K)^+$) has appeared (indicated that dendrimer had reacted with the linker, and the linker, in turn, was attached to the cysteine). The intensities of those peaks were very low compared to the intensity of the peaks corresponding to the dendrimer (Figure 25).

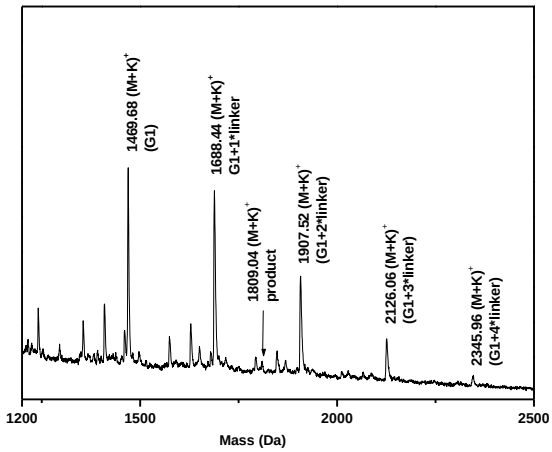


Figure 25. MALDI-TOF MS spectrum that shows the ratios of the product (G1 dendrimer+SMCC+Cys) and various impurities in the reaction mixture.

The reaction of the oligopeptide I with *N*-ethylmaleimide was studied separately (Appendix, No. 7). The MALDI TOF MS spectra indicated that oligopeptide had reacted with 1 to 3 *N*-ethylmaleimide molecules. It means that the amino groups of the peptide can successfully react with the double bond of *N*-ethylmaleimide (Figure 26).

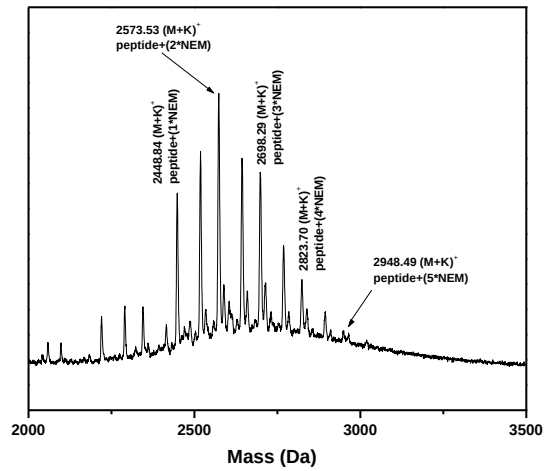
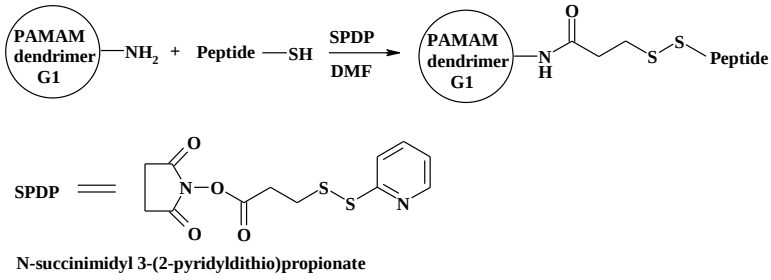


Figure 26. MALDI-TOF MS spectrum of the reaction products between N-ethylmaleimide (NEM) and peptide.

SPDP linker (see structure in Scheme 25) was also tested in the petide addition reaction. The SPDP linker is attached to the amino groups of the dendrimer outer layer analogously to SMCC linker. SPDP is also a heterobifunctional linker that carries a NHS ester group on its one end and the 2-pyridyldisulfide group (that is able to react with thiol groups) on the other end. As a result, a disulfide bond is formed between the linker and a peptide (Scheme 25, Appendix, No. 8). The drawback in using this linker is that the disulfide bond may not be stable enough, as in practical use, its proposed biological environment contains reducing agents (for example glutathione) that are able to reduce this bond.

We found that oligopeptide I was bound to the dendrimer with SPDP linker (3800.05 Da $(M+H)^+$), but the intensity of the corresponding peak was low compared, with for example, with the peak (corresponding to the dendrimer itself; Figure 27). Free oligopeptides (2285.73 Da $(M+H)^+$), conjugate of the dendrimer and the linker (1628.50 Da $(M+H)^+$) and the oligopeptide dimer (4604.84 Da $(M+K)^+$), were also observed in the spectrum. When the mixture was re-analysed in three days after the beginning of the reaction, no important changes compared to the earlier spectra were observed. Only a more intensive oligopeptide dimer peak was observed.



