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Research Article

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Spectroscopic studies of interactions of PAMAM G5-NH_2 and PAMAM G5-OH dendrimers with flutamide and cyclocreatine in aqueous solution

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Abstract Spectroscopic examinations of flutamide solubility and ^{1}H NMR measurements (flutamide and cyclocreatine) show that in aqueous solutions, molecules of the antitumor drugs tested stronger interact with macromolecules of cationic PAMAM G5-NH_2 dendrimer compared with hydroxyl PAMAM G5-OH dendrimer. Solubility measurements show that cationic PAMAM G5-NH_2 dendrimer macromolecules combine more ($n = 1.40 \pm 0.16$) flutamide molecules compared with PAMAM-OH G5 ($n = 0.45 \pm 0.15$). The results of ^{1}H NMR spectroscopic measurements imply that the combination of flutamide with both dendrimers proceeds with the macromolecule functional groups placed on their surface.

Keywords dendrimer, PAMAM G5-NH_2, hydroxyl-terminated, PAMAM G5-OH, flutamide, cyclocreatine, ^{1}H NMR spectroscopy, solubility measurements

Introduction

Polyamideamine dendrimers (PAMAM) constitute a subgroup of tree-like shape oligomers synthesized in the eighties by Donald Tomalia [1-3]. Commercially available PAMAM dendrimers of integer generations with an ethylenediamine core are terminated with the terminal amino groups: $CH_2CH_2CONHCH_2CH_2NH_2$ (in the case of cationic dendrimers) or terminal hydroxyl groups $CH_2CH_2CONHCH_2CH_2OH$ (in the case of neutral dendrimers).

Dendrimer macromolecules can be used as carriers of low-molecular ligands, including: antitumor drugs, genetic material fragments, medical imaging contrasts [4-6]. Ligand molecules can be bound with the functional groups of dendrimer macromolecules in a supramolecular (non-covalent way) through electrostatic interactions and hydrogen bonds. It is to be hoped that surface modified PAMAM dendrimers can play as carriers of smaller ligand molecules in organisms.

A developed and well defined dendrimer structure constitutes excellent nano-scaffolding, to which one can covalently connect many different functional groups showing specific biochemical functions [7]. In designing complex drug nanotransporters, it is necessary to take into consideration the types and interaction forces of the functional groups of transporter and drug. A relatively strong combination of dendrimer macromolecules and useful drug molecules is desired but at the same time the complex should be capable of dissociating drug molecules in a place of their required action [8].

Therefore an investigation takes place to find such properties of drug (ligand) molecules and to identify dendrimer binding centers that would facilitate the formation of a supramolecular dendrimer–drug complex. To our research on model PAMAM dendrimers we selected two PAMAM dendrimers of the fifth generation – cationic (amine) and

neutral (hydroxyl) dendrimers. As model ligand molecules we selected two drugs: flutamide and cyclocreatine (Fig. 1), both with a high toxicity but different solubility and acid-base character in aqueous solutions.

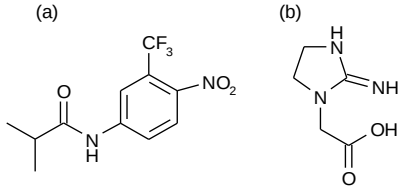

Figure 1: Structures of: a) flutamide, b) cyclocreatine

Flutamide (Flt) is an oral, non-steroidal antiandrogen drug [9-11] (Fig. 1a), used for the treatment of benign prostatic hypertrophy and prostate cancer [12-14]. This drug inhibits the binding of dihydrotestosterone, male sexual hormone, to the target cell receptors, for binding to androgen receptors in the prostate gland [15], which impedes the tumor growth [16]. In organism, flutamide is quickly metabolized to various compounds, among other things, 2-hydroxyflutamide, an active α-hydroxyl derivative contained in plasma [14-15] and excreted in the urine, the primary form being 2-amino-5-nitro-4-(trifluoromethyl)-phenol [17].

