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Research Article

PAMAM Dendrimers as Potential Carriers of Gadolinium Complexes of Iminodiacetic Acid Derivatives for Magnetic Resonance Imaging

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This is the first study describing the utilization of PAMAM dendrimers as delivery vehicles of novel magnetic resonance imaging (MRI) contrast agents. The purpose of this paper was to establish the potential of G4 PAMAM dendrimers as carriers of gadolinium complexes of iminodiacetic acid derivatives and determine imaging properties of synthesized compounds in *in vivo* studies. Furthermore, we examined the influence of four synthesized complexes on the process of clot formation, stabilization, and lysis and on amidolytic activity of thrombin. Biodistribution studies have shown that the compounds composed of PAMAM G4 dendrimers and gadolinium complexes of iminodiacetic acid derivatives increase signal intensity preferably in liver in range of 59–116% in MRI studies which corresponds with the greatest accumulation of gadolinium after administration of the compounds. Synthesized compounds affect kinetic parameters of the proces of clot formation, its stabilization, and lysis. However, only one synthesized compound at concentration 10-fold higher than potential plasma concentrations contributed to the increase of general parameters such as the overall potential of clot formation and lysis ($\uparrow CL_{AUC}$) and total time of the process ($\uparrow T$). Results of described studies provide additional insight into delivery properties of PAMAM dendrimers but simultaneously underscore the necessity for further research.

1. Introduction

Over the last quarter of century there has been considerable interest in developing biodegradable nanodevices as effective drug delivery components. Generally, nanostructures have the ability to protect drugs encapsulated within them or conjugated to them from hydrolytic and enzymatic degradation in the gastrointestinal tract and target the delivery of a wide range of drugs to various areas of the human organism [1].

The drug delivery systems, in relation to the current huge interest in nanotechnology, include liposomes, polymeric micelles, nanoparticles, dendrimers, and nanocrystals [1]. The method of preparation determines the number of properties and release characteristics of the encapsulated, dissolved, absorbed, or attached therapeutic agent [2]. It is worth mentioning that modification of nanocarriers' surface enables controlling their pharmacokinetic and pharmacodynamic properties such as increased stability, prolonged circulation time, and required biodistribution [3].

Dendrimers, a relatively new class of compounds, compared to the traditional linear polymers are characterized by unique molecular architecture and dimensions [4, 5]. Dendrimers have, in comparison to linear polymers, well-defined chemical structure and exact molecular mass [4].

Low polydispersity, three-dimensional architecture, and high functionality of dendrimers contribute to the intensified interest in these polymers [5].

Presumably the application of dendrimers as drug delivery devices is the most promising application in the field of pharmaceutical sciences. The potential virtues of dendrimers' utilization as drug vehicles include prolonging the residence time of the drug in the circulatory system, protecting the drug from its environment, increasing the stability of the active compound, and tissue targeting [6, 7].

A lot of scientific papers report utilization of dendrimers as delivery vehicles of nonsteroidal anti-inflammatory drugs (NSAIDs), anticancer drugs, and other drugs such as simvastatin, famotidine, or quinolones [6, 8]. Apart from drugs molecules dendrimers might be used as carriers of magnetic resonance imaging (MRI) contrast agents, genetic material, and targeting agents. All these molecules might be enclosed within the dendrimers structure either by encapsulation, complexation, or conjugation [6, 9].

MRI, technique capable of three-dimensional (3D), high-resolution visualization of specific tissues and organs in the body, has revolutionized diagnostic medicine. It has been extensively used in the field of diagnostics, especially in neurological, musculoskeletal, and cardiovascular diseases, mainly because of the fact that it does not cause radiation harm [10].

As the sensitivity of MRI to differences in tissue type is relatively low, the contrast agents (CAs) are usually required to shorten longitudinal (T1) and transversal (T2) relaxation times of the surrounding water protons in order to improve contrast [11].

Gadolinium (III) is the most frequently occurring paramagnetic ion in MRI CAs because of its favorable combination of a large magnetic moment (μ_{eff} 7.94 BM, seven half-filled f orbitals) and long electron spin relaxation time (10^{-8} to 10^{-9} s, from symmetric S electronic state) [12].

Gadolinium chelates, such as Gd-DTPA (gadolinium-diethylenetriaminepenta-acetic acid), constitute the largest group of paramagnetic MRI contrast media. In the Gd-DTPA complex, Gd^{3+} is linked to five carboxylate oxygen atoms and three nitrogen atoms of the DTPA molecule and to one water molecule located within the inner sphere of the complex. Despite the frequent clinical application of gadolinium-based MRI CAs such as gadopentetate dimeglumine (Magnevist) and gadodiamide (Omniscan) they are not sufficiently specific and selective [13]. Therefore, CAs showing affinity for selected tissues or cells, for example, hepatocytes, and simultaneously enabling identification and differentiation of focal changes in these tissues are still in demand [14].

Iminodiacetic acid derivatives, including mebrofenin complexed with gadolinium, are contrasting compounds, which show high affinity to hepatocytes, and enable performing high-resolution imaging of liver applying MRI [8, 14] and therefore constitute promising candidates for liver imaging.

To the best of our knowledge there is no information concerning the utilization of PAMAM dendrimers as delivery systems of gadolinium complexes of iminodiacetic acid derivatives. Therefore, the objective of this paper was to synthesize novel compounds based on G4 PAMAM dendrimers and gadolinium complexes of iminodiacetic acid derivatives and to establish the amount of gadolinium complexes occurring per one dendrimers molecule.

Introduction of a new contrast agent for clinical use depends primarily on the safety of its use, and that is why it is essential to determine its toxicity and biocompatibility with particular emphasis on effects on hemostasis; therefore we decided to examine the influence of four synthesized complexes on the process of clot formation, stabilization, and lysis. In order to elucidate their mechanism of action on coagulation and fibrinolysis we examined the influence of synthesized complexes on amidolytic activity of thrombin. For determination of imaging properties of synthesized complexes we performed animal studies.

