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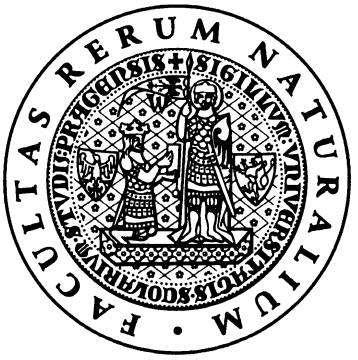

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Bimodal macromolecular MRI contrast agents with possibility of luminescence detection

Bachelor Diploma Thesis

Advisor: RNDr. Jan Kotek, Ph.D.

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I declare that I worked out this thesis solely, using the mentioned references. Neither whole thesis nor any its part has been formerly used for gaining any other academic degree.

I would like to thank my advisor Honza Kotek for his kind guidance. And I should thank him once more for his patience. I also thank Zuzka for NMR measurements. The another thanks belong to Miloš for introducing me into the techniques of work with dendrimers. Next, I would like to thank Dr. Ivan Němec for measuring of Raman and IR spectra.

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Finally I would like to thank my parents for their support and mainly for their patience.

motto[0]:

"... to je, jako když pověsíš škodovky na vánoční stromeček ..."

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1 INTRODUCTION

1.1 Dendrimers

1.1.1 History

The story of dendritic molecules began in the late 1970s when several groups performed the first attempts to prepare such compounds. The first "true dendrimers" were prepared in 1979 by the Tomalia's group in Dow Chemicals, Michigan, USA. As in the cases of many other inventions their finding was blessed with lucky coincidence. Routine preparation (reaction of ethylenediamine with methylacrylate) of linear random-coil polyamidoamine was carried out. Although no solvent was required for the reaction they added methanol to facilitate stirring. Such dilution by methanol caused reagents to form dendrimer molecules instead of long linear polymeric species.[1,2] In 1985, after few years of intensive research, Tomalia et al. published their pioneering work about preparation of polyaminoamide dendrimers (PAMAM) in Polymer Journal.[3] They called these substances Starburst™ dendrimers.

Approximately twenty articles were published about dendrimers till the end of 1990.[2] Since that time the number of works about this topic has been rising exponentially to thousands.

The invention of dendrimers is important in the way that the first time in the history of polymer chemistry it has been possible to synthesize precise abiotic macromolecules comparable to biotic ones in the mean of exact structure.

1.1.2 Structure

Dendrimers are considered to have the most regular polymeric architecture.[1,2,5] Ideally they have absolutely regular branching. From polymer point of view their tree-like molecules ("δένδρον" means "tree" in Greek) show the narrowest index of polydispersity of all synthetic macromolecular materials of similar molecular weight. They are the first man-made macromolecules with exactly defined molecular weight, topology and branching.

Dendrimer molecules consist of three main architectural components: (1) a central core, (2) interior layers (generations), composed of repeating units radially attached to the

core and (3) terminal groups attached to the outermost generation (Figure 1). The resulting molecule consist of concentric layers of monomers assembled around the single molecule (core) at the center. This pattern looks like an onion with layers of particular generations (marked G0, G1, G2, ...).

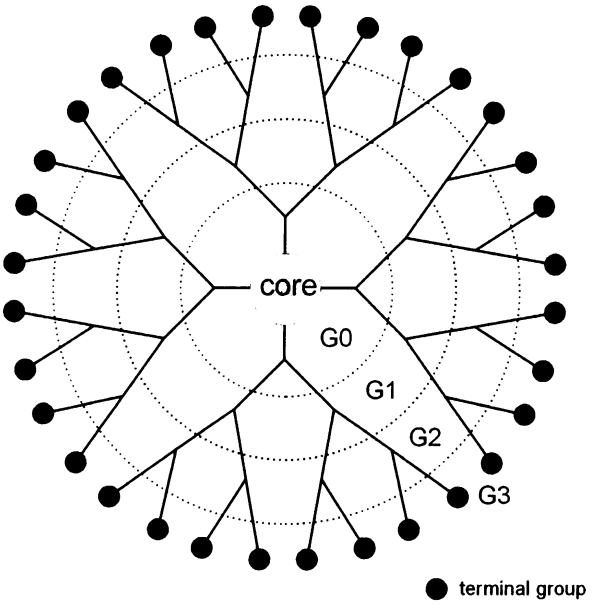


Figure 1: Schematic drawing of 3rd generation (G3) dendrimer

Although only about one hundred[14] dendritic structures have been published until now the possibilities to adjust dendritic structures by modifying terminal groups or core are nearly infinite. It seems that virtually any functional group which can be found in organic chemistry textbooks can be attached to the surface of dendrimer molecule to carry out selected tasks.

We also know great number of various core molecules. One of the components of repeating layers (like ethylenediamine in PAMAM dendrimers mentioned below) often acts as a simplest core molecule. Even one atom, e.g. nitrogen atom (the residue of ammonia), can act as a core. But it is possible to make core from almost every molecule with appropriate functional groups which can be substitued. Therefore a large number of different cores (and sometimes very unusual molecules) can be present in dendrimers, e.g. organic molecules like substituted naphtalenes, transition metal complexes of various ligands, including phenanthroline or porphyrine, porphyrine complexes of fullerenes, etc. (Figure 2).[5,7,8]

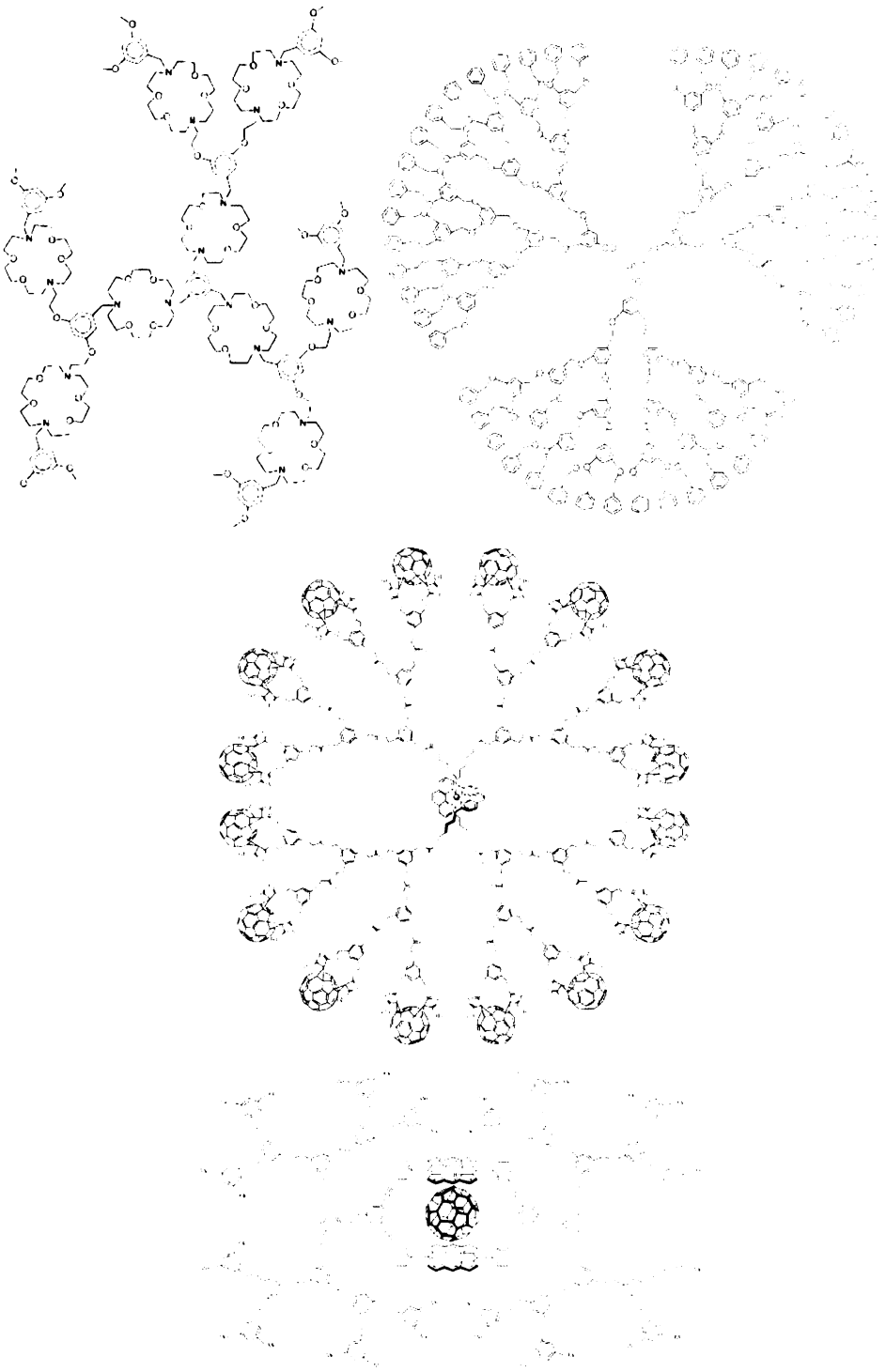


Figure 2: Examples of uncommon dendrimers. Adopted from literature.[5,8]

Due to varying composition of dendrimers it is not possible to correctly speak about their conformation in general as this property is very variable in different

dendrimers. However, it can be also dependent on the environment of the molecules, e.g. solvent, pH, temperature, ionic strength, solid surfaces, etc.[12] Nevertheless, in general it can be said that generations 3 and higher of common dendrimers have globular shape with the size rising nanometers.[5,9]

The structure of dendrimeric molecules is difficult to investigate. Usual dendrimers are amorphous, thus direct determining of their structure by X-ray diffraction is not possible. Dendrimers are just in between molecular and polymer chemistry. They pertain to molecular chemistry world by their step by step controlled synthesis, and pertain to polymer chemistry world by their repetitive structure made of monomers. Thus, the analysis of dendrimers should benefit from both worlds. The most used techniques for investigation dendrimers from the molecular point of view are usual organic analytical methods like elemental analysis, NMR spectroscopy, mass spectrometry, IR spectroscopy, Raman scattering and UV-Vis spectroscopy. The most used techniques from polymer world utilized for dendrimer analysis are scattering techniques (SAXS, SANS, LLS), microscopy (AFM, TEM), size exclusion chromatography, viscosity measurements, etc.[3,5,6] Some theoretical works using molecular dynamics were also performed on this topic.[9,13]

These investigations showed that there is not so much free space inside the molecules as has been expected. This fact is caused by the phenomenon of backfolding. It means that some branches are turn into the inner space of the molecule instead of pointing outwards from the molecule (Figure 3).[5,9] This phenomenon can be sometimes suppressed by suitable functionalization of dendrimer (e.g. by functionalization with naphtalene cores and π-π stacking of these cores[5]) or e.g. by protonation of terminal groups. Nevertheless, some cavities are usually present and they are important feature of dendrimer molecules.

In spite of declared regularity of dendrimers some defects may be present in their molecules similarly as biomacromolecules like proteins or nucleic acids should have exact structure and have defects anyway. So generational, skeletal and substitutional deviations may be present. One can find there incompletely functionalized terminal groups, missing arms, intramolecular loops or dendrimer molecules joined into dimeric structures.[3,5,10] The another fact to mention is that the size of dendrimers is limited

because there is a point at which the strain energy rises too much to enable additional uniform growth of the molecule. This fact causes that following generation cannot be completely added over the critical generation of dendrimer.[5,9]

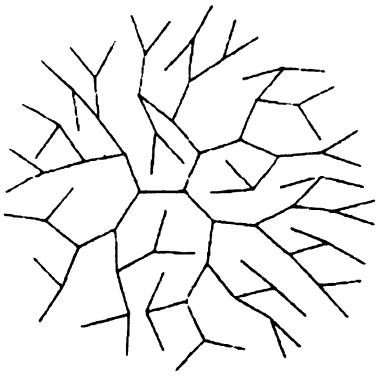


Figure 3: Schematic representation of backfolding of unspecified G4 dendrimer. Adopted from literature.[5]

As written above about 100 dendrimer architectures are known nowadays. More than 1000 of various surfaces have been reported until now.[14] Anyway, there have been only few dendrimer families widely investigated and examined upon high generations. The most well-known dendrimer families are for example Tomalia's PAMAM dendrimers, Fréchet's aromatic polyether dendrimers, polypropyleneimine dendrimers (PPI) and polyphenylacetylene dendrimers (all of them discussed below). Only very few dendrimers are commercially available (only PAMAM and PPI were commercially available in 2004[4]).

1.1.2.1 PAMAM dendrimers

As mentioned before, polyamidoamine dendrimers (PAMAM) has been the first reported and thoroughly characterized dendrimers. They are formally derived from *N*-(2-aminoethyl)acrylamide. The repeating units are added during synthesis to the molecule in two steps (mentioned below). There are two possible terminal groups of pure PAMAM: (1) amine group of the last added ethylenediamine, (2) carboxylic group of last added acrylic acid. If the branch is terminated by carboxylic group, the molecule is half-generation dendrimer. There are two most used core molecules in this type of dendrimers – ammonia and ethylendiamine (Figures 4 and 5; Table 1).[1,3,4]

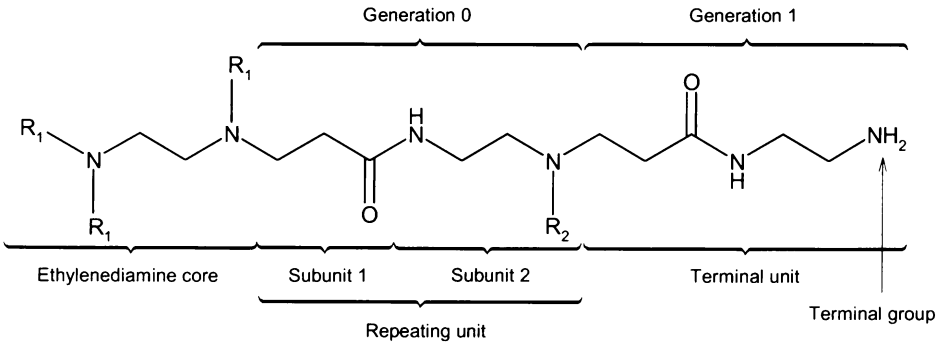


Figure 4: Structure of one branch of PAMAM G1 dendrimer with ethylenediamine core and definition of terms used for describing dendrimers. Subunit 1 comes from acrylic acid, subunit 2 comes from ethylenediamine. If the branch terminates with subunit 1, dendrimer is of half-generation. R_1 and R_2 mean another branches of dendrimer.