In an aqueous medium, flutamide molecules practically do not participate in acid-base equilibria as confirmed by the low ionization constant of amide group theoretically calculated ($pK_a = 13.1$) [17]. This drug is practically non-hygroscopic and sparingly soluble in water (S = 35 μM [15], 35±47 μM [18], 101 μM [19], 146 μM [20], 180 μM [17]). Within the UV range from 200 nm to 320 nm, flutamide in aqueous solution shows two absorption maxima [21]: the first maximum at 256 nm [22] ($\log \varepsilon = 2.13$ [22]) and the second (broad) maximum localized according to the authors at 302 nm [13] or 304 nm [21] or 306 nm [18] or 310 nm [22] ($\log \varepsilon = 3.15$ [22]). In the two-phase system: octanol–water, hydrophobic flutamide molecules are mainly accumulated in the organic phase ($\log P > 3.4$ [17], $\log P = 2.6$ [15], 3.7 [19]).

Cyclocreatine (Crt), 1-carboxymethyl-2-iminoimidazolidine (Fig. 1b) is a cyclic analogue of creatine (α-methylguanidoacetic acid). In the relatively flat cyclocreatine molecule there are carboxylic and guanidine groups (HN=R–COOH) [23]. Cyclocreatine molecules can penetrate biological membranes [24]. Cyclocreatine as amine acids can occur in various form depending on the pH value of aqueous medium. In a solution with pH = 3.5, cyclocreatine occurs in a cationic form (^+H_2N=R–COOH) [25], in which carboxyl group is neutral and the positive charge is localized on the protonated guanidine group. In a neutral medium, a zwitterionic form of the drug is dominating (^+H_2N=R–COO^-) [25, 26], in which both carboxyl and guanidine group undergo ionization. In a solution with pH = 10, cyclocreatine occurs in anionic form (HN=R–COO^-) [25], in which only carboxyl group is ionize [25].

Cyclocreatine shows antitumor effects in relation to the cells of various cancer varieties in animals and men [25, 27-28], including breast cancer [29], cervical carcimona [29], prostate cancer [30] and liver cancer [31]. The mechanism of antitumor effect has not been fully recognized. Cyclocreatine inhibits cell proliferation in a different way than by apoptosis [32]. Probably the drug action consists in penetrating and accumulating in the pathologically changed cells of cyclocreatine metabolite, phosphocyclocreatine, which causes swelling and death of cancer cells. [25, 28, 33] Cyclocreatine administrated with other antitumor drugs (including 5-fluorouracil) intensifies their antitumor action [34]. Cyclocreatine also shows antiviral and antidiabetic effects. In organism cells, creatine and cyclocreatine molecules, undergoing phosphorylation, take part in the energetic metabolism regulation [23-34]. Cyclocreatine protects organism tissues against ischemia and hypoxia effects [25]. Creatine transporter deficit in brain cells results in mental retardation, autism and language ability weakness [24]. Cyclocreatine administrated to mice showing creatine deficit symptoms improved their cognitive capabilities [24, 35], despite the fact that the therapy attempts by creatine administration were ineffective. Moreover, cyclocreatine showed protective action on the mouse nervous system [36].

The aim of the study is the analysis of interactions of two PAMAM dendrimers of integer generation: cationic PAMAM G5-NH_2 dendrimer terminated with amine groups and neutral PAMAM G5-OH dendrimer terminated with hydroxyl groups with two antitumor drugs: flutamide and cyclocreatine in aqueous medium. The experimental spectroscopic techniques used included: measurements of solubility and titration by the method of proton magnetic resonance.

Materials and Methods

Materials

PAMAM G5-NH_2 dendrimer (m.w. ~28.82 kDa, Sigma-Aldrich) with ethylenediamine core, PAMAM G5-OH dendrimer (m.w. ~28.95 kDa, Sigma-Aldrich) with ethylenediamine core, flutamide (m.w. 276 Da, Sigma-Aldrich, ≥99%), cyclocreatine (m.w. 143 Da, Sigma-Aldrich, ≥98%), water distilled three times and degassed, deuterium oxide (Sigma-Aldrich, ≥99.99%).