Scheme 25. Attaching oligopeptide I to the outer layer of dendrimer by SPDP linker.

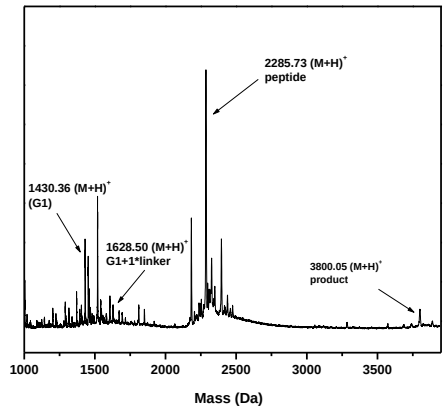


Figure 27. MALDI-TOF MS spectrum that shows the ratios of the product (G1 dendrimer+SPDP+Peptide-Cys) and various impurities in the reaction mixture.

6.3.3.1. Modification with oligopeptide II

The structure of the oligopeptide II is shown in Figure 28.

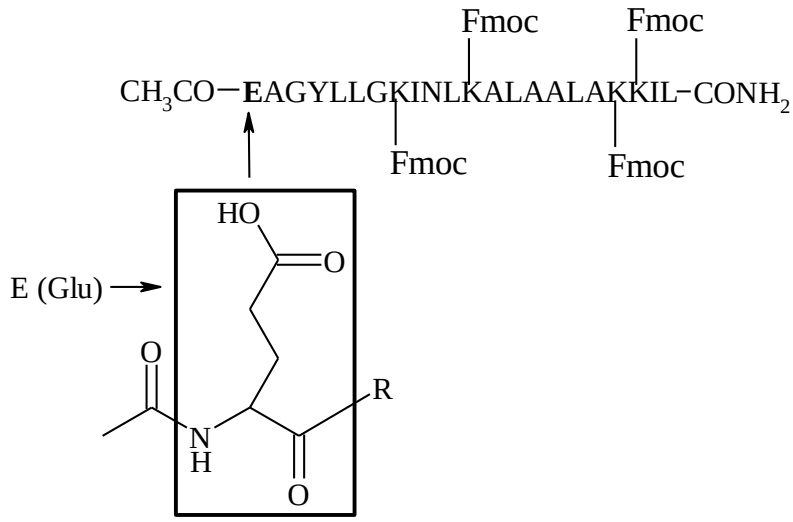
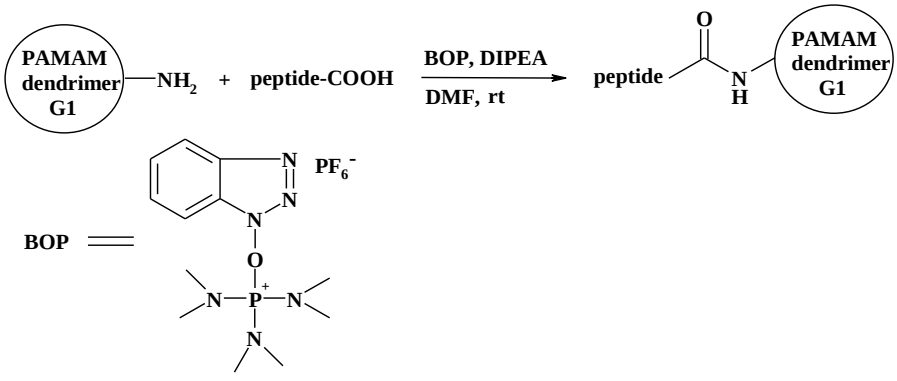


Figure 28. The amino acid sequence of the oligopeptide II. The first amino acid is *N*-acylated glutamic acid.

An important difference of oligopeptide I from oligopeptide II is that in the latter the Lys side chains are protected by Fmoc (9-fluorenylmethoxycarbonyl) groups. The amino acid sequence is also different. The oligopeptide II had glutamic acid at the end of the the chain, that has a free carboxyl group. We tried to attach the oligopeptide to the outer layer of the dendrimer *via* this carboxylic group, using BOP reagent (Scheme 26, Appendix, No. 9). The Fmoc groups were later removed with 50% piperidine / dimethyl formamide.