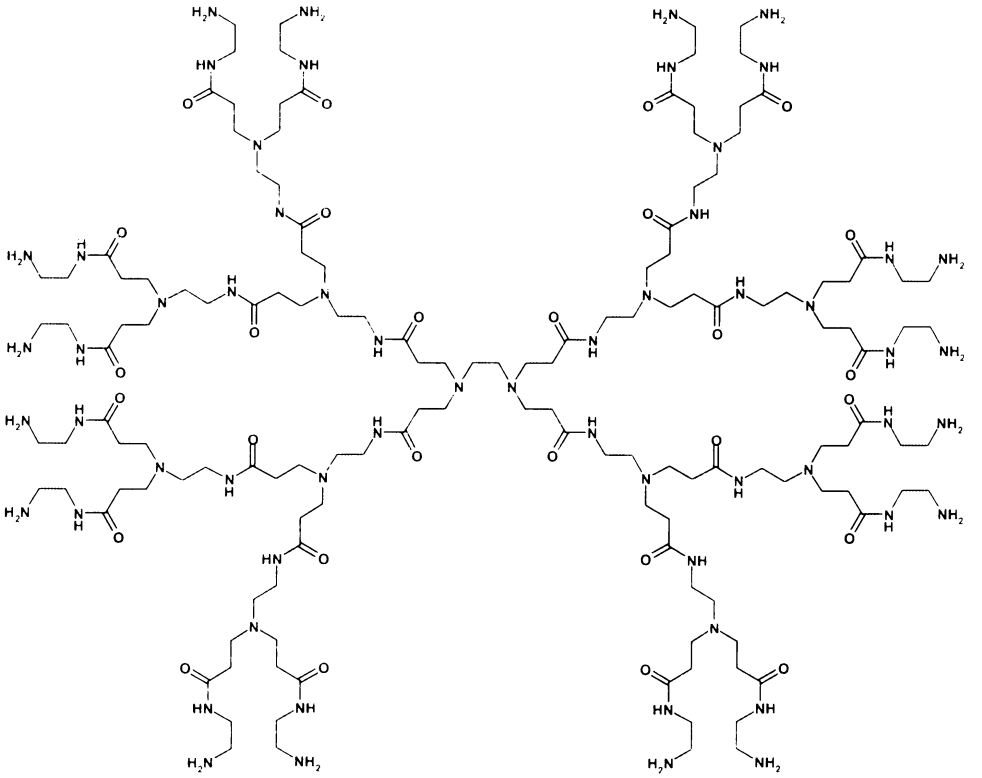


Figure 5: Structure of PAMAM G2 dendrimer with ethylenediamine core. Red colour marks parts derived from acrylic acid, blue colour marks ethylenediamine.

In fact PAMAM dendrimers are probably the most extensively studied family of dendrimers. They are commercially available in the range of generations 0–10[17] as Starburst™ dendrimers. They were prepared and characterized up to generation 10.

Higher generations than 10 are not available because of high sterical crowding (not all branches are accessible to further branching, see above).[9]

Table 1: Properties of PAMAM dendrimers with ethylenediamine core. Annotation: RU – repeating unit; EN – ethylenediamine; ENTPr – ethylenediaminetetrapropionic acid

generation	number of terminal groups	number of whole repeating units	end group	M_w	number of atoms				
					C	H	N	O	Σ
RU		1	-	113	5	9	2	1	17
EN	2	-	NH_2	60	2	8	2	0	12
ENTPr	4	-	COOH	348	14	24	2	8	48
0	4	4	NH_2	517	22	48	10	4	84
0,5	8	4	COOH	1 093	46	80	10	20	156
1	8	12	NH_2	1 430	62	128	26	12	228
1,5	16	12	COOH	2 583	110	192	26	44	372
2	16	28	NH_2	3 256	142	288	58	28	516
2,5	32	28	COOH	5 562	238	416	58	92	804
3	32	60	NH_2	6 909	302	608	122	60	1 092
3,5	64	60	COOH	11 521	494	864	122	188	1 668
4	64	124	NH_2	14 214	622	1 248	250	124	2 244
4,5	128	124	COOH	23 438	1 006	1 760	250	380	3 396
5	128	252	NH_2	28 825	1 262	2 528	506	252	4 548
5,5	256	252	COOH	47 273	2 030	3 552	506	764	6 852
6	256	508	NH_2	58 047	2 542	5 088	1 018	508	9 156
6,5	512	508	COOH	94 943	4 078	7 136	1 018	1 532	13 764
7	512	1020	NH_2	116 490	5 102	10 208	2 042	1 020	18 372
7,5	1024	1020	COOH	190 283	8 174	14 304	2 042	3 068	27 588
8	1024	2044	NH_2	233 376	10 222	20 448	4 090	2 044	36 804
8,5	2048	2044	COOH	380 962	16 366	28 640	4 090	6 140	55 236
9	2048	4092	NH_2	467 149	20 462	40 928	8 186	4 092	73 668
9,5	4096	4092	COOH	762 320	32 750	57 312	8 186	12 284	110 532
10	4096	8188	NH_2	934 694	40 942	81 888	16 378	8 188	147 396

1.1.2.2 PPI dendrimers

The first synthesis of polypropyleneimine dendrimers (PPI) was performed by Meijer in 1993. The synthesis was carried out by divergent approach (explained in chapter 1.1.3.1), based od the older works of Voegtle.[23] Today PPI dendrimers are commercially available up to generation 5 under the commercial name Astramol™.

PPI dendrimers are synthetized from acrylonitrile by repeating sequence of two reactions - exhaustive Michael addition of acrylonitrile on amine, and hydrogenation of nitrile. As the reaction starts commonly with diaminobutane (as a core) they are also called DAB dendrimers (Figure 6).

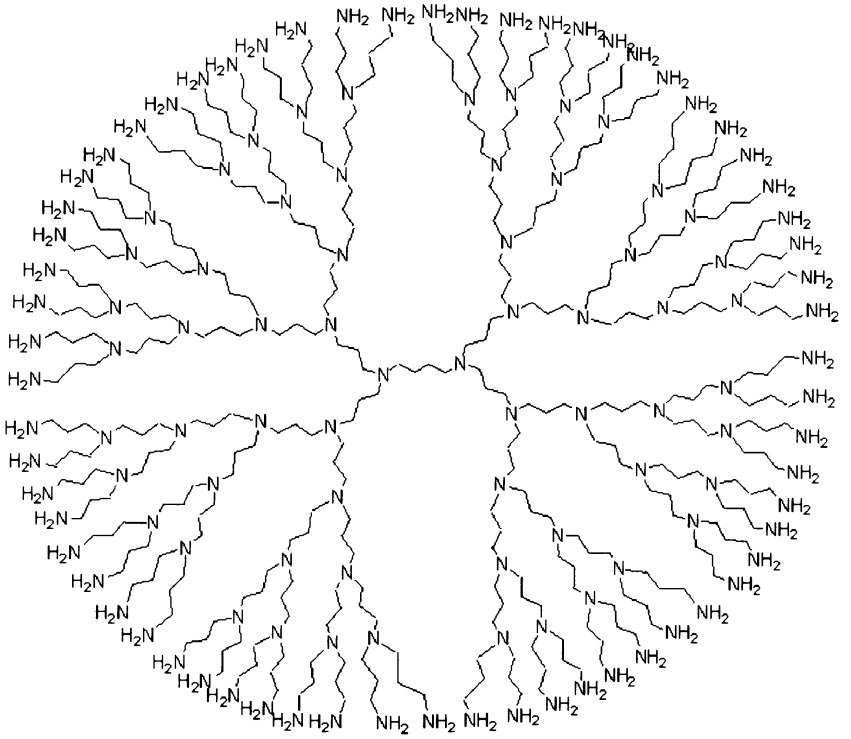


Figure 6: PPI G4 (DAB G4) dendrimer. Adopted from literature.[19]

1.1.2.3 Aromatic polyether dendrimers

Polyarylether dendrimers (Figure 7) were first prepared in 1990 by J. M. Fréchet.[23] This preparation was the first convergent synthesis (explained in chapter 1.1.3.2) of dendrimer. Because of their preparation (they are at first prepared as dendrons, which are then attached to a core) they can be used in bizarre dendrimers (see Figure 2, where 3 from 4 examples are polyarylethers).

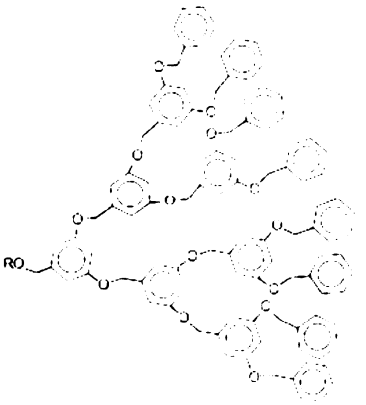


Figure 7: Fréchet-type polyaryl ether dendron. Adopted from literature.[5]

1.1.2.4 Phenylacetylene dendrimers

The first clearly hydrocarbon dendrimers has been phenylacetylene dendrimers (Figure 8). They were first prepared in the early 1990s by J. S. Moore using convergent method (chapter 1.1.3.2).[20] As they are composed from fully conjugated system of triple bonds and benzene rings they are the first shape-persistent dendrimers. Due to this feature they are widely investigated since they may possess more precisely defined three dimensional structure in contrast to PAMAM or PPI.[21] Due to conjugated π-systems they are also widely investigated as light-harvesting systems and for using in molecular electronics.

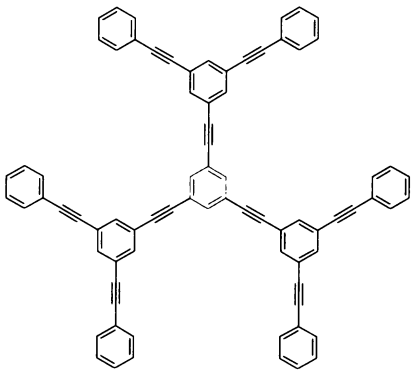


Figure 8: Phenylacetylene G1 dendrimer. The core of dendrimer is depicted by red colour.

1.1.3 Synthesis

Two conceptually different approaches of the construction of higher generation dendrimers exist – the divergent approach and the convergent approach (Figure 9). These methods provide most of dendrimer molecules.

1.1.3.1 Divergent synthesis

In divergent approach, dendrimer molecule is built out from the core layer by layer. This classical method was used e.g. by Tomalia for synthesis of PAMAMs. The procedure consists of two steps, involving (1) exhaustive Michael addition of core or previous generation of dendrimer by methyl acrylate, (2) exhaustive amidation of the resulting methylester by ethylenediamine (Figure 10).[1,3] Each particular sequence of steps (or sometimes only one particular step) adds exactly just one new layer (generation).

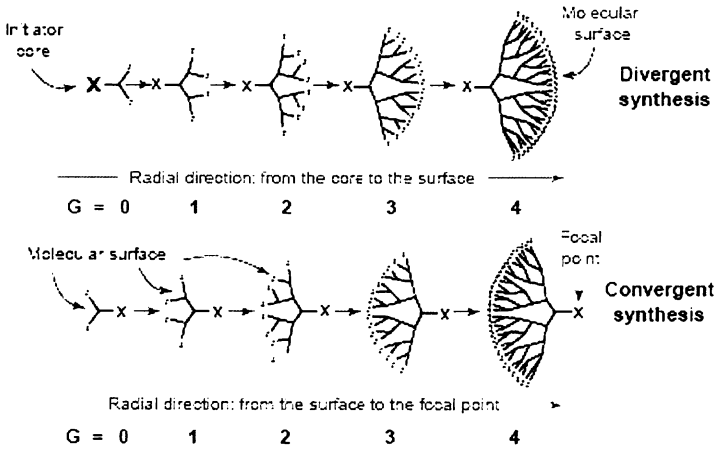


Figure 9: Differences between the convergent and divergent synthesis of dendrimers. Adopted from literature.[18]

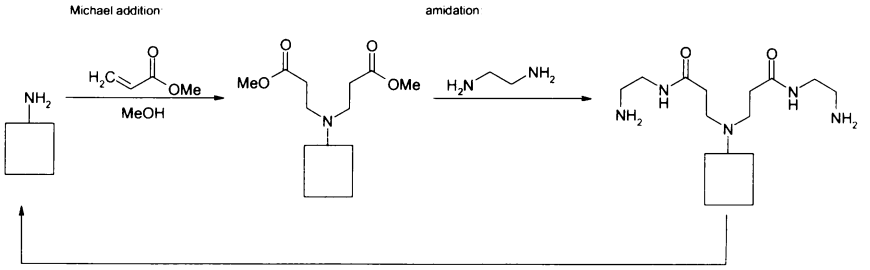


Figure 10: Synthesis of PAMAM dendrimer

This is the very straightforward approach, but some synthetic problems may be encountered. Numerous reactions have to be performed on single molecule as the dendrimer molecule increases. Even if the selectivity of reactions is high the number of reacting groups rises exponentially and thus the number of defects rises in the same way and the yield of defect-free dendrimers might be small. Since every new generation can be hardly purified (product and byproducts are very similar), the presence of statistical defects cannot be avoided. Thus the purity of dendrimers is mainly governed by statistics.[5,14,15]

However, despite mentioned disadvantage, this method is still widely used. Except of PAMAM also PPI dendrimers are synthetized in this way.

1.1.3.2 Convergent synthesis

Instead of expanding dendrimer molecule outward as in divergent method, convergent growth starts with the most peripheral part of dendrimer and continues inward to afford building blocks called dendrons that are subsequently coupled to the core or to the branching monomer to obtain next generation of dendron. Since reaction proceeds only on two sites in one time the number of side products is small and their mass differences are sufficient to purify the desired product. However, the focal point of obtained dendron with the reactive group is sterically hindered so its reactivity is decreased, causing the reactions to be slower and less efficient.

As a consequence, convergently produced dendrimers can be defect-free. Another important fact is that convergent approach allow us to obtain more curious dendrimers because of possibility of combining various molecular patterns in one dendritic molecule.[2,5,14,15]

This method was introduced by Fréchet in 1990 to create his polyarylether dendrimer. Also e.g. polyphenylacetylene dendrimers are prepared by this method.

1.1.4 Applications

Dendrimers are extensively investigated for various uses due to their unique structure. The mainly investigated utilizations take advantages of their basic features: (1) well defined molecular structure together with their macromolecular character, (2) possibility to attach desired substituents to the terminal groups, leading to high density of such attached substituents, (3) possibility to use various cores which have in high-generation dendrimers their own microenvironment isolated from the environment outside of the dendrimer molecule and (4) the availability to encapsulate some molecules into the cavities present in the molecule. There is a huge amount of dendrimer applications and it is not possible to mention all of them, thus there are shortly described some of the most important and/or interesting applications. In general, two main applications of dendrimers are reported: (1) utilization in catalysis, (2) utilization in biological systems for various purposes (particularly medicinal).

1.1.4.1 Catalysis

Catalysis seems to be a research area, in which promising applications for dendrimers may be developed. Dendrimers as the catalysts take advantage of both homogeneous and heterogeneous catalysis. They are soluble in the reaction mixture and thus they have great catalytic efficiency as homogeneous catalysts. In addition, due to their macromolecular structure they are readily removable from the reaction mixtures (similarly as heterogeneous catalysts) by ultrafiltration or dialysis. Therefore, dendrimer catalysts can be readily recovered and re-used in another reaction. This is the big advantage comparing to homogeneous catalysts because the homogeneous catalysts are often very expensive, and mostly cannot be regenerated.