Measurements of flutamide solubility in water

In articles describing the spectroscopic properties of aqueous flutamide solutions, the wavelength, at which the maximum of absorption and molar absorption coefficients occur, show very great divergences [13, 18, 21-22]. Therefore, there were carried out series of spectrophotometric measurements of aqueous flutamide solutions in pure water. The aqueous solutions of flutamid were prepared in a volume of 1 ml each with drug concentrations from 2.5 μM to 50 μM. The flutamide concentration in solutions tested was determined by the spectrophotometric method, using a Specord50 spectrophotometer from Analytic Jena.

Then, the saturated aqueous solutions of flutamid were prepared in a volume of 1 ml each. These solutions were saturated at room temperature (20 °C) for a week. After centrifuging (8 min, 14000 rpm), the samples were diluted and flutamide concentrations were determined by the spectrophotometric method and flutamide solubility in pure water was calculated.

Measurements of flutamide solubility in dendrimer solutions

Amide bonds present in the structure of PAMAM dendrimers show quite a strong absorption of UV radiation within a range up to 210 nm. Therefore the drug determined in the mixture containing dendrimer should show the maximum of absorption above this wavelength. Cyclocreatine, contrary to the other two drugs tested, does not show the maximum of absorption over 210 nm [25] and therefore its solubility in dendrimer solutions was not determined. Therefore, the measurements of drug solubility in dendrimer solutions were only performed for flutamide. The analytical wavelength selected for the determination of flutamide concentration in the aqueous and water-dendrimer mixture tested was $\lambda_2 = 228$ nm.

Flutamide concentration in PAMAM G5-NH_2 and PAMAM G5-OH dendrimers was determined by the spectrophotometric method, using a Specord50 spectrophotometer from Analytic Jena. The aqueous solutions of the dendrimers investigated were prepared in a volume of 1 ml each with concentrations from 2.5 μM to 50 μM. The crystalline drug (about 0.015 g) placed in 2 ml eppendorf tube was flooded in succession with these dendrimer solutions. These solutions were saturated at room temperature (20 °C) for a week. After centrifuging (8 min, 14000 rpm), the aqueous dendrimer aqueous saturated with the drug were diluted and determined by the spectrophotometric method. All the spectra of aqueous dendrimer-drug mixtures were recorded regarding the aqueous dendrimer solutions with identical concentration.

^{1}H NMR spectroscopy of fluamide – dendrimer and cyclocreatine – dendrimer solutions

There were carried out measurements of the spectra of proton magnetic resonance (spectrometer Bruker Avance III, 600 MHz) of the mixture of flutamide and cyclocreatine with PAMAM G5-NH_2 and PAMAM G5-OH dendrimers. During ^{1}H NMR measurements of mixtures of the drugs with dendrimers in heavy water as solvent, there was maintained a constant concentration of dendrimers equal to 70 μM.

The mixture of flutamide and dendrimers in heavy water was prepared adding excess crystalline flutamide (0.0002 g) to 70 μM solution of G5-NH_2 (G5-OH) and leaving it for 3 days at room temperature. After centrifuging the solution (5 min, 14000 rpm), the clear samples from above sediment were subjected to ^{1}H NMR measurements. There were also recorded ^{1}H NMR spectra of flutamide and both dendrimers in heavy water. The concentration of flutamide in the mixture was determined independently by the method of UV spectroscopy. The flutamide concentration in the solution with PAMAM G5-NH_2 obtained in this way was 90 μM, and that with PAMAM G5-OH – 40 μM.

There were also made ^{1}H NMR measurements of the mixtures of PAMAM G5-NH_2 and PAMAM G5-OH with a constant concentration (70 μM) in heavy water and cyclocreatine with variable concentration from 1.4 mM to 42 mM. Each ^{1}H NMR spectrum was an average of 64 scans of the given sample.