2. Materials and Methods

2.1. Reagents. Lithium hydroxide (purity ≥ 99.0%, 23.95 g/mol), gadolinium chloride hexahydrate (371.70 g/mol, 99.9%), arsenazo (III) (2,2′-(1,8-dihydroxy-3,6-disulfo-naphthylene-2,7-bisazo)bisbenzenearsonic acid), and PAMAM dendrimers (ethylenediamine core, amine terminated) were purchased from Sigma Aldrich. In the study G4 PAMAM-$(NH_2)_{64}$ dendrimers (10 wt. % in methanol; Mw = 14214.17, diameter 4.5 nm) were used. Exactly before the experiments PAMAM dendrimers were diluted with endotoxin-free water.

We used thrombin and phosphate buffer saline (PBS) produced by Biomed (Poland), and recombinant tissue plasminogen activator (t-PA) produced by Boehringer (Germany). Tris-buffered saline (TBS) was purchased from Sigma Aldrich. Calcium chloride and sodium chloride were obtained from Polish Chemical Reagents (Poland). Chromogenic substrate S-2238 (Chromogenix, Italy) was used for determination of amidolytic activity of thrombin.

2.2. Compounds Synthesis. Within this study we present synthesis of four compounds (K1–K4) based on PAMAM G4 dendrimers and gadolinium complexes of iminodiacetic acid derivatives: N-(4-methyl-acetanilide)iminodiacetate gadolinium complex (compound K1), N-(2,4-dimethylacetanilide)-iminodiacetate gadolinium complex (compound K2), N-(2,4,6-trimethylacetanilide)imino-diacetate gadolinium complex (compound K3), and N-(3-bromo-2,4,6-trimethylacetanilide)-iminodiacetate gadolinium complex (compound K4).

The first step of synthesis included preparation of substrates for the reaction, four iminodiacetic acid derivatives: N-(4-methylacetanilide)iminodiacetic acid (ligand L1), N-(2,4-dimethylacetanilide)iminodiacetic acid (ligand L2), N-(2,4,6-trimethylacetanilide)-iminodiacetic acid (ligand L3), and N-(3-bromo-2,4,6-trimethylacetanilide)-iminodiacetic acid (ligand L4). Synthesis of these substrates was described in our previous publication [8]. Briefly, the method is based on the reaction between nitrilotriacetic acid anhydride obtained *in situ* and appropriate derivative of aniline.

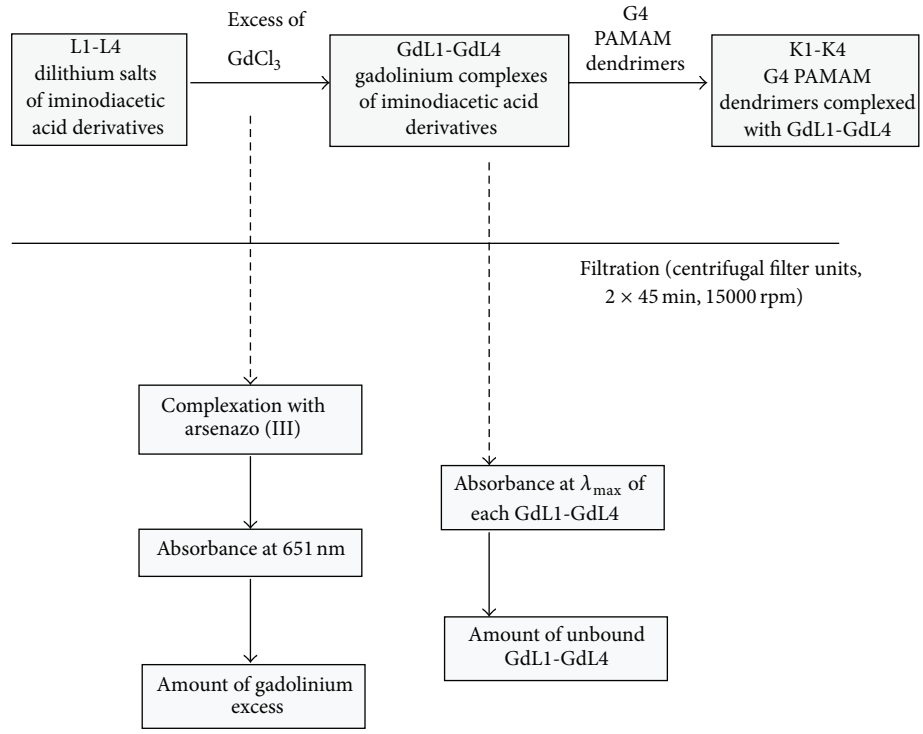
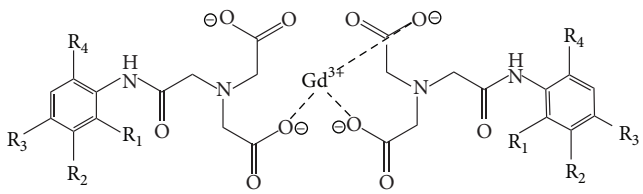


Figure 1: Synthesis of compounds (K1–K4) based on G4 PAMAM dendrimers and gadolinium complexes of iminodiacetic acid derivatives GdL1–GdL4.

Table 1: Gadolinium complexes GdL1–GdL4 (see Scheme 1).

Synthesized complex	R_1	R_2	R_3	R_4
GdL1	H	H	CH_3	H
GdL2	CH_3	H	CH_3	H
GdL3	CH_3	H	CH_3	CH_3
GdL4	CH_3	Br	CH_3	CH_3



Scheme 1

We present the scheme of the synthesis of iminodiacetic acid derivatives (L1–L4) (see Figure S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/394827).