Scheme 26. Attaching oligopeptide II to the dendrimer using the BOP reagent.

We succeeded in obtaining the corresponding oligopeptide (3182.28 Da) G1 dendrimer conjugates (3705.15 Da). This method proved quite promising for the synthesis of dendrimer-oligopeptide conjugates.

6.4. Use of G5 PAMAM dendrimer in biological experiments

G5 dendrimer has been used by molecular biologists in DNA transfection processes. For a closer study of the transfection processes, G5 dendrimer was marked with a fluorescent marker (AlexaFluor 488) and its transfection was monitored by fluorescence microscopy. It was observed that the PAMAM dendrimer enters the cells relatively quickly (ca. 15 min). A couple of hours later, most of dendrimers had accumulated around the nucleus but only very few had entered the nucleus.

It is known from the literature that a PAMAM dendrimer loses the ability to participate in transfection processes on prolonged storage [12]. One reason for this can be that the dendrimer molecules form associates on standing. Such associates were, indeed, observed in the case of the fluorescently labeled dendrimer (located outside the cell membrane) and for those dendrimers the transfection effect was extremely low.

G5 PAMAM dendrimer was preferentially suitable for transfection of oligonucleotides in cells, providing better results as compared to PEI (polyethylenimine).

G5 dendrimer with the modified outer layer (*N*-methyl- and *N*,*N*-dimethylamino groups instead of amino groups) was also tested for DNA transfection. The dendrimer with *N*,*N*-dimethylamino groups had no transfection effect. The transfection effect of dendrimer with *N*-methylamino groups was also low (lower than that of G5 itself).

7. CONCLUSIONS

In this work, the fifth generation PAMAM dendrimer with EDA-core was synthesized step-by-step and the reaction conditions for the synthesis of higher dendrimer generations (G2.5-G5) were worked out.
In the course of synthesis of half generations, suitable chromatographic methods were developed for their purification, which depending on the generation allowed a complete or partial separation of the defective dendrimers. Column chromatography on silica gel was used to purify the generations G-0.5, G0.5, G1.5 and size-exclusion chromatography on Sephadex LH-20 to purify generations G2.5, G3.5, G4.5. The HILIC and RP-HPLC were found to suit best for the separation of the full generation of dendrimers from the defective products and were successfully applied to G1 and G5 generations.

For the analysis of various generations, GC, CZE, ^{1}H and ^{13}C NMR and MALDI-TOF MS were used.

GC could only be used to analyse the generation G-0.5, which allowed closer investigation of one of the main side-reactions causing dendrimer fragmentation, namely *retro*-Michael reaction. This reaction can be observed already at 4 °C and is quite considerable at room temperature. Due to the *retro*-Michael reaction, the defective dendrimers with missed branches as well as the compounds with active double bond could be formed. The latter are able to give the Michael reaction both with ethylenediamine and ideally branched dendrimer and result in new defective denrimers.
CZE was suitable for the determination of the homogeneity of various full generations; the analysis of half generations was more complicated due to their hydrolysis. CZE enabled us to discriminate between the dendrimer full generations up to fifth.

^{1}H and ^{13}C NMR were successfully used for the analysis of dendrimers up to generation G2.5. Analysis of the higher generations was complicated, mostly because of the increasing number of defective dendrimers. Furthermore, this method did not clarify the structure of various types of defects because of the large number of overlapping signals.

The best analysis method proved to be MALDI-TOF MS. MALDI-TOF MS, allowing a detailed analysis of different generations, independent of whether it was half or full generation. Suitable matrixes were also found for the analysis of PAMAM dendrimers by MALDI-TOF MS. Up to generation G2.5 (included), the structures corresponding to all mass peaks could be devised. The method allows us to determine the purity of dendrimers and define the structures of the side-products.

The fifth generation PAMAM dendrimer was used as a carrier to investigate various biological processes (e. g. DNA transfection into cell) which were performed in cooperation with molecular genetics from the NICPB (National Institute of Chemical Physics and Biophysics). In order to vary the properties of the carrier, two types of the fifth generation dendrimer were synthesized. In one of them, the outer layer consisted of *N,N*-dimethylamino

groups, in the other, of *N*-methylamino groups. Both modifications were also used to investigate DNA transfection.