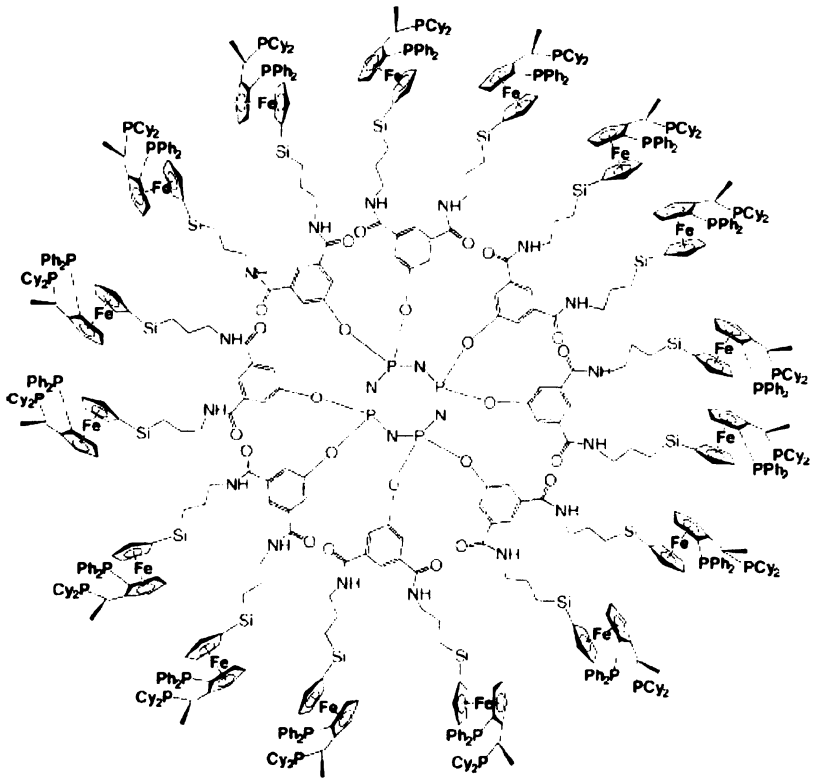


Figure 11: Example of dendrimer catalyst – dendrimer loaded with ferrocenyl terminal groups for enantioselective hydrogenation. Adopted from literature.[16]

As a catalytic site mainly act some transition metal complex, thus these dendrimers are called metallodendrimers. Catalytic sites can be bound to the terminal groups of dendrimers (Figure 11). This form possess high density of catalytic sites on the surface of macromolecule. In addition, suitable core can also act as a catalytic site. This

type of dendrimeric catalysts can possess special microenvironment of inner space of dendrimer molecule (different from outside environment), which is necessary for given reactions.[1,5,16]

1.1.4.2 Dendrimers in biological systems

As dendrimers have proper size and globular shape, they can be used to mimic some biomacromolecules. They are about the same size and shape as lots of important proteins, antibodies, nucleic acids and viruses. They are considered to be "*artificial proteins*". Within the PAMAM family, they closely match the sizes and contours of many important proteins, e.g. insulin (G3) or hemoglobin (G5).[14] Due to these properties they can pass through cell membranes using cell surface receptors, so they can be used for targeted drug delivery.

They are also often used in gene transfection. The PAMAM and PPI dendrimers can transfer oligonucleotides into the cells. Polylysine dendrimers even form complexes with DNA similar to those formed by DNA with histones. As dendrimers can be variously modified, it is possible to direct DNA-dendrimer complexes into the specific cells. This approach of delivering genetical information into the cells has an advantage over current method employing viruses in the way that virures can sometimes stimulate immune response which can destroy the viral agent before genetic material reaches its target.[1,5,14]

Dendrimers have been studied extensively as antitumor, antiviral and antibacterial agents. The dendrimers with a light harvesting groups have been studied as antitumor drugs suitable for photodynamic therapy (PDT). These dendrimers can have light harvesting cores (e.g. porphyrine) or terminal groups (e.g. 5-aminolevulinic acid).

Polylysine dendrimers modified with sulfonated naphtyl groups have shown antiviral activity against *Herpes simplex* virus. Another polylysine-based dendrimer has been found to reduce transmission of HIV. These dendrimer-based drugs inhibits early stage virus/cell absorption and later stage viral replication by interfering with reverse transcriptase.[1,14]

Utilization of dendrimer-based contrast agents (CAs, compounds enhancing the contrast of imaging methods) takes advantage of all yet mentioned features considerable

for pharmaceutical usage, e.g. high density of contrast-causing groups, bringing these groups on specific sites (targeting) or the fact, that macromolecules are not so simply eliminated from blood pool as low molecular weight compounds so they have much longer circulation times.[14]

Dendrimers loaded with radionuclides can be used as CAs for radionuclide imaging or for single photon emission computed tomography (SPECT). For example monoclonal antibodies attached to PAMAM dendrimers loaded with ^{111}In or ^{153}Gd complexes were synthetized for these purposes.[14]

In vivo oxygen imaging based on quenching of phosphorescence by oxygen requires chromophore with absorption bands different from bands of natural chromophores present in blood stream (to minimize interference between them). Such chromophores are e.g. Pd-complexes of tetrabenzoporphyrine. The complex has to be water soluble and protected from interactions with macromolecules present in blood serum. Encapsulation of Pd-complex into the dendrimer was used for this purpose to enhance the contrast.[14]

Very important is research of dendrimers as future CAs for magnetic resonance imaging (MRI). In addition to the features mentioned above, using dendrimers in MRI brings more enhanced contrast due to their macromolecular nature (as will be described in chapter 1.2.3). Dendrimeric CAs are provided by dendrimers functionalized by suitable Gd-chelates. This research is focused mainly on conjugates of PAMAM, PPI or polylysine dendrimers with derivatives of Gd-DOTA or Gd-DTPA (see chapter 1.2.2).[22,23,24] Clinical application of such macromolecular CAs is unfortunately limited by slow excretion rate (although this feature is sometimes an advantage, as mentioned before) and, as a result, their accumulation in the body. In addition, the long residence time of CA enhances the risk of potential release of toxic Gd(III).[14] However, there is even one commercial dendrimer CA – Gadomer-17™. It is polylysine dendrimer loaded with 24 Gd-GlyMeDOTA units (see chapter 1.2.2). This compound has been the first commercially used dendrimer pharmaceutical.[24] It is used in magnetic resonance angiography (MRA) because of its longer retention in blood pool comparing to low-molecular CAs.

1.2 MRI

Magnetic resonance imaging (MRI) is a non-invasive method providing three-dimensional images of human or animal body. It is mainly used for medical and biological purposes to visualize physiological or pathological alterations of living tissues. This method is very suitable for measurement of soft tissues. Compared to well-known Computed Tomography (CT), MRI has similar spatial resolution but far better contrast resolution, which allows to distinguish between two very similar, but chemically not identical tissues. However, it is also used for many other purposes, e.g. for non-destructive characterization of timber or for detecting rock permeability to hydrocarbons. But only the medicinal utilization of this method will be mentioned in following text (Figure 12).[26]

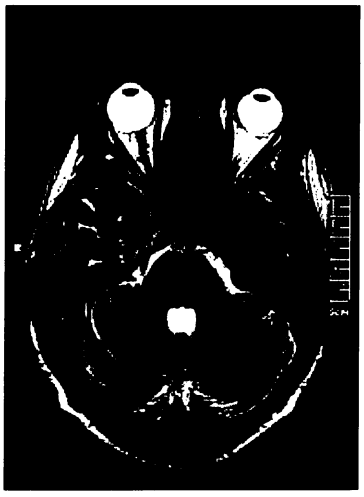


Figure 12: Picture of head acquired at 3 T MR scanner without using CA

MRI was formerly refered also as magnetic resonance tomography (MRT), nuclear magnetic resonance imaging (NMRI) or NMR-zeugmatography. The world "nuclear" was discarded from the name because of better feelings of investigated patients.

The MRI was invented in 1970s by Paul C. Lautenbur and Sir Peter Mansfield, who were awarded by Nobel prize for physiology and medicine in 2003 for their discovery. There has been a shadow of controversy on this award because more people has claimed their contributions on the invention and have been demanding to be also awarded. [25,26]

Nowadays, MRI is widely used in medicine for various purposes. However, the instrumentation is very expensive (millions of dollars) and the operation is costly too. There were 32 MRI scanners in use in Czech Republic in 2005 (compared to 5 PET scanners, 126 CT scanners and several thousands of X-ray devices).[27]

1.2.1 Principle

The fundamental physical principle of MRI is the same as the principle of nuclear magnetic resonance (NMR). The atomic nuclei of suitable isotopes (those with nonzero nuclear spin) placed in strong magnetic field can absorb certain energy and thus reach higher energy level. The energy difference between the excited state and the ground state is proportional to the strength of field applied and lies in the range of energies of radiofrequency radiation. It is possible to measure the energy difference (which is mainly used in chemistry in NMR and is quantified as chemical shift), signal intensity and the times required to return to ground state of nucleus (relaxation times). The most widely measured nuclei in NMR are ^{1}H and ^{13}C. The fact that human body consists of more than 70% of water and ^{1}H has predominant natural abundance supports ^{1}H utilization in MRI. As chemical shift of water protons is similar within whole body, it cannot provide almost any resolution. But the intensity of signal, which is inversely proportional to the relaxation times (that vary considerably within the body) can be used for imaging.[28,29,30]

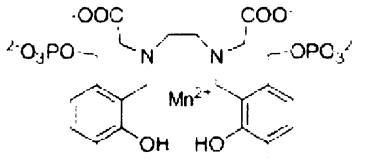
The spatial resolution of MRI is provided by using gradients of magnetic field. As the resonance frequency is proportional to the strength of magnetic field the spatial information is encoded in the frequency and the resolution is provided by different intensity. It is necessary to perform Fourier transformation of received signal to obtain an image. Three-dimensional picture is rendered by measuring of many slices. As there is orthogonal system of magnetic gradients, it is possible to measure slices under almost every desired angle.[26,30]

1.2.2 Contrast agents

A contrast acquired by MRI can be further enhanced by using suitable contrast agent (CA). Unlike CA used in X-ray methods or nuclear medicine, MRI CA are not directly visualized in the image and only their effects are observed. They cause dramatic

variation of water proton relaxation times (and consequently the intensity of NMR signal) and thus provides physiological information beyond the impressive anatomical resolution of uncontrasted images. Generally, the purpose of a CA is to reduce $T_{1,2}$ relaxation times in order to bring higher contrast in shorter times and better signal-to-noise ratio (T_1, longitudinal relaxation time, expresses time required for returning of magnetization vector back to the initial value in the z-axis orientation; T_2, transversal relaxation time, expresses time required for relaxation in directions perpendicular to z-axis). CAs can be divided into two groups: (1) T_1 CAs, sometimes called *positive CAs*, which mainly reduce T_1 relaxation time and thus increase the intensity of signal, and (2) T_2 CAs, called *negative CAs*, which heavily affect T_2 relaxation time and thus decrease the intensity of signal.[30]

Since the relaxation times are largely affected by unpaired electrons, research of CAs is targeted on highly paramagnetic compounds. Therefore the first investigated CA was Cr(III) complex of H_4EDTA. The most paramagnetic transition metal ions are Mn(II) and Fe(III), both with 5 unpaired electrons. Mn-DPDP complex is commercially used as hepatotropic CA (Figure 13). Fe(III) is used in the form of Fe_2O_3 micro- and nanoparticles as T_2 CA. However, CAs based on these ions are not so common as Gd-based CAs mentioned below.[30]



Mn-DPDP
TESLASCAN®

Figure 13: Commercially used Mn-DPDP. Adopted from literature.[30]

The most paramagnetic ion is Gd(III) with 7 unpaired electrons. This feature is very suitable for MRI CA. However, Gd(III) is very toxic and so it must be entrapped into thermodynamically and kinetically very stable complexes before it can be administered to a patient. The most investigated group of complexes has been that, which form Gd(III) with polyaminocarboxylic acids (both linear and cyclic). Such CAs are based primarily on Gd(III) complexes of H_4DOTA or H_5DTPA derivatives (Figure 14). These octadentate ligands form complexes with one coordination place occupied by

exchangeable water molecule. Its presence is necessary for contrast enhancement, as it provides linkage between CA and bulk water molecules. As mentioned in the chapter 1.1.4.2 the dendrimer-based CA Gadomer-17 is in clinical use. This CA is also based on DOTA-like ligand – GlyMeDOTA (Figure 15).[24,30]

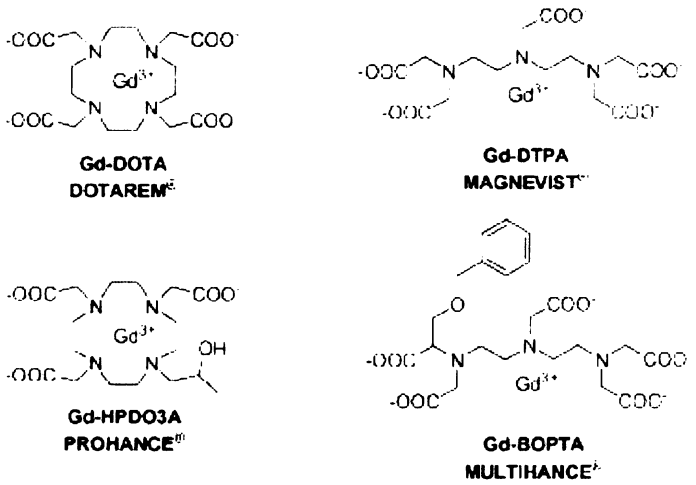


Figure 14: Examples of commercially used Gd-based CA. Adopted from literature.[30]

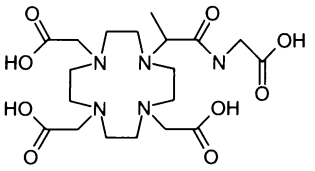


Figure 15: GlyMeDOTA – macrocyclic ligand contained in Gadomer-17.

One part of research of new MRI CAs is focused on synthesis of high relaxivity agents. This is performed by tuning properties of CAs (described below). Another part of this research is developing of responsive agents, which should be sensitive to pH, temperature, enzymatic activity, etc. This feature would be very helpful for direct diagnosis. The research of targeted CA, which bind selectively on specific sites in the body is also very intensive. Such agents are now represented mainly by Gd-complexes bound to the suitable biomacromolecules like monoclonal antibodies.[24,30]

1.2.3 Factors influencing effectivity of CA

Lanthanide ions causes the effect of Lanthanide Induced Relaxation (LIR) in the NMR spectra, which is a merit of function of CA. According to the theory of relaxation it

is possible to evaluate the efficacy of CA expressed as milimolar relaxivity, r_1 (relaxation rate observed in presence of 1 mmol of CA). The observed longitudinal relaxation rate R_1^{obs} of the water proton in the solution of paramagnetic complex is expressed as

$$R_1^{obs} = R_{1d} + R_{1p}^{is} + R_{1p}^{os} = R_{1d} + r_1 c_{Gd},$$

where R_{1d} is the diamagnetic contribution, that value corresponds to proton relaxation rate measured in the presence of diamagnetic complex of the same ligand, R_{1p}^{is} is the paramagnetic contribution related to the water exchange from the inner sphere of the metal complex to bulk water, R_{1p}^{os} is paramagnetic contribution related to the water diffusion in outer coordination sphere and c_{Gd} is concentration of Gd(III).