Iminodiacetic acid derivatives (L1–L4) were transferred into dilithium salts. The following step of the synthesis included a reaction between dilithium salts of ligands L1–L4 and slight excess of gadolinium chloride hexahydrate. Obtained in this way lithium salts of gadolinium complexes of iminodiacetic acid derivatives (GdL1–GdL4, Table 1) were mixed with G4 PAMAM dendrimers in molar ratio (PAMAM dendrimer: gadolinium complexes) 1 : 96. All reactions were conducted in methanol and water at room temperature. Received complexes between GdL1–GdL4 and G4 PAMAM dendrimers (compounds K1–K4) were purified by filtration by means of centrifugal filter units with ultracel 3K membrane (Millipore). Utilization of these devices allowed for the filtration of the excess of gadolinium ions and, simultaneously, verification that free gadolinium ions do not remain in the solution of synthesized compounds. Furthermore, filtration allowed determining the amount of unbound gadolinium complexes GdL1–GdL4. Figure 1 presents the procedure of synthesis.

2.3. Establishment of Stoichiometry between Dendrimers and Gadolinium Complexes of Iminodiacetic Acid Derivatives. The colorimetric assay of determination of the amount of gadolinium ions in filtrate is based on the large bathochromic shift of the absorbance of arsenazo III in the presence of transition metals, such as gadolinium, due to the formation of a stable complex [15]. The free dye absorbs strongly at 548 nm; but when it is complexed with heavy metals such as gadolinium it absorbs at 651 nm.

The concentration of gadolinium ions in the filtrate was analyzed as follows: an aliquot of the filtrate was diluted with water and added to the arsenazo III stock solution. Calibration curve ($y = 4.976163 * x$, in concentration range 0.009–0.120 mg/mL; $R^2 = 0.9899$) was prepared to calculate the concentration of the arsenazo III-gadolinium complex. It was obtained by measuring the absorbance at 651 nm of a series of standard solutions of the arsenazo III-gadolinium complex formed by equimolar quantities of gadolinium chloride hexahydrate and arsenazo III [15]. The concentration

of this complex corresponds to the amount of gadolinium ions excess added to the reaction mixtures. The amount of complexed gadolinium was obtained by subtracting the excess of gadolinium ions determined by colorimetric studies from the total amount of gadolinium added to the reaction mixture.

The amount of unbound GdL1–GdL4 was also determined in the filtrate. An aliquot of filtrate was diluted and examined spectrophotometrically. The amounts of GdL1–GdL4 were calculated on the basis of measured absorbance and the appropriate calibration curve. The concentration of GdL1–GdL4 bound to dendrimers was obtained by subtracting their amount determined in filtrate by spectrophotometric studies from the total amount of GdL1–GdL4 formed in the reaction (the amount of complexed gadolinium).

2.4. Plasma Preparation. Blood was obtained from healthy donors in Blood Donation Centre in Lodz. Blood was collected into vacuum tubes containing 3.2% buffered sodium citrate. Poor platelet plasma (PPP) was obtained by blood centrifugation (3000 ×g, 20 min, room temperature) in Micro 22R centrifuge (Hettich ZENTRIFUGEN). PPP was pooled and stored in small quantities at −30°C until the measurements were taken. Immediately prior to each measurement, the plasma was restored in a water bath at 37°C for 15 minutes. Plasma was thawed only once and was not frozen again.

2.5. Test of Clot Formation and Lysis (CL-Test). The CL-test [7, 16], in conjunction with computer program, was used for the measurements of kinetic parameters of clot formation and lysis [17]. This test is based on the evaluation of the global assay of coagulation and fibrinolysis by continuous measurements of the alterations of optical transmittance. The method is a modification of optical measurement of coagulation and fibrinolysis based on the spectrophotometric studies of Glover et al. [18] and He et al. [19].

Assays based on the overall hemostasis potential (OHP) were evaluated in connection with hypercoagulation in some thrombophilias, diabetes, stroke, coronary heart attack, and hypocoagulability. The results indicate also the possible usefulness of the method in monitoring anticoagulant treatment which gives evidence that applied assay may be a good candidate for clinical purposes [20].

All experiments were conducted in plasma diluted three times with TBS buffer. Thrombin at concentration of 0.5 IU/mL was added in order to initiate clot formation and t-PA at concentration of 240 ng/mL was added to start the process of fibrinolysis.

Measurements of clot formation and fibrinolysis process were conducted by means of a spectrophotometer (Cecil CE2021, London, England) at 37°C and with stirring. The process was monitored continuously at 405 nm. General experimental conditions were arranged earlier and described in our previous papers [7, 16].

Our computer program enables estimating the parameters of process of clot formation (phase I), clot stabilization (phase II), and fibrinolysis (phase III). The kinetic parameters are as follows: T, total time of the process of clot formation and fibrinolysis; CL_{AUC}, overall potential of clot formation and lysis and parameters of phase I; T_t, thrombin time; F_{max}, maximum clotting; T_f, plasma clotting time; Fvo, initial plasma clotting velocity; phase II, T_c, clot stabilization time; and phase III: L_{max}, maximum lysis; Tl, fibrinolysis time; Lvo, initial clot fibrinolysis velocity [16].

2.6. Amidolytic Activity of Thrombin. The amidolytic activity of thrombin was determined by means of the method described by Lottenberg et al., [21] which is based on the measurements of the hydrolysis of chromogenic substrate D-Phe-Pip-Arg-pNA. All experiments were conducted in TBS buffer at pH 7.4. Thrombin (0.075 IU/mL) and synthesized complexes K1–K4 at 4 concentrations ranging from 0.04 μmol/mL to 4 μmol/mL or appropriate volume of TBS (control) were added to the cuvette and incubated for 3 min at 37°C. To start the enzymatic reaction, 20 μL of 2 mM chromogenic substrate was added. The changes in absorbance at a wavelength of $\lambda = 405$ nm were recorded continuously by means of a spectrophotometer (Cecil CE2021, England) at 37°C for 10 min. The obtained curves were evaluated by computer program which is useful for evaluation of thrombin activity. Our computer program enables evaluating the following parameters: dA/dt: initial velocity of reaction and A_{max}: maximum of enzymatic activity.