Initial conditions for synthesising dendrimer conjugates were worked out on model compounds. Among the dendrimers, the chosen model compound was G1; among the saccharides, D-glucose, D-*N*-acetylglucoseamine and hyaluronic acid disaccharide were used. The corresponding saccharides were linked to G1 *via* Schiff base, which was then reduced with $NaCNBH_3$.
The HA oligosaccharide (16-mer) was linked to G1 dendrimer outer layer by employing carbodiimide chemistry. The oligosaccharide was coupled by using EDC alone and also in combination with *N*-hydroxysuccinimide.

To attach the oligopeptides to the dendrimer outer layer, different linkers or carboxyl group activating reagents (SMCC, SPDP, BOP) were used. The BOP reagent was found to be a more efficient as compared to SMCC and SPDP.

8. REFERENCES

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9. ACKNOWLEDGEMENTS

I'm very grateful to my supervisor Margus Lopp who offered me a possibilty to do my Ph. D. in this laboratory, for his patience and for giving me excellent support and inspiration that I needed.

I'm grateful to my colleagues and co-authors: Juhan Subbi, Tõnis Pehk, Arkadi Ebber, Merike Vaher, Priit Kogerman, Anders Valkna and Anne Meikas, for smooth and fruitful collaboration. I also thank all my other colleagues from the Department of Organic Chemistry and from Prosyntest Ltd.

I wish to thank Veiko Allikmaa, Kalle Kaljuste and Tõnis Kanger for their excellent collaboration and all kind of help during these years.

I would also like to thank my teachers at Tartu University for helping me to make my first steps in science.

I am grateful to my parents for their support during all these years.

10. APPENDIX
Experimental

1. *HILIC.* 10 × 150 mm glass column was filled with 55 mg Silasorb Diol (10 μm; Chemapol Prague-Czechoslovakia). 1 mg G1 was dissolved in 100 μl acetonitrile/water (1:1) mixture and inserted into the column. Air pressure *(0.1 bar)* was applied to the column, and the first fraction was collected. The next fractions were collected analogously, using 100 μl of the same eluent, 2 × 100 μl of distilled water, 2 × 100 μl of distilled water acidified with acetic acid to pH = 3.39, and finally, with 2 × 100 μl of distilled water/acetic acid (pH = 2.98). The resulting fractions were lyophilized and analysed by MALDI-TOF MS.
HPLC. HPLC analysis was carried out on the Shimadzu system: LC 10AT vp HPLC pump, SPD-10A VP UV-VIS detector and SCL-10A VP system controller. Samples of the reaction mixtures were analysed in a Symmetry C_{18} column (4.6 × 250 nm, 5 μm), detection at 218 nm. The eluent system was: A - 0.1% trifluoroacetic acid in water, B - acetonitrile. The linear gradient 0-50% of B in 20 min was used (1 ml/min).

2. a) Synthesis of G5 dendrimers modified by *N,N*-dimethylamino groups in the outer layer

G4.5 (0.081 g, 0.0032 mmol) was dissolved in 1 ml of methanol. The solution of *N,N*-dimethylethylene diamine (5.43 g, 61 mmol) in methanol (2.2 ml) was prepared separately. Both solutions were cooled in a refrigerator at approximately -20 ºC for an hour. Then the solutions were mixed and the temperature of the reaction mixture was allowed to rise to the room temperature. The air was replaced with argon and the reaction mixture was left to stand at room temperature for 14 days. The excess of *N,N*-dimetylenediamine was distilled off as an azeotrope with n-butanol (75 ml). The reaction product was analysed by MALDI-TOF MS. The analysis of spectra revealed that the desired product was in the correct mass range (Figure 11).

b) Synthesis of G5 dendrimers modified by *N*-methylamino groups in the outer layer

The synthesis and analysis were carried out analogously to the previous part (*N,N*-dimethylamino modified dendrimer) with the next amounts of reagents:
G4.5 0.077 g, 0.003 mmol in 0.6 ml methanol; *N*-dimethylethylene diamine 4.4 g, 0.058 mmol in 2.7 ml methanol (Figure 11).