The inner sphere contribution is expressed as

$$R_{1p}^{is} = \frac{q c_{Gd}}{55.5(T_{1M} + \tau_M)},$$

where q is hydration number of metal ion in the complex, τ_M is the mean residence lifetime of water molecule in the complex and T_{1M} is relaxation time of its protons.

We can espress T_{1M} in the equations

$$\frac{1}{T_{1M}} = \frac{2}{15}\left(\frac{\mu_0}{4\pi}\right)^2 \frac{\gamma^2 g_e^2 \mu_B^2 S(S+1)}{r_H^6}\left(\frac{7\tau_{c2}}{1+\omega_S^2\tau_{c2}^2} + \frac{3\tau_{c1}}{1+\omega_I^2\tau_{c1}^2}\right),$$

$$\tau_{ci}^{-1} = \tau_R^{-1} + \tau_M^{-1} + T_{iE}^{-1}, \text{ where } i=1, 2$$

where r_H is the distance between water proton and metal ion, τ_R is rotational correlational time, T_{iE} means electronic relaxation times (dependent on applied magnetic field strength), g_e is electronic g-factor, γ is nuclear gyromagnetic ratio, μ_B is Bohr magneton, S is spin quantum number, ω_I is nuclear Larmor frequency, ω_S is electron Larmor frequency and μ_0 is permeability of vacuum.

The outer sphere contribution depends on T_{iE}, on the distance between water and paramagnetic metal ion, relative diffusion coefficients and again on the applied magnetic field strength.

As can be seen the relaxivity is dependent on four main quantities – T_{iE}, τ_R, τ_M and q. The only τ_R, τ_M and q are accessible for chemists to improve the efficiency of CA. Increasing of hydratation number causes in most cases undesired lowering of complex stability (releasing of toxic Gd(III)) and thus preclude its using in living organisms. However, some suitable ligands forming complexes with $q = 2$ were synthetized (Figure 16). The two remaining properties are the parameters that are mostly improved. Water exchange time represented by τ_M, that should be in optimal range (between 10–20 ns), can be improved by ligand design. For example Gd(III) forms with DOTA-like ligands generally two diastereomeric complexes traditionally called *m* (minor) and *M* (major). It was found that *m* isomers possess much better water exchange lifetimes than *M* isomers. Thus, ligand design leading to high abundance of *m* isomer is required.

Rotational correlation time, τ_R, that should be as long as possible, depends on rotation of molecule of CA. Thus it can be enhanced by attaching Gd(III) complex to some macromolecule (that slows down molecular motion).[30,31]

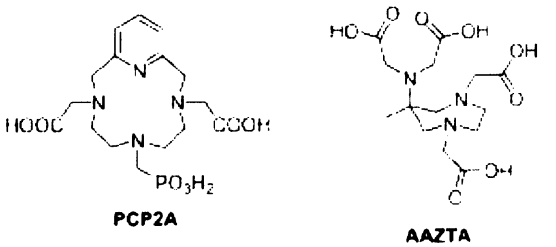


Figure 16: Examples of potential heptadentate ligands for CA possessing two bound water molecules. Adopted from literature.[30]

1.3 Fluorescence microscopy

Fluorescence microscopy is a very useful method to investigate cells and intracellular space. Since individual molecules can be labeled by fluorescent reagent, fluorescent microscopy provide a method to evaluate intracellular distribution of specific molecules. The method is also used in medicine for diagnostics.[26,32]

Fluorescence occurs when electron previously excited to higher electronic state returns back to its ground level and releases energy in the form of photon. After the initial absorption of radiation (and excitation) the upper vibrational states undergo (but not necessarily) radiationless decay, giving energy to surroundings. A radiative transition then occurs from the lower vibrational state, thus fluorescent light has lower energy and longer wavelenght. The various types of non-radiative and radiative transitions that can occur in molecule may be represented on a schematic Jablonski diagram (Figure 17).[27]

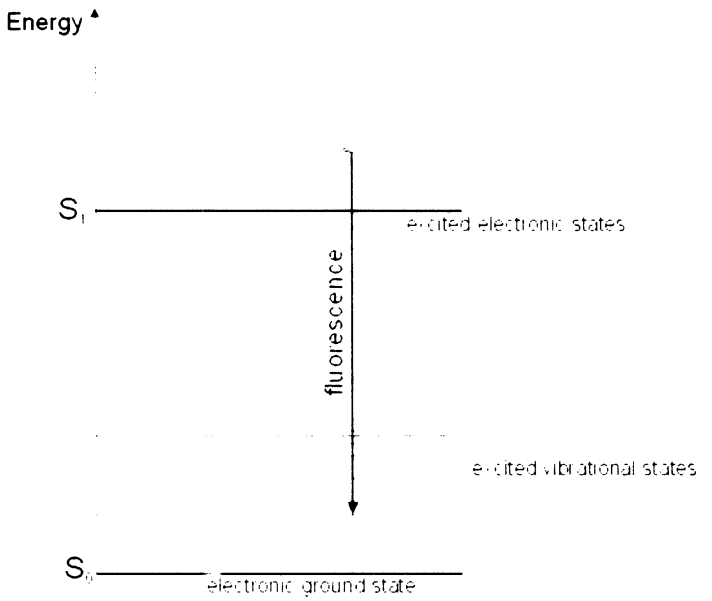


Figure 17: Jablonski diagram for simple fluorescence

Although fluorescence of naturally occurring molecules is sometimes employed, in most cases the addition of some fluorescent agent is necessary. As an examples of agents commonly used for labelling, we can mention fluoresceinisothiocyanate (FITC), tetramethylrhodamineisothiocyanate (TRITC) and dansylchloride. Substances produced

directly in the cell, e.g. green fluorescent protein (GFP), are sometimes used as fluorescence agents. Also combinations of fluorescent dyes are used to represent various objects within the cell.

Fluorescent microscopes can have various different constructions. The classical one consists of source of light (Xe or Hg lamp, laser), excitation filter, dichroic mirror, emission filter, optics and radiation detector (Figure 18). The devices can operate in visible or infrared range.[26,32]

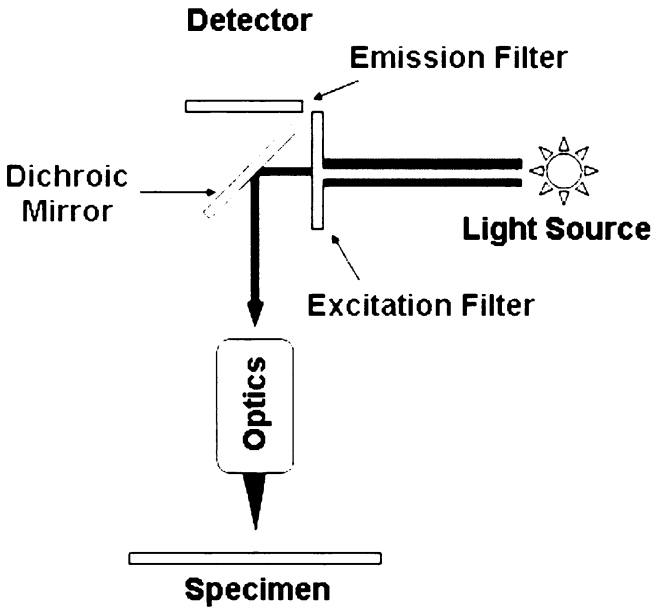


Figure 18: Schematic depiction of classical construction of fluorescent microscope. Adopted from literature.[26]

2 MOTIVATION AND THE AIM OF THE WORK

The interest in nuclear magnetism and applications of this phenomena is very atractive and currently highly investigated part of research. The importance of this part of science can be expressed also by several Nobel prizes awarded in this field (see following list).[25]

Physics:

1944 – Isidor Isaac Rabi *for his resonance method for recording the magnetic properties of atomic nuclei.*

1952 – Felix Bloch *and* Edward Mills Purcell *for their development of new methods for nuclear magnetic precision measurements and discoveries in connection therewith.*

Chemistry:

1991 – Richard. R. Ernst *for his contributions to the development of the methodology of high resolution nuclear magnetic resonance (NMR) spectroscopy.*

2002 – *half of the price was awarded to* Kurt Wüthrich *for his development of nuclear magnetic resonance spectroscopy for determining the three-dimensional structure of biological macromolecules in solution.*

Medicine and Physiology:

2003 – Paul C. Lautenbur *and* Sir Peter Mansfield *for their discoveries concerning magnetic resonance imaging.*

The real background of this work is a research cooperation between the Department of Inorganic Chemistry and Institute of Experimental Medicine (IEM), Academy of Sciences. Dr. Jendelová and her group at IEM perform a research dealing with stem cells. MRI represents useful method to trace stem cells previously loaded by CA in the body, but provides neither cellular nor sub-cellular resolution. Suitable method for tracing various molecules within the cell (having spatial resolution in the sub-cellular range) is fluorescence microscopy. Thus the aim of this work is to prepare bimodal CA suitable for both MRI and fluorescence microscopy.

The relaxivity of Gd-based CA can be considered as a measure of contrast-enhancing abilities of this CA. It depends on three factors suitable to be well modified by current chemistry – τ_R, τ_M, q (see chapter 1.2.3). When we focus only on the complexes with one coordinated molecule of water, we can infuence only τ_R and τ_M. Slow rotational correlational time and water exchange rate on the metal ion in optimal range are required for preparing high relaxivity CA. The first condition is in our case fulfilled by dendrimeric (macromolecular) merit of desired compound. The second condition is fulfilled by choosing appropriate Gd-chelate with fast water exchange reported.[33] Similar CA, based on PAMAM G2 and fully loaded with H_4DO3AP^{ABn}-derived ligand was investigated by J. Rudovský and possessed optimal parameters.[36]

Mentioning all previously written facts, the aim of this work is to prepare bimodal fluorescence/MRI active macromolecular agent. PAMAM G2 dendrimer was chosen as a macromolecular carrier, H_4DO3AP^{ABn} as a Gd(III) chelator with optimal water exchange rate and fluoresceinamine as luminescence label. The aim was to prepare macromolecular ligand with approx. 2 fluorescein-derived groups and the rest of terminal groups being H_4DO3AP^{ABn}-derived (Figure 19) and to make complex of this macromolecular ligand with Gd(III), which will be provided for experiments with stem cells.

The second important part of the work is to optimize various analytical methods allowing thorough analysis of prepared macromolecular compound.

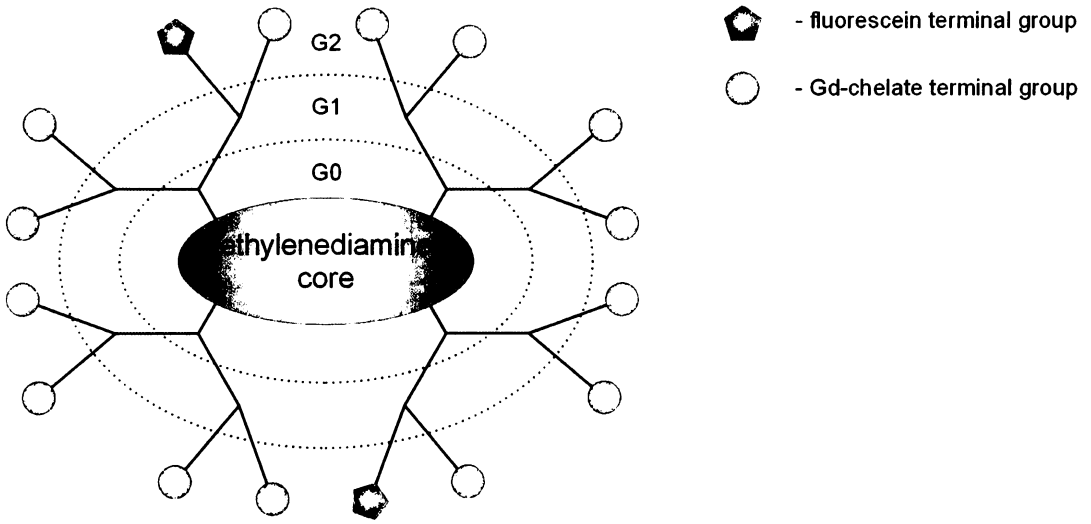


Figure 19: Sketch of PAMAM G2 derived CA with 2 fluorescent terminal groups and 14 Gd-chelate terminal groups.

3 EXPERIMENTAL

3.1 List of chemicals

acetic acid, 99%, p.a., Penta

acetone, p.a., Penta

ammonia, 25% aqueous solution, p.a., Lachner

ammonium hypophosphate, older material recrystalized from water and dried in dessicator over P_2O_5

argon, gas, pressure bomb, Linde Gas, 99.9999%

bromothymol blue, indicator, Lachema

dichloromethane, p.a., Penta, dried by refluxing with P_2O_5 if needed

N,N-dimethylformamide, pure, Lachema

disodium ethylenediaminetetraacetic acid dihydrate, p.a., Lachema

t-butanol, Roanal

tBu_3DO3A·HBr, pure, prepared and characterized previously in our laboratory

dysprosium chloride hexahydrate, 99.9%, Aldrich

ethanol, azeotropic, denatured with hexane, Penta

europium chloride hexahydrate, 99.9%, Strem

fluoresceinamine, isomer I, Aldrich

gadolinium chloride hexahydrate, 99.9%, Aldrich

hexamethyldisilazane, >98% GC, Fluka

hydrogen, gas, pressure bomb, Linde, 99.9%

hydrochloric acid, p.a., 35%, Penta

chloroform, p.a., Lachner

nitrobenzylbromide, >97% HPLC, Fluka

palladium on active charcoal, 10%, Aldrich

PAMAM G2 (Starburst™ dendrimer, ethylenediamine core), 20% methanolic solution, Aldrich

paraformaldehyde, filtered from old formaldehyde solution, Lachema

potassium hydroxide, pure, Lachner

tetrahydrofuran, p.a., Penta

tetrachloromethane, pure, Lachema

thiophosgene, 90% GC, Fluka

toluene, pure, Lachema

trifluoroacetic acid, 99%, Acros

NMR solvents:

DMSO-d^6, 99.8%,

D_2O, 98%, Russian origin

NaOD/D_2O, 1 M, prepared from Na and D_2O

3.2 Purification and characterization methods

Elemental organic analysis

Contents of C, H, N, S and Cl were determined by elemental analysis service at Institute of Macromolecular chemistry, Academy of Sciences in Prague. The results showed are averages of two measurements. Values in brackets are counted according to referred theoretical formula.

IR spectroscopy

IR spectra of samples in the form of nujol mull were recorded on a Nicolet Magna 760 FTIR spectrometer (2 cm^{-1} resolution, Happ-Genzel apodization). IR spectra were used for identifying isothiocyanate compounds, that possess signal at 2000 cm^{-1}.