Results and discussion

Measurements of flutamide solubility in water

In articles describing the spectroscopic properties of aqueous flutamide solutions, the wavelength, at which the maximum of absorption and molar absorption coefficients occur, show very great divergences [13, 18, 21, 22]. Therefore, there were carried out series of spectrophotometric measurements of aqueous flutamide solutions in pure water with drug concentrations from 10 μM to 50 μM (Fig. 2). It was found that aqueous solutions of flutamide show three absorption maxima, for which the molar coefficients of absorption amount to:

$\lambda_1 = 197$ nm $\quad\quad \varepsilon_1 = 26630 \pm 660$ M^{-1} cm^{-1}

$\lambda_2 = 228$ nm $\quad\quad \varepsilon_2 = 12250 \pm 200$ M^{-1} cm^{-1}

$\lambda_3 = 302$ nm $\quad\quad \varepsilon_3 = 7630 \pm 40$ M^{-1} cm^{-1}

respectively.

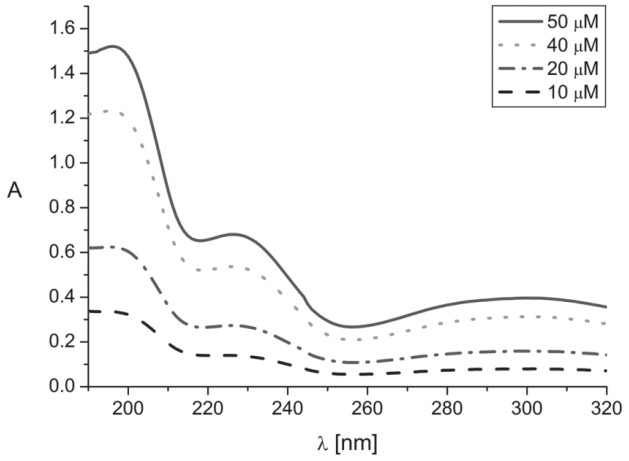

Figure 2: UV spectra of aqueous flutamide solutions

To determined flutamide solubility in pure water, the saturated aqueous drug solution was centrifuged, diluted and the drug spectrum was recorded in relation to water as reference. The measurements were repeated 6 times. The averaged drug absorption at $\lambda_2 = 228$ nm was recalculated to the flutamide solubility in water: $S = 110$ μM ± 10 μM.

Measurements of flutamide solubility in dendrimer solutions

The flutamide solubility in aqueous solutions of the dendrimers investigated (PAMAM G5-NH_2 and PAMAM G5-OH) was determined by the spectrophotometric method. The dendrimer concentration in the mixtures tested ranged

from 2.5 μM to 50 μM. The spectra of the dendrimer-drug mixture were recorded in relation to the aqueous dendrimer solution with the same concentration.

The solubility measurements indicate a slight linear increase in solubility of this drug with increasing polymer concentration (Fig. 3). The free term of both dependence (110 μM for G5 and 104 μM for G5-OH) is similar to the flutamide solubility in pure water (110 μM ± 10 μM) independently determined by us. The direction coefficient describes the number of flutamide molecules combined with a dendrimer molecule [37, 38]. The molecule of cationic PAMAM-NH_2 G5 dendrimer combines more flutamide molecules ($n = 1.40 \pm 0.16$) than that of hydroxyl PAMAM-OH G5 dendrimer ($n = 0.45 \pm 0.15$).

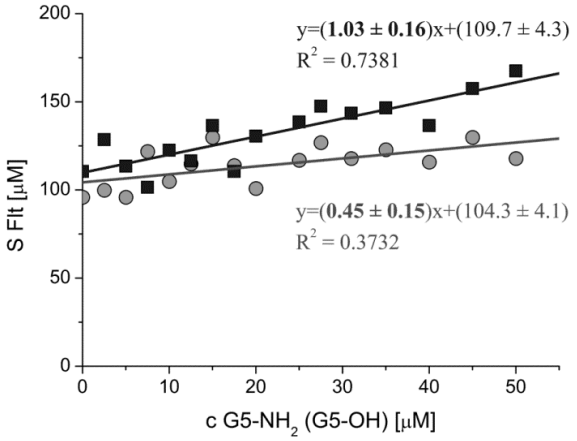

Figure 3: Dependence of flutamide solubility on the concentration of PAMAM-NH_2 G5 (□) and PAMAM-OH G5 (○) dendrimer solution.