2.7. Animals Studies. All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments. All studies were conducted in accordance with guidelines for the use and care of laboratory animals set forth by Medical University in Gdansk.

Bioimaging properties of synthesized complexes K1–K4 were determined in male Wistar rats (300 g, $n = 3$ for each complex). Rats were injected intravenously (lateral tail vein) with 23 mg–29 mg of complexes K1–K4 in 0.2 mL of PBS, corresponding to 4 mg of gadolinium per rat, simultaneously with the calibrated range of proportionality 2–7 mg per rat.

Biodistribution studies were carried out on GE Signa 1.5T T1 FS SE sequence, 4 mm thick coronal slices. 4 organs were selected: liver, kidney, brain, and testes, in which the signal intensity was monitored. The study was performed in three replicates; 30 minutes of observation was conducted in loops every 5 minutes.

We calculated the percentage of gadolinium in the accumulation in the following organs. The calculations were based on a standard rat model [22, 23], where the % share of the weight for the liver is 5%, 0.7% for kidneys, 0.6% for brain, and 0.6% for testes.

The percentage of administered gadolinium in the accumulation in the following organs corresponds to the increase in the intensity in the organs divided by the increase in the intensity of the whole body and then multiplied by the percentage of organ in the whole rat body.

3. Results

3.1. Synthesis and Establishment of Stoichiometry between Dendrimers and Gadolinium Complexes of Iminodiacetic

Table 2: The results of spectrophotometric studies concerning the establishment of unbound amount of GdL1–GdL4 (n = 2-3).

Complex	Amount of unbound GdL1–GdL4 in filtrate	% of the total amount of GdL1–GdL4	Molar ratios in complexes (GdL-PAMAM)
K1			
GdL1-PAMAM G4	$4.97 * 10^{-5}$ [mol]	29.06%	68.08 : 1
K2			
GdL2-PAMAM G4	$4.77 * 10^{-5}$ [mol]	29.26%	67.94 : 1
K3			
GdL3-PAMAM G4	$4.56 * 10^{-5}$ [mol]	29.23%	67.85 : 1
K4			
GdL4-PAMAM G4	$3.65 * 10^{-5}$ [mol]	29.20%	67.84 : 1

Acid Derivatives. Synthesis of compounds (K1–K4) based on PAMAM dendrimers and gadolinium complexes of iminodiacetic acid derivatives GdL1–GdL4 is presented in Figure 1.

Applied colorimetric assay, based on the large bathochromic shift of the absorbance of arsenazo III in the presence of transition metals, provides the quantitative analysis of the excess of gadolinium ions (detected as its complex with arsenazo III) and enables establishing the sufficient duration of centrifugation to obtain stable compound [15].

The total amount of gadolinium used for the syntheses was calculated as 110% of stoichiometric amount of gadolinium; thus, the excess of gadolinium was approximately 9% of the amount used for the reaction. Obtained results are in agreement with theoretical calculations; in the case of all complexes, the amount of gadolinium ions constituted approximately 9% of the total amount used in the reactions.

The results of spectrophotometric experiments conducted to establish the amount of unbound gadolinium complexes GdL1–GdL4 are presented in Table 2.

The results of these studies revealed that approximately 68 molecules of GdL1–GdL4 occur per one molecule of dendrimer in the case of complexes with G4 PAMAM dendrimers.

It can be concluded on the basis of obtained results that the employed procedure allowed for accurate determination of the composition of synthesized complexes (the amount of GdL1–GdL4 per one PAMAM dendrimer molecule). Moreover, the abovementioned method confirmed that all gadolinium ions in synthesized compounds were complexed.

3.2. Test of Clot Formation and Lysis (CL-Test). Figures 3–6 present the effects of complexes K1–K4 on kinetic parameters of clot formation, stabilization, and fibrinolysis. Incubation of human plasma with tested compounds K1–K4 did not lead to the spontaneous coagulation or any changes with plasma proteins. In the case of complex K4 (formed between G4 PAMAM dendrimer and GdL4) at the highest concentration, continuous measurement of absorbance was impossible because addition of 10 μL methanolic solution of K4 to the diluted plasma led to the precipitation of K4 and turbidity of the sample.

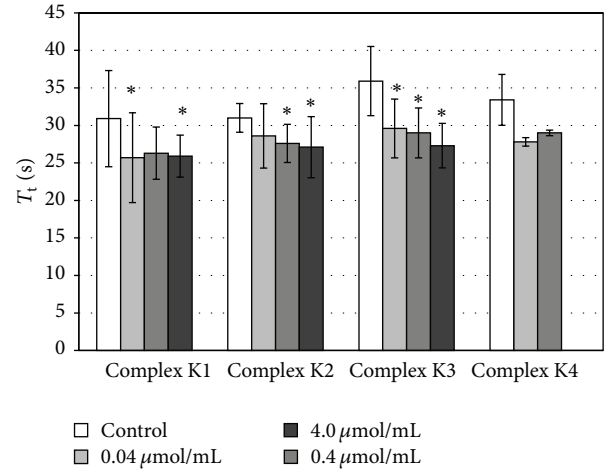


Figure 2: Influence of complexes K1–K4 on thrombin time (T_t); $^*P < 0.05$; $n = 5$.