3. General procedure for the synthesis of dendrimer and saccharide conjugates

G1 was dissolved in water (concentration of G1 in solution 0.087 M), then saccharide was added (concentration of saccharide in the reaction mixture 0.7 M). One day later, $NaCNBH_3$ was added (concentration of $NaCNBH_3$ in

reaction mixture 0.3 M). Five hours after addition of the reducer, the reaction mixture was chromatographed with RP-HPLC.

4. Attaching of the hyaluronic acid oligosaccharide to G1 dendrimer by EDC

G1 dendrimer (0.2 mg, 0.14 μmol) dissolved in water (0.35 μl), EDC (0.01 mg, 0.056 μmol) was added to the resulting solution, followed by the solution of HA oligosaccharide (16-mer, 0.15 mg, 0.051 μmol) in water (0.35 μl). The mixture was left standing overnight and chromatographed by RP-HPLC (Figure 19). The collected fractions were lyophilized and analysed by MALDI-TOF MS (Figure 20).

5. Attaching of the hyaluronic acid oligosaccharide to G1 dendrimer by EDC and NHS

G1 dendrimer (0.4 mg, 0.3 μmol) was dissolved in water (0.3 μl). To the resulting solution, EDC (0.014 mg, 0.075 μmol), NHS (0.0086 mg, 0.075 μmol) and the solution of HA oligosaccharide (16-mer, 0.2 mg, 0.069 μmol) in water (1.1 μl) were added. 2.5 hours later the reaction mixture was chromatographed by RP-HPLC (Figure 21). The resulting fractions were lyophilized and analysed by MALDI-TOF MS (Figure 22).

6. Attaching of oligopeptide to dendrimer by SMCC

G1 dendrimer (0.2 mg, 0.14 μmol) was dissolved in 11 μl of 0.5 M phosphate buffer (pH = 7.5). SMCC (0.05 mg, 0.14 μmol) was separately dissolved in 2 μl of THF or DMF and added to the dendrimer solution. The reaction mixture was allowed to stand at room temperature for 1.5 h. Thereafter an oligopeptide (0.3 mg, 0.14 μmol, dissolved in 7 μl of 0.5 M phosphate buffer pH = 7.5) and TCEP (0.04 mg, 0.14 μmol, dissolved in 4.4 μl of 0.5 M phosphate buffer pH = 7.5) were added. After the mixing, the pH of the solution was 7.2-7.5 (by indicator paper). The reaction mixture was left to stand at room temperature for an hour and then stored overnight at 4 ºC. The resulting reaction mixture was analysed by MALDI-TOF MS.

7. The reaction between *N*-ethylmaleimide and oligopeptide

N-ethylmaleimide (0.01 mg, 0.087 μmol) was dissolved in 3 μl of THF. The resulting solution was added to the solutions of oligopeptide (0.2 mg, 0.087 μmol) and TCEP (0.025 mg, 0.087 μmol) in 8 μl of 0.5 M phosphate buffer (pH = 7.5). The reaction mixture was left to stand at room termperature for 24 hours and later analysed by MALDI-TOF MS (Figure 26).

8. Attaching of oligopeptide to dendrimer by SPDP

G1 dendrimer (0.12 mg, 0.087 μmol) was dissolved in 7 μl of water, and 0.027 mg SPDP dissolved in 1.8 μl DMF was added. Half an hour later, the peptide (0.2 mg, 0.087 μmol) was added to the reaction mixture. The reaction mixture

was left standing at room temperature. The first specimen to analyse the reaction mixture by MALDI-TOF MS was taken three hours from the starting of the reaction (Figure 27).

9. Attaching of oligopeptide to dendrimer by BOP

Oligopeptide (1.8 mg, 0.58 μmol) was dissolved in 10 μl of DMF, where BOP (0.37 mg 0.838 μmol) and DIPEA (0.22 mg, 1.68 μl) were added. The solution of G1 dendrimer (0.4 mg, 2.2 μmol) in DMF (5 μl) was added to the resulting solution. The reaction mixture was left standing overnight. On the next day, 15 μl of piperidine was added to the reaction mixture (to remove Fmoc groups) and left standing for five hours. The reaction mixture was chromatographed with RP-HPLC, collected fractions were lyophilized and analysed by MALDI-TOF MS.

ARTICLE I

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