^{1}H NMR spectroscopy of dendrimer–flutamide solutions

The ^{1}H NMR spectra of PAMAM G5-NH_2 dendrimer with a concentration of 70 μM in D_2O and its mixtures with the same concentration and flutamide (90 μM) were recorded (Fig. 4). In the same way measurements were made with PAMAM G5-OH recording the spectra of 70 μM PAMAM G5-OH dendrimer solution mixtures with flutamide (40 μM) in heavy water (Fig. 5). Flutamide was dissolved in aqueous (D_2O) mixtures of the dendrimers investigated for 72 h in the presence of excess crystalline drug. The concentration of flutamine in the mixtures with the dendrimer investigated was determined by the spectrophotometric method (UV).

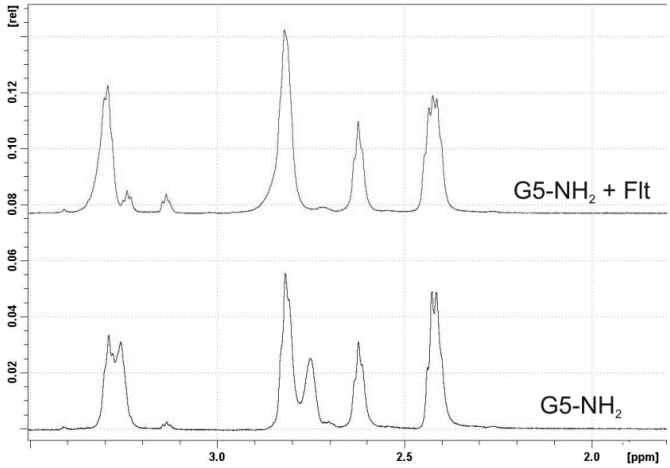

Figure 4: ^{1}H NMR spectra recorded for 70 μM solution of PAMAM G5-NH_2 dendrimer and PAMAM G5-NH_2 dendrimer solution (70 μM) and flutamide (90 μM) in heavy water as solvent.

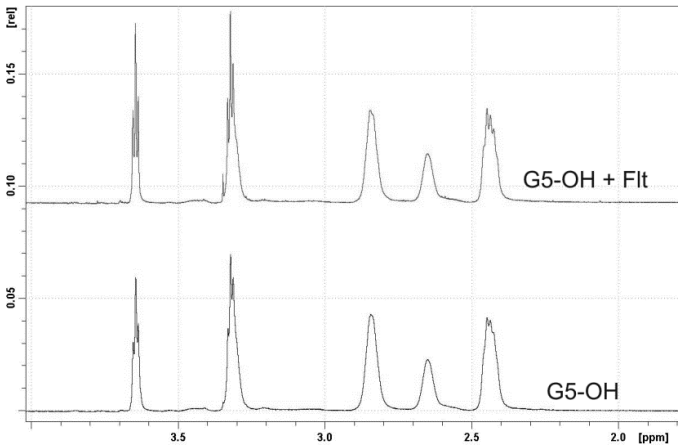

Figure 5: [1]H NMR spectra recorded for 70 μM solution of PAMAM G5-OH dendrimer and PAMAM G5-OH dendrimer solution (70 μM) and flutamide (40 μM) in heavy water as solvent.

To the signals in ^{1}H NMR spectra of the mixtures containing PAMAM-NH_2 G5 and PAMAM-OH G5 dendrimer in heavy water (Fig. 4, Fig. 5) one may ascribe four proton groups (for PAMAM-NH_2) or five proton groups (for PAMAM-OH), which is consistent with literature data [39-43] (Fig. 6).