NMR spectroscopy

^1H, ^{17}O and ^{31}P NMR spectra were measured on Varian ^{UNITY}*INOVA* 400 spectrometer. Spectra were measured at 399.95 MHz for ^1H, 54.23 MHz for ^{17}O and 161.92 MHz for ^{31}P. All measurements were carried out at 25 °C. Chemical shifts are written in ppm while interaction constants are in Hz.

Samples for dysprosium induced shifts (DIS) and bulk magnetic susceptibility (BMS) measurements consisted of solution of complex in D_2O/H_2O (20% D_2O) with small amount of *t*BuOH.

Concentration of Ln(III) was determined from the difference of δ_H of *t*BuOH in the sample and in the insert tube (Evans method). Concentration is expressed as

$$c_{Ln} = \frac{2.84^2 \Delta\delta_H T}{4 \cdot 10^3 s\pi\mu_{eff}^2},$$

where $\Delta\delta_H$ is difference of δ_H of *t*BuOH in the sample and in the insert tube, T is temperature, s is geometric factor (in our measurements $s = 1/3$) and μ_{eff} is effective magnetic moment of particular Ln(III) ($\mu_{eff}^{Gd} = 7.94$, $\mu_{eff}^{Dy} = 10.4$).[44]

The number of coordinated water molecules was determined by DIS. The dependence of induced δ_O on concentration of complex was plotted into a graph. Comparison of dependence slope of $[Dy(H_2O)_8]Cl_3$ with the slope of measured complex gives the number of coordinated water molecules. δ_O was determined with deconvolution.

Raman spectroscopy

Raman spectra of concentrated aqueous solutions were recorded on a Nicolet Magna 760 FTIR spectrometer equipped with Nicolet Nexus FT Raman module (2 cm^{-1} resolution, Happ-Genzel apodization, 1064 nm Nd:YVO_4 laser excitation, 1 W power at the sample) in the 100–3700 cm^{-1} region. The obtained data were worked up by OMNIC software. This method was used for determination of fluorescein/dendrimer ratio in dendrimer labeled by FITC. The mixtures of aqueous solution of PAMAM G2 and the solution of fluoresceinamine in 1 M KOH were used for acquiring a calibration curve.

The determination was based on comparison of signal height (over the baseline) of two signals. Broad band signal at 1310 cm^{-1} belongs to vibrations of C–H bonds of aliphatic skeleton of dendrimer while narrow signal at 2945 cm^{-1} belongs to fluorescein groups.

Thin layer chromatography - TLC

Two kinds of TLC plates were used – Silica Gel 60 F_{254}, Merck and Silufol, Kavalier. Chomatograms were dried by hot-air flow from heat gun. Mobile phases used are written at each particular used chromatography. Abbrevitation IPAV 7:3:3 means mixture of isopropanol, conc. ammonia (25% aq.) and water with ratio 7:3:3.

Ultrafiltration

Separations of macromolecular compounds from low-molecular compounds were carried out by ultrafiltration in Milipore ultrafiltration cells using Milipore membranes with 3 kDa cut-off. Final purities of obtained solutions of macromolecular compounds mentioned at particular preparations are written in the form of total dilution of low-molecular species (100% efficiecy expected).

Example: the solution of original volume 50 cm^3 was concentrated to 10 cm^3, then diluted to 50 cm^3 and concentrated again to 10 cm^3. Therefore, the dilution of low-molecular compounds was

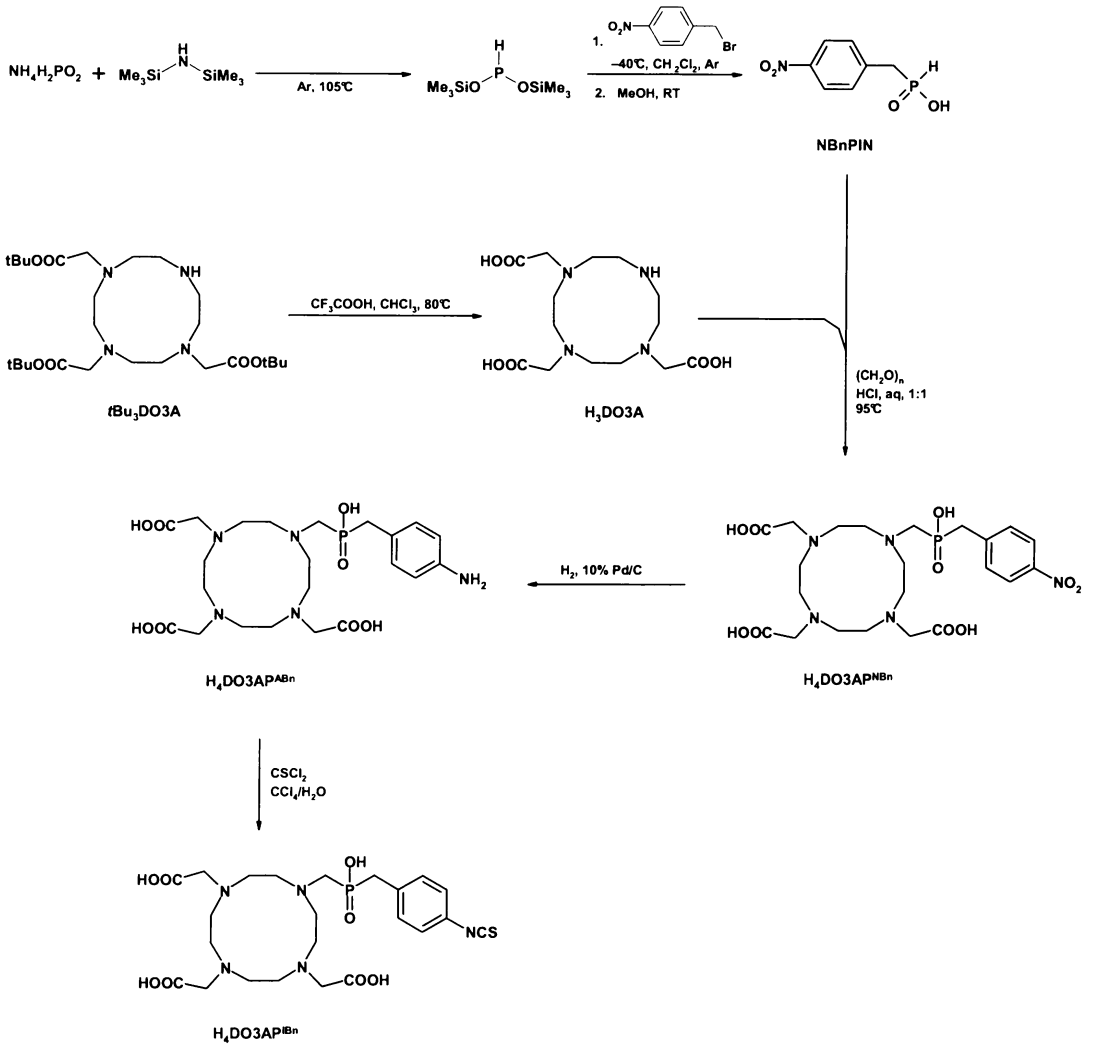
$$\frac{10}{50} \cdot \frac{10}{50} = 0.04 = 4\%$$

of original concentration.

UV-Vis spectroscopy

Measurements were carried out on the UNICAM UV 300 spectrometer. All measurements were performed in quartz cuvettes. The obtained data were worked up by Vision 32 software. This method was used for determination of number of fluorescein groups attached to dendrimer molecule. Fluoresceinamine was used for acquiring a calibration curve. Fluorescein and its derivatives can act as acidobasic indicators. Their molar absorption coefficients are highly dependent on pH. Thus the pH of samples was adjusted to 9.15–9.45.

3.3 Ligand preparation



4-nitrobenzylphosphinic acid, NBnPIN

The reaction was performed according to the procedure ordinarilly used in our laboratory.[34] A 1000 ml three-neck flask was equipped with gas inlet tube, spiral condenser (equipped with another gas inlet tube connected to oil rotary vacuum pump) and stopper. Magnetic stirring bar and $NH_4H_2PO_2$ (20.0 g) were charged into the flask. The apparatus was evacuated properly and subsequently filled by argon. This procedure was carried out four times. Then gas inlet tube on the condenser was exchanged for a bubble counter (with nujol) and the stopper was exchanged for a septum (both in strong flow of argon). Hexamethyldisilazane (100 cm^3) was added to the flask through the

septum from syringe. Argon flow was reduced to 2–3 bubbles/5 s. The reaction mixture was heated and stirred at oil bath (bath temperature approx. 95–105 °C) for 18 hours. After that time the argon flow was increased and the apparatus was taken out from the bath and let to cool to ambient temperature.

Dry CH_2Cl_2 (200 cm^3) was added to the flask through the septum. The apparatus was cooled to –40 °C in the cooling bath (solid CO_2/EtOH). The solution of 4-nitrobenzylbromide (23.6 g, 0.109 mol) in dry CH_2Cl_2 (200 cm^3) was added slowly (during 20 min) to the reaction mixture through the septum at continuous stirring and cooling. Then the whole apparatus including cooling bath was let to warm up to ambient temperature and the mixture was stirred for additional 18 hours.

Erlenmeyer flask (2000 ml) was loaded with EtOH (500 cm^3) and magnetic stirring bar. One side of long stainless cannula was inserted through the septum to a three-neck flask under the surface of reaction mixture and the second side was immersed to the Erlenmeyer flask just above the surface of ethanol. Then the stopcock on the gas inlet tube was closed and the pressure of argon was adjusted in such way that it transferred the reaction mixture into the stirred EtOH. The resulting mixture was evaporated to dryness.

HCl (6 M, 300 cm^3) and CH_2Cl_2 (300 cm^3) were added to the flask with resulting solid and the material was thoroughly mixed. Undissolved material was then filtered off by S4 glass frit. This solid was the first fraction of crude NBnPIN.

Subsequently, the organic phase was separated in separatory funnel. The resulting water phase was extracted with CH_2Cl_2 (5x200 cm^3). All organic fractions and the crude solid NBnPIN were combined and evaporated to dryness. Obtained solid material was dissolved in small amout of EtOH and evaporated to dryness.

The crude solid product was partially dissolved in boiling water (100 cm^3). Hot mixture was quickly filtered through S3 glass frit. The obtained solution was let to cool to ambient temperature and crystallize overnight. The resulting crystalic NBnPIN was filtered off, washed by cold water and dried by air flowing through the glass frit. Product was then finally dried in vacuum dessicator. Weight of dry NBnPIN from this fraction was 5.0 g.

The material, which had remained on the glass frit in previous attempt to dissolve crude NBnPIN in water, was again extracted in boiling water (110 cm^3) and the previously described crystallization was performed in the same way. Weight of dry NBnPIN obtained from this fraction was 5.3 g.

Another separation of desired product from the undissolved material that had remained in previous crystallization procedure was carried out in the same way as described above. Weight of dry NBnPIN obtained from this fraction was 2.3 g.

The purity of all four fractions was examined by ^{31}P NMR. The spectra showed no significant signals instead of NBnPIN phosphorus signal in all three cases. The fractions were of approx. same purity (~ 98% according to integration of ^{31}P NMR; impurity was the byproduct: bis(nitrobenzyl)phosphinic acid, 28.8 ppm) and therefore they were combined. Total yield was 12.6 g (0.063 mol, 57%).

Characterization:

Elemental analysis: C: 42.35% (41.80%); H: 4.01% (4.01%); N: 6.88% (6.96%); theoretical formula: $C_7H_8NO_4P$

^{31}P NMR (in EtOH:NH_3,1:1): 21.8 (dt, 1P, $^1J_{PH}$=510.5; $^2J_{PH}$=17.5), 28.8 (bs, 0.02P)

^1H NMR (in NaOD/D_2O): 8.09 (d, 2H, J_{HH}=8.4); 7.33 (m, 2H, $\sum J$=78); 6.86 (d, 1H, $^1J_{PH}$=522.7), 3.00 (d, 0.1H, $^2J_{PH}$=17.0, signal of practically exchanged methylene hydrogen atoms)

^1H NMR (in DMSO-d^6): 8.19 (d, 2H, J_{HH}=8.4); 7.59 (dd, 2H, J_{HH}=8.4, J_{HH}=2.4); 6.98 (d, 1H, $^1J_{PH}$=541.2), 3.22 (d, 2H, $^2J_{PH}$=19.2)

1,4,7,10-tetraazacyclododecane-4,7,10-tri(carboxymethyl)-1-[methyl(4-nitrobenzyl) phosphinic acid], H_4DO3AP^{NBn}

The reaction was performed according to slightly modified[35] literature procedure.[33] *t*Bu_3DO3A·HBr (5.00 g, 6.18 mmol) was added to the mixture of $CHCl_3$ (25 cm^3) and CF_3COOH (99%, 25 cm^3) in 100 ml round-bottom flask. Reaction mixture was refluxed overnight in oil bath (bath temperature approx. 80 °C). A small sample of the mixture was evaporated to dryness and dissolved in small amount of water. This procedure was performed three times. Resulting solid was dissolved in D_2O and ^1H NMR

spectra of this sample was measured. Disappearing of *tert*-butyl hydrogen atom signals (approx. 1 ppm) indicated complete hydrolysis of the reactant. Therefore, whole reaction mixture was evaporated to dryness, dissolved in small amount of water and evaporated again. This procedure was repeated twice. Pale yellow crystalline product (H_3DO3A) remained in the flask after last evaporation. Next reaction step was started without any other purification of this intermediate.

Obtained H_3DO3A was dissolved in 100 ml round-bottom flask in HCl 1:1 (40 cm^3) and NBnPIN (5.00 g, 3 eq.) was added. The mixture was heated in oil bath (temperature of bath 90–98°C). Paraformaldehyde (1.00 g, 4 eq.) was added in 10 doses during 4 hours (0.10 g every approx. 20 min). Subsequently, the mixture was stirred overnight at the same temperature. All solid materials present in the reaction mixture disappeared after 14 hours of heating. New portion of paraformaldehyde (0.50 g, 2 eq) was added in one dose after 24 hours of heating.

The progress of reaction was monitored by ^{31}P NMR. Samples from the reaction mixture were taken after 2, 4, 8, 16 and 44 hours. The last spectrum indicated that the reaction is complete (signal of NBnPIN [~37 (dt, 1P, $^1J_{PH}$~580)] disappeared while signal of product appeared [46.5, bs]; other signals in spectra belong to byproducts: ~28 ppm – nitrobenzylphosphonic acid, ~50 ppm – hydroxymethyl(nitrobenzyl)phosphinic acid) and thus the reaction was quenched after approx. 48 hours of heating.

Resulting mixture was evaporated to dryness. Solid material was dissolved in water and evaporated again. This procedure was once repeated. The mixture significantly made a foam during the first evaporation and brown solid, insoluble in water (hydroxymethyl(nitrobenzyl)phosphinic acid), precipitated on the wall of flask. This solid was filtered off and discarded.