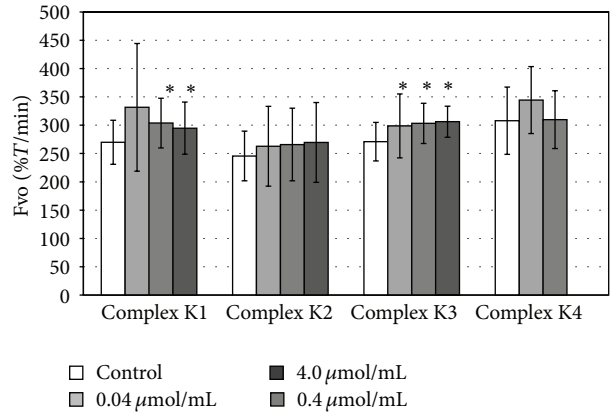


Figure 3: Influence of complexes K1–K4 on initial plasma clotting velocity (Fvo); $^*P < 0.05$; $n = 5$.

As presented in Figure 2, all examined complexes (K1–K4) shortened thrombin time ($\downarrow T_t$) in a statistically significant manner. However, statistically significant differences for all investigated concentrations were reported for complex K3.

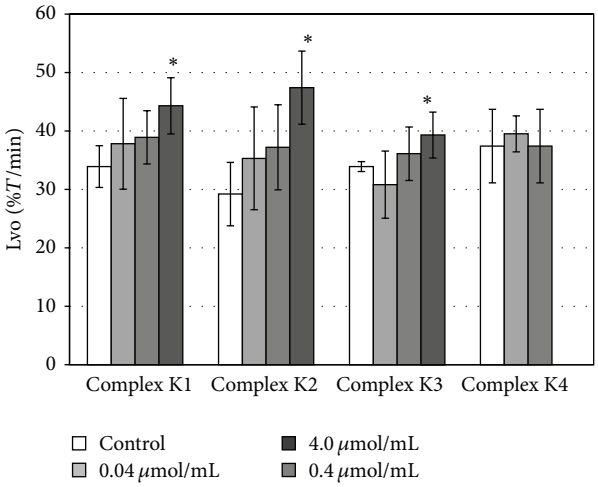


Figure 4: Influence of complexes K1–K4 on initial clot fibrinolysis velocity (Lvo); $^{*}P < 0.05$; $n = 5$.

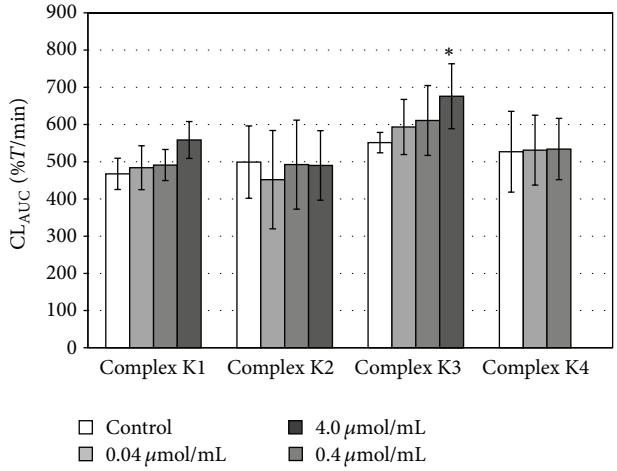


Figure 5: Influence of complexes K1–K4 on overall potential of clot formation and lysis (CL_{AUC}); $^{*}P < 0.05$; $n = 5$.

Complexes K1 and K3 contributed to statistically significant increase in maximum clotting ($\uparrow F_{max}$), K1 at concentration of 0.4 μmol/mL and 4.0 μmol/mL, and K3 at concentration of 4.0 μmol/mL. F_{max} depends on the diameter of the filaments of fibrin. Therefore reported statistically higher values of F_{max} suggest that filaments of fibrin were thicker (Figure S2).

Complexes K1–K3 shortened plasma clotting time ($\downarrow T_f$); however, only in the case of K2 and K3 the effect was statistically significant. The observed changes in plasma clotting time were associated with the increase in initial plasma clotting velocity (↑Fvo) (Figure 3).

In the case of clot stabilization phase the results of the studies showed that only complex K3 at concentration of 4.0 μmol/mL contributed to the prolongation of clot stabilization time ($\uparrow T_c$) in statistically significant manner (Figure S3). The observed changes in parameters of clot stabilization suggest that complex K3 influenced the structure of clot and, importantly, delayed the process of fibrinolysis.

In the case of fibrinolysis phase of the study, it is worth emphasizing that unchanged or increased values of maximum lysis (L_{max}) for complexes K1–K4 were related to the values of maximum clotting (F_{max}) and provided evidence for total lysis of previously formed clots.

As presented in Figure 4, complexes K1–K3 at the highest concentrations increased initial clot fibrinolysis velocity in a statistically significant manner (↑Lvo) and as a result shortened fibrinolysis time (↓Tl).

Similarly to the phase of clot formation and stabilization, complex K4 did not contribute to any statistically significant changes in the values of kinetic parameters of fibrinolysis.

Generally, the most significant changes in the overall potential of clot formation and lysis ($\uparrow CL_{AUC}$) were caused by complex K3 (Figure 5). Moreover, K3 at the highest concentration was associated with statistically significant increase in the total time ($\uparrow T$) of the process of clot formation and fibrinolysis. In the case of other complexes, no changes in CL_{AUC} were observed.

3.3. Amidolytic Activity of Thrombin. In order to elucidate the mechanism of synthesized compounds based on PAMAM dendrimers and gadolinium complexes with iminodiacetic acid derivatives on blood coagulation and fibrinolysis we determined their effects on amidolytic activity of thrombin. Results presented in Figure 6 prove that compounds K1–K3 do not affect the enzymatic activity of thrombin. A statistically significant increase in initial velocity of amidolytic reaction was documented only for the concentration of 1.6 μmol/mL and 4.0 μmol/mL of compound K4.