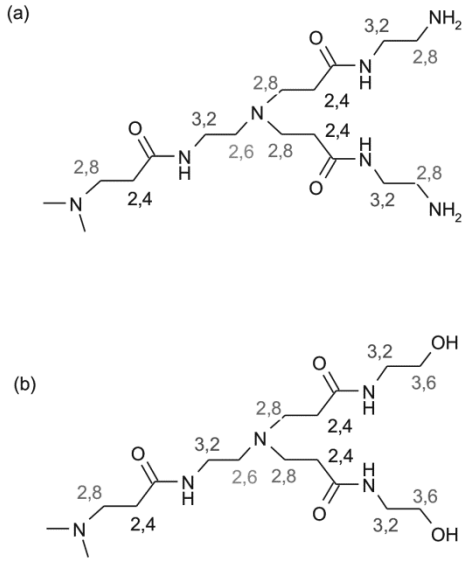

Figure 6: Ascribing the values of chemical shifts to the groups of protons in dendrimer macromolecules: a) PAMAM-NH_2, b) PAMAM-OH.

Comparing the spectra recorded for PAMAM-NH_2 G5 solution and its mixture with flutamide in heavy water (Fig. 4), one can observe changes in signal position at 2.8 ppm and 3.2 ppm in the ^{1}H NMR spectra of mixtures containing PAMAM-NH_2 G5 dendrimer. Bands at 2.4 ppm and 2.6 ppm practically are not shifted. Flutamide solutions do not show in ^{1}H NMR spectrum signals with a high intensity ranging from 2 ppm to 4 ppm, so they do not impede the interpretation of the signals derived from proton present in the dendrimer structure.

Three methylene groups combined with the tertiary nitrogen atom in the dendrimer structure show two peaks: at 2.6 ppm (methylene group placed on the PAMAM macromolecule core side) and at 2.8 ppm (methylene group placed on the PAMAM macromolecule surface side). These signals behave differently after adding flutamide molecules into the PAMAM G5-NH_2 solution. The band at 2.6 ppm, corresponding to the proton of methylene groups placed at

the tertiary amine groups (nearer the macromolecule core) are not distinctly shifted. The band at 2.8 ppm corresponds to two groups of protons: apart from the protons of methylene groups at tertiary amine groups, it also comprises the signals of methylene protons directly combined with the amine terminal groups of dendrimer (nearer the surface). This band (2.8 ppm) is clearly shifted after the addition of flutamide. This indicates a surface mechanism of interaction between flutamide molecules and PAMAM G5-NH_2 macromolecules.

Similar protons of the methylene group combined with PAMAM amide groups give two groups of signals depending on the side of amide bond where they are placed: on the PAMAM core side (signal at 2.4 ppm) or on the PAMAM surface side (signal at 3.2 ppm). Introducing flutamide molecules into the PAMAM G5-NH_2 solution causes no changes in the position of peak at 2.4 ppm, but affects the position of signal at 3.2 ppm.

The lack of clear changes in shifting protons at 2.4 ppm additionally confirms that the combination of flutamide molecules with the interior of PAMAM G5-NH_2 macromolecule does not occur. Distinct changes in the band position at 3.2 ppm (Fig. 4) additionally confirm the surface mechanism of bonding flutamide with PAMAM-NH_2 G5 macromolecules.

Analyzing the spectra of flutamide-PAMAM G5-OH mixtures compared with to those of the mixtures with G5-NH_2, one can observe an additional peak at 3.6 ppm describing the protons of the methylene groups directly combined with terminal hydroxyl groups in the macromolecule of PAMAM G5-OH dendrimer (Fig. 5). Comparing the spectrum of PAMAM G5-OH dendrimer in heavy water with that recorded for the mixture of PAMAM G5-OH dendrimer and flutamide, one can notice that the presence of flutamide molecules in heavy water does not distinctly influence the chemical shift of methylene group protons in macromolecules of this PAMAM G5-OH dendrimer. This indicates weaker interactions of flutamide with hydroxyl PAMAM G5-OH dendrimer compared to that of its equivalent as confirmed by the result of solubility and equilibrium dialyses of these systems.