Crude product was dissolved in water (approx. 20 cm^3) and the solution was poured dropwise on the column of strong cation exchanger Dowex 50 (height 25 cm, diameter 4 cm) in a H^+-cycle. The column was washed with 200 cm^3 of water. Then aqueous ethanol (50%) was used till total volume reached 2.3 dm^3. Subsequently, the product was eluted with aqueous ammonia (5%). It was important to follow the head of eluating ammonia (by observing of the moving site, where neutralization heat was being producted and slight colour change of cation exchanger was occurring). Product-

containing fraction (approx. 150 cm^3) started to be caught as soon as the head reached the end of column. Further fractions were also caught. It was found out that the most of desired product was present in the first ammonia fraction (by comparing TLCs of eluates with standard).

The material from the first ammonia fraction was evaporated and dissolved in small amount of water. The solution was poured dropwise on the column of strong anion exchanger Amberlite IRA 420 (height 26 cm, diameter 4 cm) in an acetate cycle. The column was washed with 1 dm^3 of water and 1 dm^3 of AcOH (5%). Consequently, the desired product was eluted by 1 dm^3 of HCl (3%). The fractions were combined and evaporated to dryness. Resulting solid was dissolved in small amount of water and evaporated again. Crude solid product was treated with THF by stirring for one week. Product was collected on glass frit and dried in vacuum dessicator. Yield was 2.96 g (4.20 mmol, 68%).

Characterization:

Elemental analysis: C: 37.45% (37.49%); H: 6.16% (6.08%); N: 9.48% (9.94%); Cl: 12.42% (12.58); theoretical formula: $H_4DO3AP^{NBn}\cdot 2.5HCl\cdot 3H_2O$; $C_{22}H_{42.5}N_5O_{13}PCl_{2.5}$

$^{31}P\{^1H\}$ NMR (in H_2O): 30.1, bs

TLC (silufol, IPAV 7:3:3, ninhydrine detection): $R_f = 0.75$, mauve spot

1,4,7,10-tetraazacyclododecane-4,7,10-tri(carboxymethyl)-1-[methyl(4-aminobenzyl) phosphinic acid], H_4DO3AP^{ABn}

The reaction was performed according to the slightly modified[35] literature procedure.[33] $H_4DO3AP^{NBn}\cdot 2.5HCl\cdot 3H_2O$ (1.70 g, 2.4 mmol) was dissolved in 100 ml round-bottom flask in water (40 cm^3). The flask was flushed three times with argon. Pd/C (10%; 0.25 g) was added and the flask was again flushed once with argon and twice with hydrogen. Balloon with hydrogen was connected to the flask and the mixture was stirred at ambient temperature. After three days of hydrogenation (complete reduction was detected by TLC and by disappearing of reactant signal in $^{31}P\{^1H\}$ NMR) the mixture was filtered through S4 glass frit. The obtained solution was evaporated to dryness. The

residue was dissolved in small amount of water and evaporated again. The resulting material was dissolved in HCl 1:1 and evaporated. This procedure was once repeated. Crude solid product was treated with THF by stirring for two days. Product was collected (S3) and dried in a vacuum dessicator. Yield was 1.55 g (2.2 mmol, 92%).

Characterization:

Elemental analysis: C: 37.10% (37.15%); H: 6.41% (6.50%); N: 9.60% (9.85%); Cl: 17.57% (17.45); theoretical formula: $H_4DO3AP^{ABn}\cdot 3.5HCl\cdot 3H_2O$; $C_{22}H_{45.5}N_5O_{11}PCl_{3.5}$

$^{31}P\{^1H\}$ NMR (in H_2O): 33.1, bs

(parent compound: 30.1, bs)

TLC (silufol, IPAV 7:3:3, ninhydrine detection): Rf = 0.7, dark pink spot

(parent compound: R_f = 0.75, mauve spot)

1,4,7,10-tetraazacyclododecane-4,7,10-tri(carboxymethyl)-1-[methyl(4-isothiocyanatobenzyl)phosphinic acid], H_4DO3AP^{IBn}

The reaction was performed according to the literature procedure.[31] $H_4DO3AP^{ABn}\cdot 3.5HCl\cdot 3H_2O$ (3.5 g, approx. 4.9 mmol) was dissolved in 250 ml round-bottom flask in water (30 cm^3). The solution of $CSCl_2$ (3.00 g in 20 cm^3 of CCl_4) was added and the flask was quickly capped. Biphasic system was intensively stirred for one day. Then the heavier organic layer was removed and the aqueous phase was evaporated. The product was obtained as a pale brown oily material (5.42 g). No other purification was performed and the product was immediately used in further reaction.

Characterization:

IR: signal at approx. 2000 cm^{-1} belonging to isothiocyanate group

3.4 Fluorescent label preparation

5-aminofluorescein FITC

5-isothiocyanato-3',6'-dihydroxy-spiro[isobenzofuran-1(3H),9'-[9H]xanthen]-3-one, 5-fluoresceinisothiocyanate, FITC

The reaction was performed according to the slightly modified literature procedure.[37] 5-aminofluorescein (0.50 g, 1.44 mmol) was dissolved in 50 ml pear-shape flask in DMF (1.25 cm^3). Acetone (2.5 cm^3) was added and stirred solution was cooled in cooling bath (NaCl:ice 1:1) to –15 °C. After cooling, 0.75 cm^3 of thiophosgene solution (approx. 42%, prepared by mixing 0.60 g of $CSCl_2$ and 0.82 g of CCl_4) was added during 20 min, and the solution was stirred for further 10 min.

Yellow solid material precipitated from the solution. This product was collected on S3 glass frit. Product was washed with acetone (15 cm^3), $CHCl_3$ (10 cm^3) and water (10 cm^3). Solid product was dried in a vacuum dessicator. Yield was 390 mg.

Acetonic filtrate was poured into the water (200 cm^3) and stirred for 30 min. Formed precipitate was collected by filtration. Yellow solid material was washed with water (10 cm^3). The product was dried in vacuum dessicator. Yield was 40 mg.

The both fractions were found to be pure (TLC check, one spot), therefore, they were combined. Total yield was 430 mg (1.11 mmol, 77%).

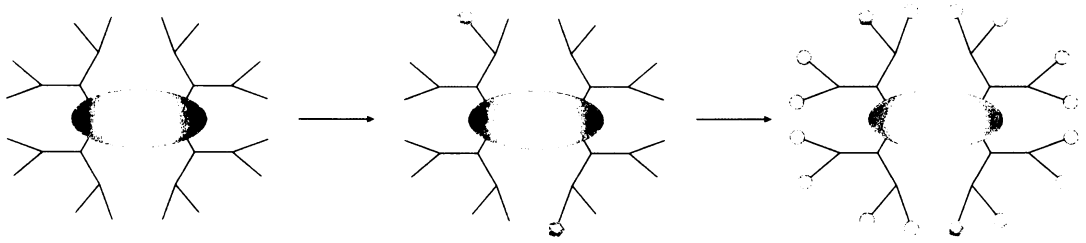
Characterization:

TLC (Merck, THF:toluene 1:1, visual detection): $R_f = 0.7$, orange spot

(parent compound: $R_f = 0.45$, yellow spot)

IR: strong signal at 2000 cm^{-1} belonging to isothiocyanate group

3.5 Preparation of conjugates



PAMAM G2 $D\text{-}F_2$ $D\text{-}F_2\text{-}L_{14}$

– fluorescein terminal group

– Gd-chelate terminal group

$D\text{-}F_2$

The reaction was performed according to the slightly modified literature procedure.[39] Methanolic solution of PAMAM G2 (4.70 g of 20%, i.e. 0.96 g of dendrimer, 0.29 mmol) was mixed with water (150 cm^3) in 500 ml round-bottom flask. Aqueous HCl (0.5 M) was added dropwise to adjust pH of the solution to 7.5 (measured by calibrated electrode). The solution of FITC (130 mg, 0.33 mmol in acetone/50 ml, 1.13 eq.) was added dropwise to intensively stirred reaction mixture during 20 min. The mixture was stirred for one day and then another portion of FITC (120 mg, 0.31 mmol in acetone/50 ml, 1.07 eq.) was added. Subsequently, the solution pH was readjusted to 7.7. The mixture was stirred for three days. Acetone was removed from the mixture by concentration on vacuum rotary evaporator (VRE) at bath temperature 35 °C and the solution was filtered through S4 glass frit. The filtrate was concentrated and purified by ultrafiltration (up to the dilution of low-molecular-weight compounds to approx. 0.35% of original concentration). The resulting solution was carefully concentrated on VRE to approx. 20 cm^3 and this solution was lyophilized. Resulting product was dark orange glassy material (1.17 g).

Characterization:

Elemental analysis: C: 46.34%; H: 7.49%; N: 16.53%; S: 2.34%

^1H NMR (in D_2O): 6.1–7.9 (m, 22.3H); 2.1–4.1 (m, 228H)

D-F_2-L_{14}

The reaction was performed by analogy to the literature procedure.[36] D-F_2 (1.03 g) was dissolved in 100 ml round-bottom flask in 25 cm^3 of water. Oily H_4DO3AP^{IBn} (5.42 g, obtained from previous reaction) was dissolved in water to obtain 19.8 g of solution. Part of this solution (12.6 g) was added dropwise to the dendrimer solution. Resulting solution pH was checked by calibrated electrode and kept in neutral or slightly acidic pH-range (5–7) during the addition of H_4DO3AP^{IBn} by simultaneous dropwise addition of 1M KOH. After addition of all H_4DO3AP^{IBn} solution, pH was adjusted to 8.8. Reaction mixture was stirred overnight at 30–35 °C. This intermediate was purified by ultrafiltration (up to the dilution of low-molecular-weight compounds to approx. 3.0% of original concentration)

The rest of H_4DO3AP^{IBn} solution was added to intermediate solution (30 cm^3) in the same way as decribed above. pH was adjusted to 9.0 and the solution was stirred at approx. 30 °C for 13 hours. The solution of desired product was purified by ultrafiltration (up to the dilution of low-molecular-weight compounds to approx. 0.5% of original concentration). The resulting solution of dendrimer was carefully concentrated at VRE to approx. 20 cm^3 and this solution was lyophilized. Resulting product was orange glassy material (2.16 g).

Characterization:

Elemental analysis: C: 42.44%; H: 6.18%; N: 12.24%; S: 3.69%

^{1}H NMR (in D_2O): 7.0–7.5 (bd, 10.00H); 6.45–6.65 (bs, 1.21H); 2.2–4.2(m, 95.18H)

3.6 Complexations

Complexes with Eu, Gd and Dy were prepared. All three complexations followed the same preparation scheme, according the literature procedure.[36] The preparation described below belongs to Eu, while data written in the square brackets belongs to [Gd/Dy]. Conjugate D-F_2-L_{14} (100 mg [500 mg/300 mg]) was dissolved in 25 ml [100 ml/50 ml] round-bottom flask in water (5 cm^3 [20 cm^3/12 cm^3]). A small amount of bromothymol blue indicator was added and the solution went dark green. $LnCl_3 \cdot 6H_2O$ (115 mg [590 mg/350 mg], approx. 2.5 eq.) was added to intensively stirred solution and

the mixture slowly went orange. The pH was adjusted to approx. 6.5–7.0 (green colour of the solution) by dropwise addition of 1M KOH (solid material precipitated from the solution during this operation). The mixure was stirred overnight at 50 °C. Na_2H_2EDTA (175 mg [750 mg/ 520 mg], 1.5 eq. per total amount of Ln(III)) was added and pH was readjusted again to 6.5–7 and the solution was stirred for additional 10 hours. Solid material dissolved almost immediately after addition of Na_2H_2EDTA. The resulting green solution was purified by ultrafiltration (up to the dilution of low-molecular-weight compounds to approx. 0.01% [0.01%/0.03%] of original concentration). The last portions of filtrates were absolutely colourless or only very slightly yellow. The resulting solution of dendrimeric complex was carefully concentrated at VRE to approx. 20 cm^3 and this solution was lyophilized. Resulting product was bright orange floss (106 mg [540 mg /310 mg]).

Characterization:

Gd complex:

Elemental analysis: C: 38.25%; H: 5.56%; N: 11.04%; S: 3.41%

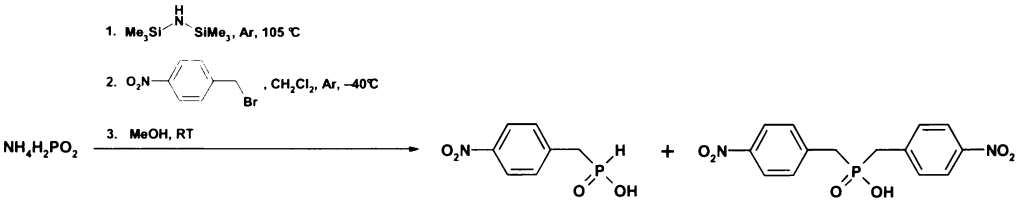
Eu complex:

^1H NMR (in D_2O): see appendix 1

4 DISCUSSION AND RESULTS

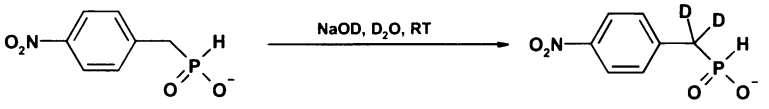
4.1 Ligand preparation

NBnPIN



The reaction was carried out according to the procedure optimalized and routinely used in our laboratory. Yield was 57% (with respect to nitrobenzylbromide) and the product had high purity (approx. 98% according to integration of ^{31}P NMR). Further crystallization performed according to the previously described procedure (chapter 3.3) did not gave any solid product. No other attempts were carried out due to small abundance of product and high abundance of predominant byproduct – bis(nitrobenzyl) phosphinic acid – in mother liquor (product/byproduct ratio was approx. 1/3 according to the integration of $^{31}P\{^{1}H\}$ NMR in EtOH:NH_3, 1:1).

The interesting event occurred when ^{1}H NMR (in NaOD/D_2O) was measured. The signal of methylene hydrogen atoms practically disappeared. Unexpected exchange of these hydrogen atoms for deuterium atoms from solvent was suggested to explain this fact. The hypothesis was confirmed by ^{1}H NMR in DMSO-d^6 where expected integral intensities were found. Another interesting fact is that despite the exchange of methylene hydrogen atoms, P–H proton remained unexchanged.



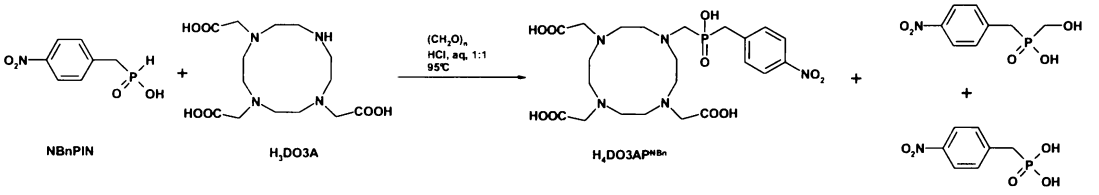
H_4DO3AP^{NBn}

The reaction was performed according to slightly modified[35] literature procedure.[33] The main modification was work-up procedure. Strong cation exchanger

was used to separate macrocyclic compounds containing amino-groups from non-aminic ones. Strong anion exchanger in acetate cycle separated strong acids from weak ones (separation of compounds containing P–OH from compounds with only carboxylic acid groups). Also treatment of solid product with THF was used instead of precipitation product by anhydrous EtOH from aqueous solution. The result of such modification was that $H_4DO3AP^{NBn}\cdot 2.5HCl\cdot 3H_2O$ was gained instead of $H_4DO3AP^{NBn}\cdot 3H_2O$.