Our computer program enables also estimation of maximum activity (A_{max}) which corresponds to maximal amount of p-nitroaniline released from chromogenic substrate in enzymatic reaction. As presented in Figure 7 all tested compounds in the whole concentrations range do not influence significantly maximum activity (A_{max}).

3.4. Animal Studies. The results of bioimaging studies are presented in Table 3.

Biodistribution studies have shown that the compounds composed of molecules of dendrimers PAMAM G4 and gadolinium complexes of iminodiacetic acid derivatives, N-(4-methyl-acetanilide)iminodiacetate gadolinium complex (compound K1), N-(2,4-dimethylacetanilide)-iminodiacetate gadolinium complex (compound K2), N-(2,4,6-trimethylacetanilide)imino-diacetate gadolinium complex (compound K3), and N-(3-bromo-2,4,6-trimethylacetanilide)-iminodiacetate gadolinium complex (compound K4), increase signal intensity by 24, 17, 20, and 15%, respectively. In the case of particular organs we observed increased signal intensity in the liver (from 59 to 116%) and in kidneys (from 68 to 136%), which enabled a good enhancement of normal liver tissue in T1 and T1 FATSAT images.

The greatest accumulation of gadolinium after administration of the above compounds was observed in the liver. In the case of compound K1 24% of the administered gadolinium accumulated in the liver, for the other compounds (K2, K3, and K4) this amount was, respectively, 22, 15, and 20%. We

Table 3: Biodistribution of four compounds (K1–K4) examined intravitally in rats.

SE T1	Imaging enhancement, the whole body		Imaging enhancement, liver		Imaging enhancement, kidneys		Imaging enhancement, brain		Imaging enhancement, testes	
	%	% share	%	% share	%	% share	%	% share	%	% share
K1	24	100	116	**24**	107	3	6	0.15	16	0.4
K2	17	100	76	**22**	99	8	12	0.4	16	0.6
K3	20	100	59	**15**	136	5	13	0.4	21	0.6
K4	15	100	59	**20**	68	3	2	0.08	35	1.4

% share, percentage of gadolinium dose administered to animal.

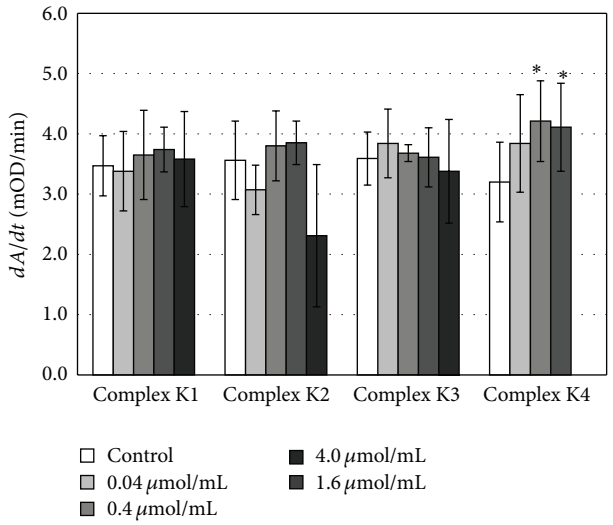


Figure 6: Influence of complexes K1–K4 on initial velocity of reaction (dA/dt), $^{*}P < 0.05$; $n = 5$.

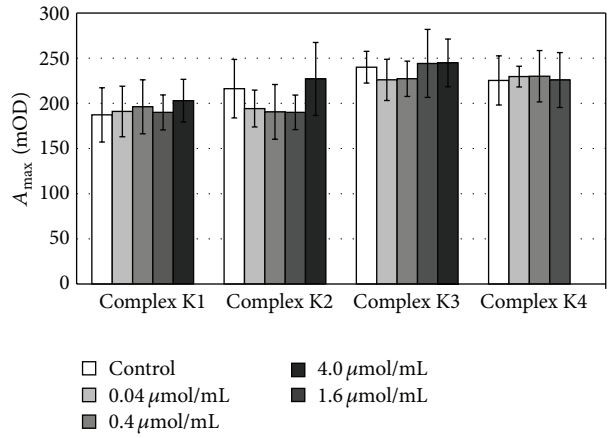


Figure 7: Influence of complexes K1–K4 on maximum reaction (A_{max}); $n = 5$.

can assume that the accumulation of the above compounds in liver cells is due to the affinity of gadolinium complexes of iminodiacetic acid derivatives to hepatocytes [14]. In the case of the kidney we reported that gadolinium accumulated therein an amount from 3 to 8 percent, contributing to the contrast enhancement in range of 68–136%.

The data presented in Table 3 were taken from the point of maximal enhancement in the liver tissue, but the shape of curve did not presented the typical peak enhancement (Figure S4). During 30 minutes of observation we noted a plateau after initial 10 minutes enhancement rise.

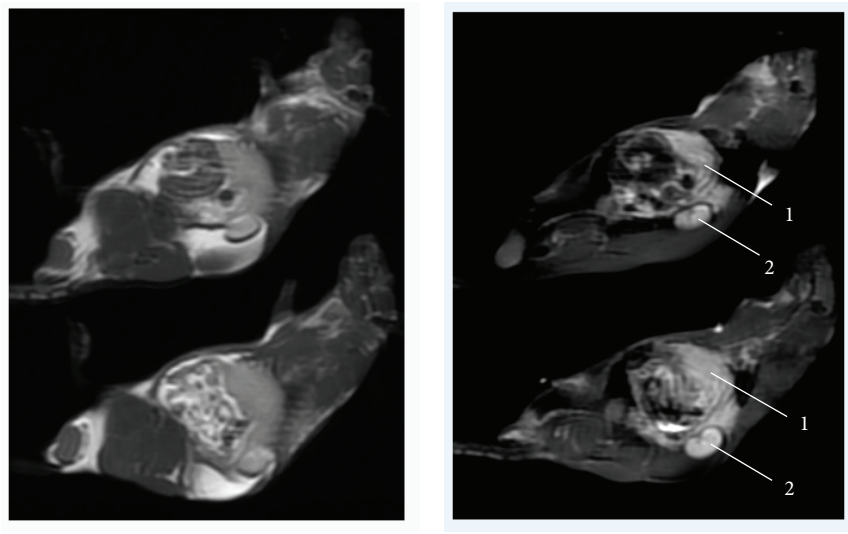
Figure 8 illustrates the MRI imaging of the body of the rat prior to administration of the compound (A) and after intravenous administration (B) of compound K4.