^{1}H NMR spectroscopy of dendrimer–cyclocreatine solutions

There were recorded the ^{1}H NMR spectra of 70 μM of PAMAM G5-NH_2 solution and the mixtures of PAMAM G5-NH_2 (70 μM) and cyclocreatine (from 1.4 mM to 42 mM) in heavy water as solvent (Fig. 7). Similarly were performed measurements with PAMAM G5-OH, recording the spectrum of 70 μM PAMAM G5-OH solution with cyclocreatine (from 1.4 mM to 42 mM) in heavy water (Fig. 8). To the signals in the ^{1}H NMR spectra of the mixtures containing the dendrimer investigated in heavy water (Fig. 7, Fig. 8) one can ascribe four groups of protons (for PAMAM-NH_2) or five groups of protons (for PAMAM-OH) [39-43] similarly as for the mixtures of dendrimers and flutamide (Fig. 6).

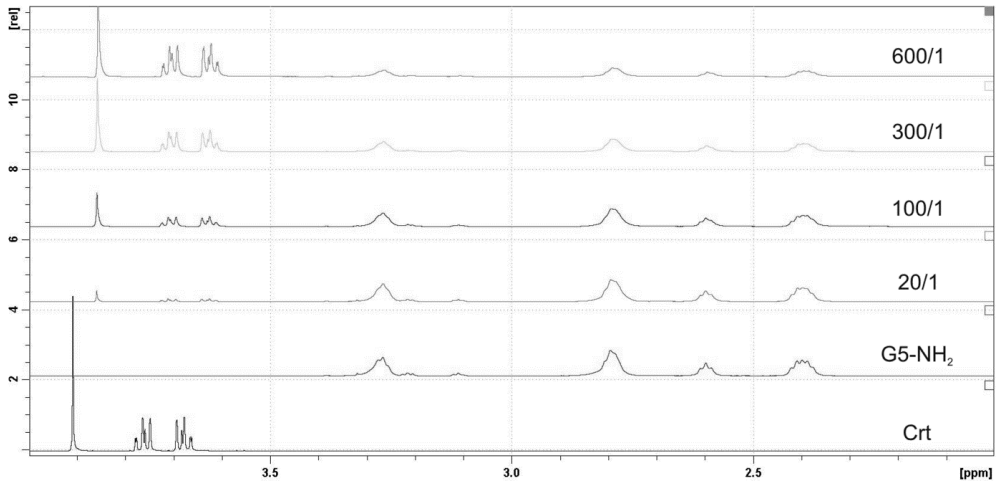

Figure 7: ^{1}H NMR spectra recorded in heavy water for 8.4 mM cyclocreatine, 70 μM PAMAM G5-NH_2 dendrimer and the mixtures of cyclocreatine with PAMAM G5-NH_2 dendrimer (70 μM) with the given molar ratio [Crt]/[G5-NH_2].