^{31}P NMR spectra showed high dependence of δ_P on pH of the sample. Signals of the sample in the reaction mixture (in fact HCl 1:1) was at 46.5 ppm, while signal in water was at 30.1 ppm.

Yield was 68% (with respect to H_3DO3A).



H_4DO3AP^{ABn}

The reaction was performed according to slightly modified[35] literature procedure.[33] No chromatography was carried out for purification and also treatment of solid product with THF was used instead of product precipitation by anhydrous EtOH from aqueous solution. The result of such modification was that $H_4DO3AP^{ABn}\cdot 3.5HCl\cdot 3H_2O$ was obtained instead of $H_4DO3AP^{ABn}\cdot 3H_2O$. Yield was 92%.

H_4DO3AP^{IBn}

The reaction was performed according to the modified literature procedure.[31] Although it was performed twice under practically same conditions the results seemed different. The product of the first preparation was pale blue glassy product, but in the second preparation the product was pale brown oily substance. The two visages were probably caused by impurities. As this compound was only intermediate which was immediately used for further reactions, the appearance of product was not important and

no further purification was performed. Conversion of reactant to desired product was confirmed by IR spectroscopy (Figure 20).

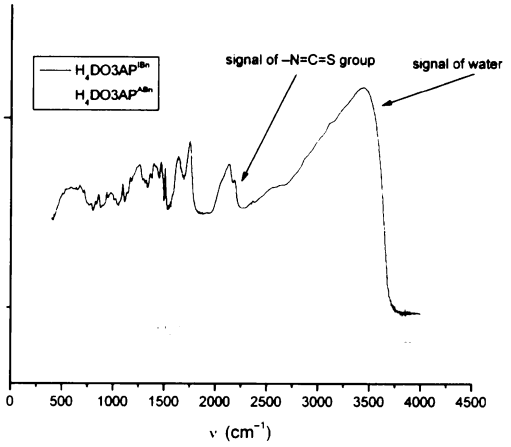


Figure 20: IR spectra of H_4DO3AP^{ABn} and H_4DO3AP^{IBn}

4.2 Fluorescent label preparation

FITC

The first attempt to prepare FITC was performed according to the Czechoslovak patent.[37] The reaction did not give desired result. It was probably caused by dilution of the reaction mixture as it was carried out at 1000x smaller scale than described in patent.

Thus, further reaction was carried out according to the another Czechoslovak patent.[38] The yield was 77%. Loss of product was caused by its extremely adhesion to all equipment used. Some mass also passed through the glass frit during the collection of second fraction as this precipitate was too fine for collection even on S4 glass frit. This portion of product was discarded due to acceptable yield of FITC obtained in previous fractions. Conversion of reactant to desired product was confirmed by IR spectroscopy (Figure 21).

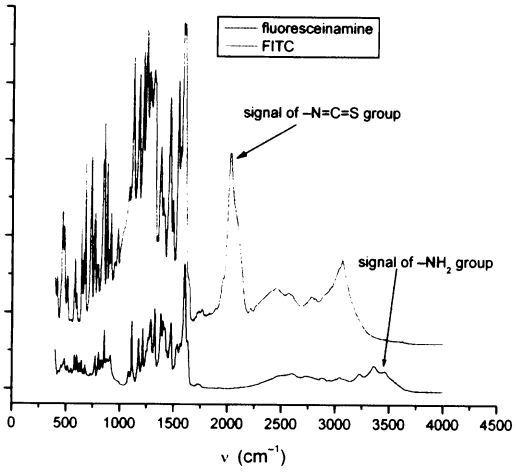


Figure 21: IR spectra of fluoresceinamine and FITC

4.3 Conjugates

4.3.1 Preparation

Both conjugates, D-F_2 and D-F_2-L_{14}, were prepared at same conditions introduced in literature.[36,39] The amount of FITC corresponding to 2.2 eq. of fluoresceinamine was reacted with dendrimer and then excess of H_4DO3AP^{IBn} was used to substitute all remaining terminal amino groups. The conjugation proceeded without any problems.

When trial batch of conjugate was prepared, the filtrate from ultrafiltration was orange but went more and more brighter as the ultrafiltration continued. During preparation of second batch of conjugates the filtrate had nearly constant colour during whole ultrafiltration. This was probably caused by unintentional using of damaged membrane. However, this event did not have large influence on purity of conjugates. It had only influence on the amount of conjugate obtained. Another loss of product can be also caused by partial hydrolysis of thiourea bridges and consequent lowering of molecular weight.

4.3.2 Characterization

Elemental analysis of D-F_2 *- determination of attached fluorescein groups amount*

C/N, C/S and N/S ratio can be used for this determination. The resulting amounts of attached fluorescein groups for particular ratios are: C/N – 2.7; C/S – 4.4; N/S – 3.8 fluorescein groups/dendrimer molecule. The best result is the number obtained from C/N ratio. However, the result is only very approximate (but still shows roughly the expected ratio), because only 2.2 eq of FITC was added to dendrimer. Moreover, some loss of fluorescein groups occurred (leaking of fluorescein during ultrafiltration) so the amount of attached fluorescein groups must be even smaller. The results based on the amount of S are too far from possible results. This huge inaccuracy can be explained by small abundance (and consequently low precision of analysis) of S in the sample. Another possible explanation is that used solution of dendrimer had lower concentration than was declared and thus higher abundance of fluorescein groups is correct. Therefore, the obtained ratio is only very approximate (but still shows roughly the expected ratio).

^{1}H NMR of D-F_2 *- determination of attached fluorescein groups amount*

Integration of ^{1}H NMR (in D_2O) was used to determine amount of fluorescein groups present in D-F_2 conjugate. All signals in aromatic range of spectrum belong to fluorescein while all signals in aliphatic range belong to ethylene hydrogen atoms of PAMAM. Observed ratio of fluorescein/dendrimer is 2.5 while total amount of added FITC was only 2.2 eq. Moreover, some loss of fluorescein groups occurred (leaking of fluorescein during ultrafiltration) so the amount of attached fluorescein groups must be even smaller. Such inaccuracy can be explained by difficult baseline fitting and phasing of spectra. Another possible explanation is that used solution of dendrimer had lower concentration than was declared and thus higher abundance of fluorescein groups is correct. Therefore, the obtained ratio is only very approximate (but still shows roughly the expected ratio), consistently with elemental analysis.

Raman spectroscopy of D-F_2 *- determination of attached fluorescein groups amount*

The determination was based on comparison of signal height (over the baseline) of two signals – broad band signal at 1310 cm^{-1} belonging to vibrations of C–H bonds of

dendrimer and narrow signal at 2945 cm^{-1} belonging to fluorescein groups. The calibration curve was based on the mixtures of fluoresceinamin and unsubstitued PAMAM. Despite the calibration was without problems, the determination was very problematic and gave only very inaccurate result (see Appendix 2). The calculated ratio is only very approximate: 2.0–2.5 fluorescein/dendrimer (it still shows roughly the expected ratio). Problematic evaluation of spectrum of D-F_2 was caused by too weak signal and consequently too low signal/noise ratio.

Elemental analysis of D-F_2-L_{14} *- determination of attached groups ratio*

Solving the equation system (mentioning the abundances of C, N and S) did not give acceptable results (the sum of numbers of attached groups was quite higher than 16). The inaccuracy of analysis can be explained by small abundance (and consequently low precision of analysis) of S in the sample. Mentioning only the ratio C/N (and subsequent supposing of full substitution) also did not bring acceptable result. Resulting ratio n_F / n_L (where n_F is the number of fluorescein groups and n_L is the number of ligands attached to dendrimer) is 3.4/12.6 – much higher than can be. Counting with C/S and N/S ratios give even worse results.

^{1}H NMR of D-F_2L_{14} *- determination of ratio of attached terminal groups*

Integration of ^{1}H NMR (in D_2O) was used to determine amount of fluorescein groups present in D-F_2-L_{14} conjugate. Signals in aromatic range of spectrum belong to fluorescein and ligand while signals in aliphatic range belong to ethylene hydrogen atoms of PAMAM and ligand. The ratio of intensity of aliphatic/aromatic signals was 8.49. The result of the integration is linear function

$$n_F = 2.984 - 0.104 n_L ,$$

where n_F is the number of fluorescein groups and n_L is the number of ligands attached to dendrimer. If complete substitution of dendrimer is supposed ($n_F + n_L = 16$), then the n_F / n_L ratio is 1.47/14.53.

UV-Vis spectroscopy of $D\text{-}F_2$ and $D\text{-}F_2L_{14}$ - determination of fluorescein concentration

Amount of fluorescein groups was determined for both conjugates. The determination was carried out without any problems. Fluoresceinamine used for calibration has absorption maximum at 489 nm while the absorption maximum for conjugates was at 499 nm. But this fact probably did not affect the results as the absorption bands are broad and the extinction coefficients are similar. Amount of fluorescein groups contained in certain weight of conjugates was obtained.

compound	contains of fluorescein groups (mol/g)
$D\text{-}F_2$	$3.77 \cdot 10^{-4}$
$D\text{-}F_2\text{-}L_{14}$	$1.04 \cdot 10^{-4}$

If we suppose exactly 2 fluorescein groups contained in $D\text{-}F_2$ then the solid form (according to above mentioned results) contains 24% of water and $D\text{-}F_2\text{-}L_{14}$ contains 37% of water.

4.4 Complexes

4.4.1 Preparation

Complexes with Eu, Gd and Dy were prepared. When trial batches of Dy and Gd complexes were prepared, unknown intensively orange material precipitated. Attempts to dissolve formed precipitate using even strongly acidic or strongly basic conditions failed. The only result of these attempts was significant destruction of thiourea bridges, which was indicated by impossibility to gain any acceptable result from DIS measurement, by the strong loss of orange colour during further ultrafiltration and by the smell of H_2S leaking from the solution. This problem was solved by using Na_2H_2EDTA to dissolve precipitate as mentioned in literature.[36]

The second attempt to prepare complexes proceeded without problems. Filtrates from ultrafiltrations were green in the beginning because of bromothymol blue indicator used. If acidified, these filtrates turn orange, that indicated some amount of fluorescent label was present in filtrates. But later filtrates were colourless or only very slightly yellow. Some hydrolysis of thiourea bridges probably occurred during complexation, as can be indicated by leaking fluorescent label. Complexes were obtained as bright orange floss and were easy to handle.

4.4.2 Characterization

Elemental analysis of Gd-D-F_2L_{14} *- determination of attached groups ratio*

Solving the equation system (mentioning the abundances of C, N and S) did not give acceptable results (the sum of numbers of attached groups was much higher than 16). The inaccuracy of analysis can be explained by small abundance (and consequently low precision of analysis) of S in the sample. Moreover, elemental analysis of complex compounds by burning method is not in generally considered to be much reliable. Mentioning only the ratio C/N (and subsequent supposing of full substitution) also did not bring acceptable result. Resulting ratio n_F / n_L is 3.4/12.6 – much higher than can be. Counting with C/S and N/S ratios give even worse results. The obtained ratio is (similarly to C/S, N/S and C/N ratios) same as for D-F_2-L_{14}. This fact was not properly explained.

^{1}H NMR *- determination of attached terminal groups ratio on Eu complex*

Integrations of ^{1}H NMR spectra of Eu complex were tried for determination of attached terminal groups ratio. The results showed fluorescein/dendrimer ratio between 0.6 and 3.9. Such inaccuracy can be explained by difficult baseline fitting and phasing of spectrum. Spectrum is shown at Appendix 1.

BMS *- determination of Ln(III) concentration*

Concentrations of Gd(III) and Dy(III) were determined by measuring BMS. The determination was carried out without any problems. Amount of complexes contained in certain weight of conjugates was obtained.

complex of	contains of Ln(III) (mol/g)
Gd	$7.03 \cdot 10^{-4}$
Dy	$7.97 \cdot 10^{-4}$

UV-Vis spectroscopy *- determination of fluorescein concentration*

Amount of fluorescein groups was determined for all 3 complexes. The determination was carried out without any problems. All three complexes showed similar

abundance of fluorescein groups. Fluoresceinamine used for calibration has absorption maximum at 489 nm while the absorption maximum for conjugates was at 499 nm. But this fact probably did not affect the results as the absorption bands are broad and the extinction coefficients are similar. Amount of fluorescein groups contained in certain weight of conjugates was obtained.

complex of	contains of fluorescein groups (mol/g)
Eu	$9.83 \cdot 10^{-5}$
Gd	$9.37 \cdot 10^{-5}$
Dy	$1.07 \cdot 10^{-4}$

Combination of UV-Vis and BMS - *determination of fluorescein/ligand ratio*

The results of UV-Vis and BMS can be combined and n_F / n_L ratio may be obtained. The last column suppose full substitution.

complex of	n_F / n_L	n_F / n_L, if $n_F + n_L = 16$
Gd	1/7.50	1.88/14.12
Dy	1/7.42	1.90/14.1

If we suppose full substitution of complexes then according to above mentioned results they contains about 30% of water.

Eu-luminescence - *determination of number of water molecules coordinated to the Eu(III) cation*

This measurement was impossible due to strong luminescence of fluorescein groups present in the measured complex. The luminescence of Eu(III) is completely overlaid by the luminescence of fluorescein.

^{1}H NMR - *determination of geometry of Eu-complex*

As has been reported in literature,[33,40,41,42] DOTA and DOTA like ligands form complexes having two isomers – *m* and *M*. These isomers can be distinguished by

measurement of ^{1}H NMR of complexes. Hydrogen atoms of ethylene units belonging to cyclene ring shows different δ_H according to the geometric arrangement of these isomers. Comparing the integral intensities of axial hydrogen atoms of both isomers is the way to determine ratio of *m* and *M* isomer. As our ligand has four different axial hydrogen atoms, average integral values of all signals of both isomers were taken into account. Average integral intensity of hydrogen atoms signals of *m* isomer (δ_H = 11.6; 13.0; 17.9; 22.4) was compared with average value of intensities of *M* isomer (δ_H = 27.4; 33.4; 36.3, 38.2). The measurement showed that 38% of Eu(III) complexes was in the form of *M* isomer, while 62% was *m* isomer (Figure 22).