4. Discussion

Despite the frequent use, the range of applications of low molecular weight CAs, such as Magnevist or MultiHance, is determined by their pharmacokinetic properties. Their limiting factors are as follows: (i) rapid extravasation from blood vessels to the interstitial fluids upon intravenous application, (ii) short circulation time, (iii) insufficient contrast between the healthy and unwell tissues (low sensitivity for recognizing the pathologic lesions), and (iv) poor contrast at high magnetic fields [24].

Therefore, scientific teams started to investigate the potential of macromolecules as Gd^{3+} vehicles. Studies concerning utilization of nanocarriers such as dendrimers [25], polymers [26], liposomes [27], and inorganic nanoparticles (NPs) [28] as Gd^{3+} chelators are available. Increase in the number of Gd^{3+} atoms per molecule in comparison with low molecular weight CAs and reduction of molecular tumbling contribute to a significant shortening of relaxation times and, as a result, contrast enhancement. In addition, high molecular weight CAs provide more favorable signal-to-noise ratio and improved pharmacokinetic properties (e.g., increase of the blood half-life) [26]. Furthermore, a macromolecular ligand gives the opportunity to act as a scaffold and allows inclusion of targeting moieties [26, 28].

Dendrimers appear to be useful in the field of MRI CAs delivery because the conjugation of Gd chelates with dendrimers leads to a substantial improvement in the water proton relaxation rates and increases their half-life time in circulatory system [29]. Dendrimers possess several advantages over other polymer carriers which are as follows: spherical and highly branched structure, low polydispersity, and large modifiable surface functionality. PAMAM dendrimers are the most commonly investigated, mainly because of their



(a) (b)

Figure 8: MRI imaging of the body of the rat prior to administration of the compound K4 (a) and after intravenous administration (b) of compound K4; 1: liver and 2: kidney.

water solubility, biocompatibility, and nonimmunogenicity [30]. Apart from PAMAM dendrimers, PPI and PLL dendrimers are frequently investigated types of dendrimers as CA vehicles [31].

To the best of our knowledge this is the first study which describes the application of PAMAM dendrimers as carriers of gadolinium complexes of iminodiacetic acid derivatives. It should be emphasized that the size (generation) of dendrimers determines pharmacokinetic and pharmacodynamic properties of CAs. Alteration of dendrimers diameter affects their vascular permeability, retention in circulatory system, excretion route, and recognition by reticuloendothelial system (RES). Therefore, we decided to use PAMAM dendrimers of fourth generation because they are quickly eliminated *via* kidneys; thus they might be utilized as potential agents for renal imaging and possess the ability to selectively leak through hyperpermeable tumor vessels [32].

In our study we decided to establish whether PAMAM dendrimers may act as delivery vehicles of gadolinium complexes of iminodiacetic acid derivatives. Based on the results of the study Darras et al. [15] we decided to use the excess of gadolinium in order to establish how much of it is stable complexed with iminodiacetic acid derivatives. For this purpose we applied the colorimetric assay of determination of the amount of gadolinium ions which is based on the large bathochromic shift of the absorbance of arsenazo III in the presence of gadolinium, due to the formation of a stable complex [15]. Applied colorimetric assay enabled confirming that synthesized compounds were stable since the excess of Gd^{3+} was totally recovered by ultrafiltration. Therefore, we may conclude that in the presence of PAMAM dendrimers gadolinium complexes with iminodiacetic acid derivatives are stable.

Determination of the amount of gadolinium complexes with iminodiacetic acid derivatives in the filtrate after ultrafiltration was used for establishment of encapsulation capacity of dendrimers. Performed synthesis of compounds K1–K4 proved that G4 PAMAM dendrimers may carry 68 molecules of GdL1–GdL4 through electrostatic or hydrophobic interactions.

Analysis of biodistribution of synthesized compounds was performed after intravenous administration of 23 mg–29 mg of compounds. We studied the increase of signal intensity in four regions: liver, kidneys, brain, and testes. Biological examination of synthesized compounds showed small differences in tissue biodistribution. All examined compounds contributed to the whole body contrast enhancement in similar percentage; however, we can distinguish two compounds K1 and K3 which presented better imaging properties; they strengthened MRI image by 24 and 20%, respectively. Two other compounds increased signal intensity to the lower extent. For liver, the greatest properties towards imaging of this organ had compound K1 which contributed to the 116% increase in the signal intensity. This value corresponds to the percentage of the accumulated gadolinium in the liver after injection of compound K1. Other tested compounds presented good liver imaging properties as well. These results appear to be in agreement with our previous study in which we assessed the bioimaging properties of some gadolinium complexes of iminodiacetic acid derivatives [20]. Conducted in 2008, experiments showed affinity of iminodiacetic acid derivatives towards hepatocytes. Furthermore, it was found that imaging properties depended on the ligand located in the aromatic part of the complex [14].

In the same manner [14] we reported that synthesized compounds K1–K4 increased the signal intensity in

the kidneys in the range of 68–136%. This good contrast enhancement might have been obtained due to the presence of G4 PAMAM dendrimers which are excreted by the kidneys.

All examined compounds, similarly to the compounds of previous study [14], did not cause any increase of the signal intensity for brain, probably due to the specific properties of the blood-brain-barrier and fast blood flow.

It is crucial to mention that within the present study during 30 minutes of observation we noted a plateau after initial 10 minutes enhancement rise, whereas in the case of previous study [14] we reported an initial growth and after 15 minutes we registered a decrease in signal intensity with simultaneous growth in the intestines and bladder.

It is of extreme importance to stress that in our previous paper [14] compounds were administered in amount corresponding to 1 mg of gadolinium per rat whereas within these experiments we had to increase the quantity of synthesized compounds to the equivalent of 4 mg of gadolinium in order to obtain good signal intensities. We presume that it might be due to the restricted access of water to the coordination sphere of gadolinium in synthesized compounds with dendrimers, similarly to Gd-DTPA complexes encapsulated within the structure of biodegradable poly(D,L-lactide-co-glycolide) (PLGA) particles [33]. This macromolecular complex presented several drawbacks such as rapid release of the chelate from PLGA particles and diminished r1 relaxivity due to the restricted access of water to the PLGA-Gd-DTPA particles [34]. This problem has been solved by Ratzinger et al. [26] who by means of different spacers coupled covalently chelating ligands (DTPA (diethylenetriamine-N,N′,N′,N″,N″-penta-acetic acid) and DOTA (1,4,7,10-tetra-azacyclododecane-1,4,7,10-tetra-acetic *acid*)) to the particle surface and afterwards loaded with gadolinium. This procedure enabled unrestricted water access to the gadolinium chelates which resulted in higher proton relaxivities in comparison with low molecular weight CAs [26].

The last decade has brought many examples of utilization of dendrimers as drug delivery systems; however, their utilization in medicine and pharmacy is limited because of inherent toxicity associated with them.

Conducted experiments revealed that upon addition of exogenous thrombin all examined complexes K1–K4 shortened thrombin time ($\downarrow T_t$) and plasma clotting time ($\downarrow T_f$), increased maximum clotting ($\uparrow F_{max}$), and increased initial plasma clotting velocity (↑Fvo). Abovementioned alterations in kinetic parameters of clot formation ($\downarrow T_t$, $\uparrow F_{max}$, $\downarrow T_f$, and ↑Fvo) of complexes K1–K4 seem to be the result of G4 PAMAM dendrimers influence on the process of coagulation ($\downarrow T_t$, $\uparrow F_{max}$, $\downarrow T_f$, and ↑Fvo) [7].

In order to elucidate compounds' mechanism of action we conducted experiments concerning the influence on the amidolytic activity of thrombin. However, we found that in the case of compounds K1–K3 there was no influence both on the initial velocity of the reaction (dA/dt) and maximum activity (A_{max}). A statistically significant increase in initial velocity of amidolytic reaction was documented only for compound K4 which could explain observed changes in kinetic parameters ($\downarrow T_t$, $\downarrow T_f$, and ↑Fvo) of the clot formation process.

In the case of Phase II of examined process, clot stabilization, we did not report the influence of synthesized compounds on clot stabilization time (T_c) apart from the highest concentration of complex K3 which contributed to the prolongation of clot stabilization time ($\uparrow T_c$) in statistically significant manner. On the basis of these results we may conclude that the examined compound do not influence the structure of the previously formed clot.

When considering the process of fibrinolysis, the influence of PAMAM dendrimers might be also observed, because examined complexes in the highest concentrations increased initial clot fibrinolysis velocity (↑Lvo) and as a result shortened fibrinolysis time (↓Tl).

In the case of general parameters of clot formation and fibrinolysis we may observe that for the concentration 0.4 μmol/mL which could be also average circulating plasma concentration there are no statistically important changes (CL_{AUC} and T without change). It can lead to the conclusion that for systemic administration examined complexes K1–K4 could be regarded as safe when considering plasma hemostasis system.

It is extremely hard to discuss the obtained results because, as far as we are concerned, there is no information concerning the influence of dendrimer based CAs on the hemostasis. The only data available are those for biomedical polymers such as dextran and hydroxyethyl-starch. Precisely speaking, these polymers increase the rate of polymerization and fibre diameter and density because of a rise in fibrin concentration by a decrease in the effective aqueous volume [35].

So far, interactions of the dendrimer based CAs with various biomacromolecules, cells, tissues, and organs have not been fully understood. Development of novel CAs for clinical use requires the knowledge on their interactions with biological components, including hemostasis system, at cellular and molecular levels. Therefore, in further studies concerning evaluation of the influence of synthesized complexes K1–K4 on the hemostasis system such as generation of endogenous thrombin, the activity of plasmin is needed.

On the basis of the results of the study evaluating the influence of synthesized complexes K1–K4 on the process of clot formation and fibrinolysis and *in vivo* studies, it may be concluded that complex K2 due to good liver imaging properties and inconsiderable influence on the process of clot formation and fibrinolysis appears to be the most promising among the all synthesized complexes. On the other hand, the most favorable imaging properties present compound K1; it strengthens images of liver and kidneys highly.

5. Conclusion

In summary, in the presented study we proved that PAMAM dendrimers might be regarded as efficient vehicles of MRI contrast agents. Applied colorimetric assay with arsenazo III enabled establishing that all gadolinium ions are stable complexed and, therefore, minimizing the side effects associated

with them. The synthesized compounds made of dendrimers PAMAM G4 and gadolinium complexes of iminodiacetic acid derivatives have an affinity for the liver cells and good imaging properties, which make them a promising group of hepatotropic MRI contrast media. Experiments concerning the influence of synthesized compounds on the process of clot formation and fibrinolysis give evidence that in the case of possible plasma concentrations examined compounds do not affect the overall hemostasis potential, but further studies are needed to optimize the use of PAMAM dendrimers as carriers of gadolinium complexes.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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