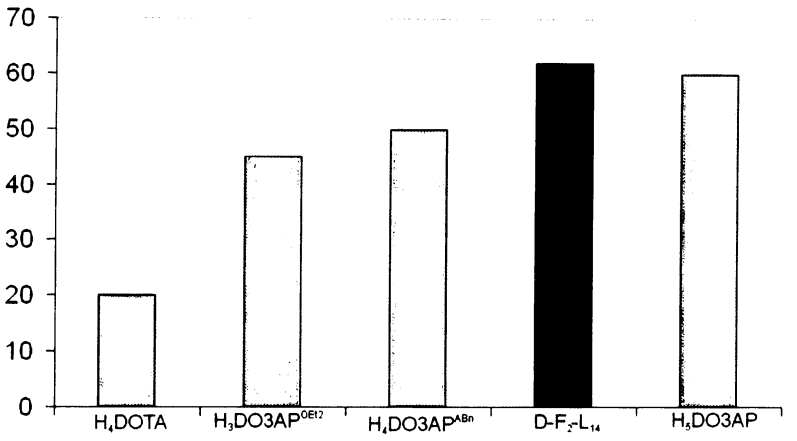


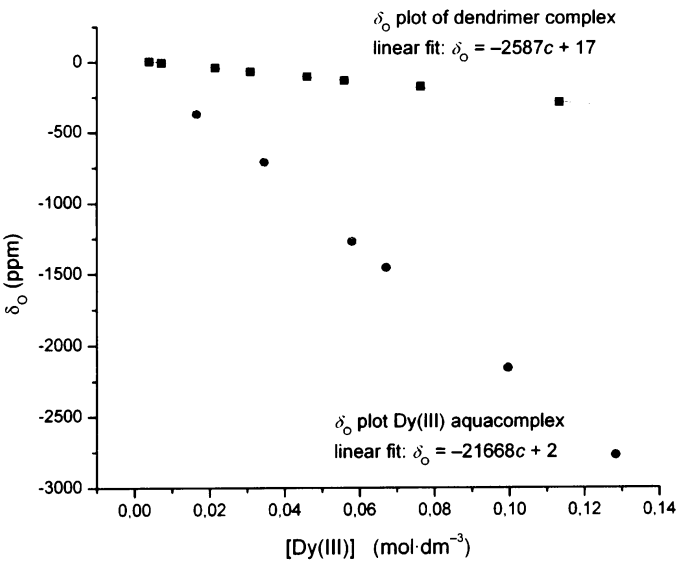
Figure 22: Molar fraction (%) of *m* isomer of Eu complexes with various DOTA-like ligands. Complexes are represented only by particular ligands. Data for low molecular ligands are adopted from literature.[33,40,41,42]

***DIS** - determination of number of water molecules coordinated to the Dy(III) cation*

Number of coordinated water molecules is important property of Gd-based MRI CA. The number was determined by comparing slope of linear fit of δ_O dependence on concentration of dendrimer complex with slope of linear fit of δ_O dependence on concentration of Dy(III) aquacomplex. Concentrations was measured by the BMS method.

Values obtained from DIS and BMS measurements.

$[Dy(H_2O)_8]^{3+}$			dendrimer complex		
$\Delta\delta_H$ (ppm)	[Dy(III)] ($mol \cdot dm^{-3}$)	$\Delta\delta_O$ (ppm)	$\Delta\delta_H$ (ppm)	[Dy(III)] ($mol \cdot dm^{-3}$)	$\Delta\delta_O$ (ppm)
3.23	0.0165	–241.3	0.80	0.00398	141.1
6.30	0.0345	–581.8	1.42	0.00725	131.0
11.34	0.0579	–1141.0	4.19	0.0214	96.0
13.12	0.0670	–1325.3	6.05	0.0308	70.4
19.49	0.0996	–2027.5	9.01	0.0460	34.9
25.11	0.1285	–2643.9	10.94	0.0558	7.1
			14.92	0.0762	–35.6
			22.17	0.113	–147.5



The number of water molecules bound to the Dy(III) ion was acquired by dividing the slope of octaaquacomplex by the factor 8 and then by comparing with slope of dendrimer complex:

$$q = \frac{-2587 \cdot 8}{-21688} = 0.96$$

Presence of one coordinated water molecule was proved. This fact confirmed our expectations.·

5 CONCLUSIONS

Bimodal fluorescence/MRI active macromolecular contrast agent was managed to prepare during the working on this thesis. This CA was prepared in quantity sufficient to provide it for experiments with stem cells. Synthetic route for preparation of this agent was found and optimized for gram scale. Because slight leaking of fluorescent label was observed during every reaction step, it is necessary to use more FITC than is desired amount of fluorescent label. The leakage is caused by hydrolysis of thiourea bridges. As some cleavage of thiourea bridges can occur, the resulting CA does not necessarily be completely substitued.

Various analytical techniques were employed for analysis products. But none of them was precious enough to provide some more accurate information. The most methods provided only (if provided) information that approximately 1.5–2.5 fluorescein groups is present in the dendrimer molecule and the rest of terminal amino groups of dendrimer are substitued by ligand (complex). Only combination of UV-Vis and BMS of Gd and Dy complexes provide more exact information. But it was only fluorescein/ligand ratio, not the absolute number of attached terminal groups. As has been found out, this compounds have absorption maximum at slightly different wavelenght than fluoresceinamine. No problems with this fact were encountered, but in some future analysis model conjugate of FITC with suitable amine (e.g. propylamine) will be used instead of pure fluoresceinamine (absorption maximum should be closer to maximum of macromolecular conjugates) for calibration.

Despite the determination of fluorescein/ligand/dendrimer ratio was not sufficiently precious, the investigation of geometry of complexes attached was successful. It was determined, that one water molecule is coordinated to complexes. This fact is necessary for using the compound as CA. Also the abundance of *m* and *M* isomers of Eu(III) complex was determined. As each of these two isomer (analogically as similar complexes) possess different relaxivity, this is an important information for CA. The measurement showed that 38% of Eu(III) complexes was in the form of *M* isomer, while 62% was *m* isomer. As has been mentioned before (chapter 1.2.3), the water exchange lifetime of Gd(III) complexes with DOTA-like ligands depends on the abundance of *m*

isomer. Because of high abundance of this isomer, prepared compounds should possess high relaxivity.

The interesting, not very relating to the CA, event was observed. Unexpected proton exchange occurred on methylene group of NBnPIN in basic conditions. The most interesting fact is that no exchange occurred on P–H proton.

This work will probably continue as Master diploma thesis. Further analyses and measurements of obtained conjugates will be performed. For example: abundance of Gd will be determined by ICP-MS, relaxometric characterization, preparation of novel conjugates with different ligands, etc.

6 LITERATURE

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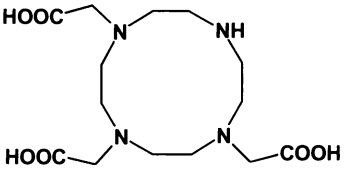
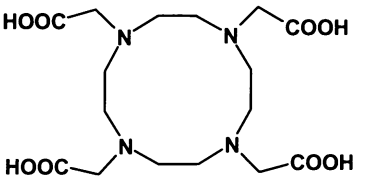
7 LIST OF ABBREVITATIONS

BMS	Bulk Magnetic Susceptibility
CA	Contrast Agent
DIS	Dysprosium Induced Shift
DMF	*N,N*-dimethylformamide
DMSO	Dimethylsulfoxide
H_3DO3A	1,4,7-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecane



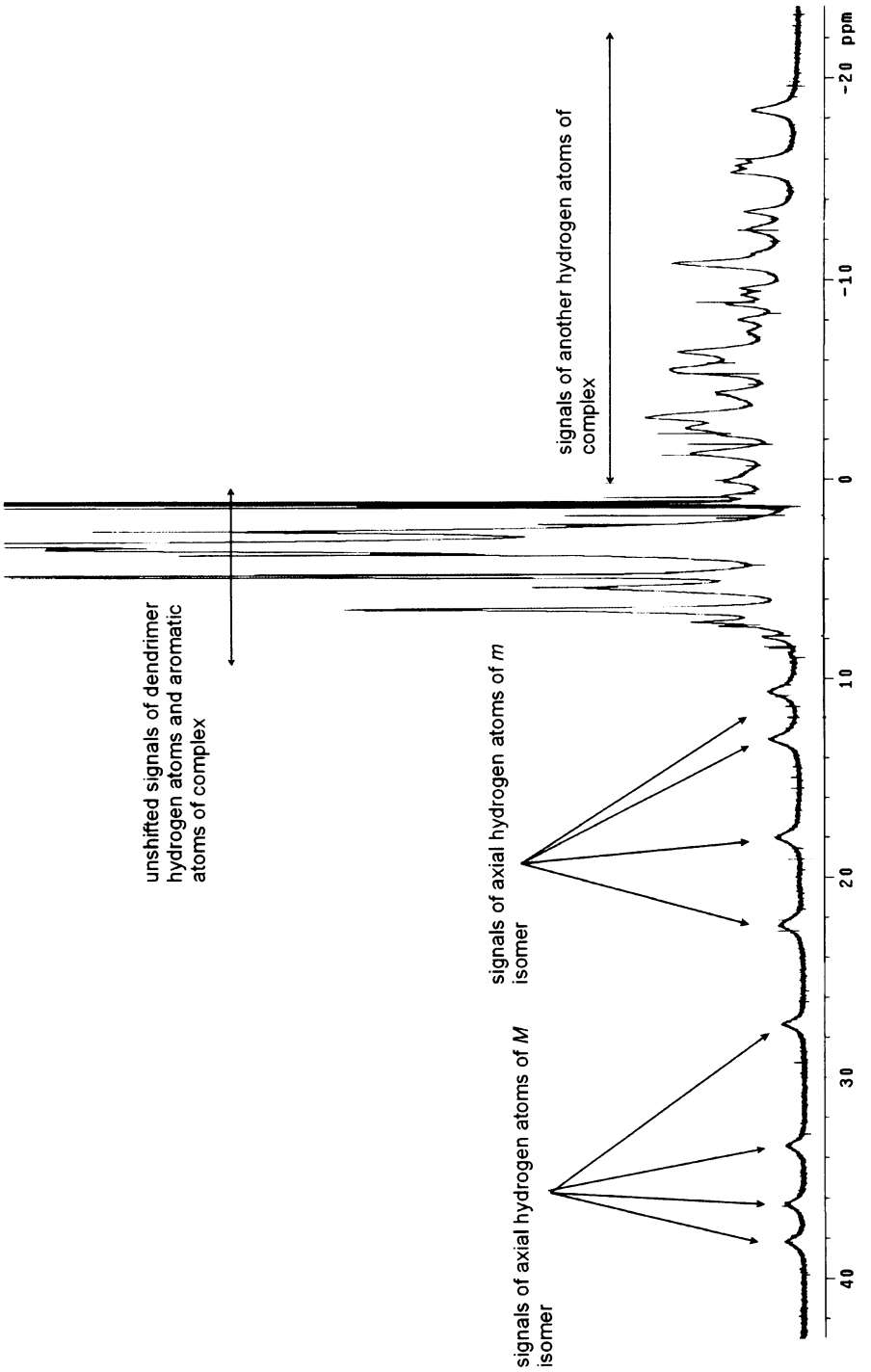
H_4DOTA	1,4,7,10-tetrakis(carboxymethyl)-1,4,7,10-tetraaza cyclododecane



H_5DTPA	Diethylenetriaminepentaacetic acid
H_4EDTA	Ethylenediaminetetraacetic acid
FITC	Fluoresceinisothiocyanate
IPAV	Isopropanol:ammonia:water
IR	Infrared spectroscopy
MRI	Magnetic Resonance Imaging
NMR	Nuclear Magnetic Resonance
PAMAM	Polyamidoamine dendrimer
THF	Tetrahydrofuran
UV-Vis	Ultraviolet/visible light spectroscopy
VRE	Vacuum Rotary Evaporator

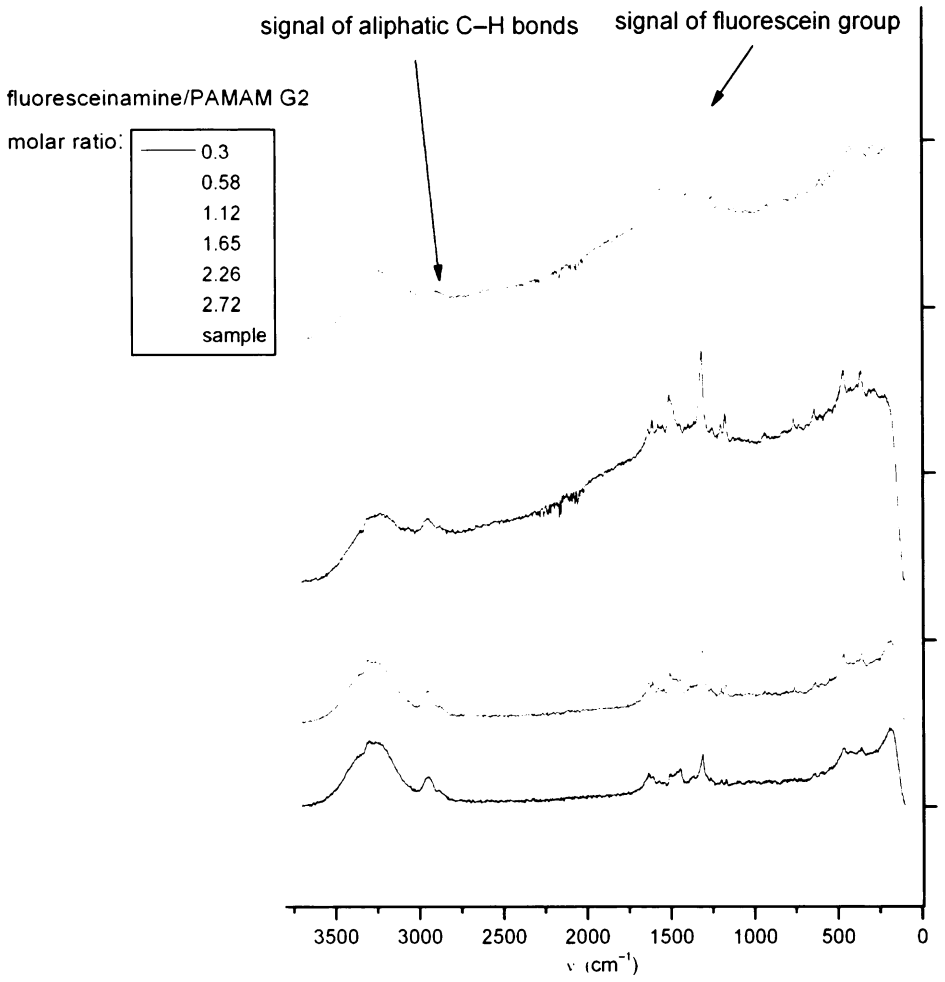
7 APPENDIX 1

^{1}H NMR spectrum of Eu complex (in D_2O)**:**



7 APPENDIX 2

Raman spectra of calibration solutions and sample and obtained calibration curve:



Calibration curve for Raman spectroscopy: I_F/I_{C-H} means the ratio of signal intensities, while n_F/n_D means the molar ratio of fluorescein groups and dendrimer.