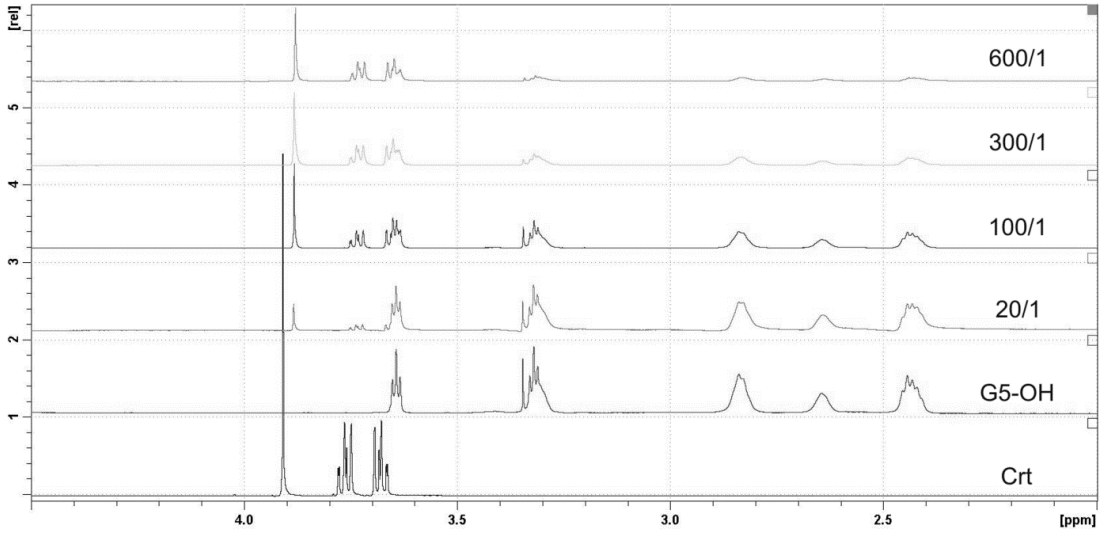

Figure 8: [1]H NMR spectra recorded in heavy water for 8.4 mM cyclocreatine, 70 μM PAMAM G5-OH dendrimer and the mixtures of cyclocreatine with PAMAM G5-OH dendrimer (70 μM) with the given molar ratio [Crt]/[G5-OH].

The spectra of the mixtures of cyclocreatine and the dendrimers investigated ranging from 3.5 ppm to 4 ppm also show the signals of cyclocreatine protons: a singlet at about 3.9 ppm from the proton of NH group in cyclocreatine heterocycle [25]. Within the range from 3.5 ppm to 3.8 ppm, one can observed two groups of signals partly overlapping each other: a group of AA'BB' multiplets on the side of the methylene group protons of the cyclocreatine heterocycle and signals of α-methylene group protons (in relation to carbonyl group) of the drug [23, 25]. In the mixtures of PAMAM-OH G5 dendrimer and cyclocreatine, the band at about 3.6 ppm corresponds to two groups of signals: methylene protons placed at the terminal hydroxyl groups of PAMAM G5-OH dendrimer and protons of methylene groups in the cyclocreatine heterocycle.

As in the case in of the dendrimer-flutamide mixtures investigated, the spectra of the mixtures of cyclocreatine and the dendrimers do not show significant changes in the peak positions of the methylene group protons of both dendrimers after the addition of cyclocreatine. With increasing the concentration of cyclocreatine in the mixture with PAMAM G5-NH_2 dendrimer we observe a slight effect of screening off the protons of the dendrimer methylene groups amounting for the extreme compositions to about 0.003 ppm. A similar effect of screening off one can observe in the mixture of cyclocreatine and PAMAM G5-OH dendrimer. The effects of screening off observed are connected with a slight increase in the electron density of the methylene groups of dendrimer localized near the active dendrimer sites saturated with cyclocreatine. Such small changes in the position of the peaks of methylene group protons of both dendrimers indicate very weak interactions of this drug molecules with both dendrimers tested. It can be also noticed that with increasing the concentration of cyclocreatine in both mixtures the height of the proton peaks of methylene groups in both dendrimers decrease. This indirectly confirms the relatively weak interactions of the zwitterions cyclocreatine molecules with both dendrimers.

Conclusion

The results of solubility and ^{1}H NMR spectroscopy of flutamide – dendrimers solutions unanimously indicate that the interactions of flutamide molecules with cationic macromolecules of PAMAM G5-NH_2 dendrimer are stronger than those with neutral hydroxyl macromolecules of PAMAM G5-OH dendrimer. The solubility of flutamide in water is very low and amounts to $S = 110$ μM ± 10 μM. Cationic PAMAM G5-NH_2 dendrimer macromolecules combine more ($n = 1.40 \pm 0.16$) flutamide molecules compared with PAMAM-OH G5 ($n = 0.45 \pm 0.15$), slightly increasing the solubility of this sparingly soluble drug. The increase in the solubility of neutral flutamide molecules in the solutions of both dendrimers (PAMAM G5-NH_2 and PAMAM G5-OH) is however weak but could find some

practical application. So, it may be concluded that PAMAM dendrimers terminated with amine or hydroxyl groups are as efficient carriers of neutral (i.e. undergoing no ionization in solution) or anionic ligand molecules as macrocyclic nanocarriers, especially cyclodextrines [13]. The results of ^{1}H NMR spectroscopic measurements suggest that the combination of flutamide with both dendrimers proceeds with the macromolecule functional groups placed on their surface.

The results of ^{1}H NMR spectroscopic examinations indicate stronger interactions of the zwitterion molecules of cyclocreatine with cationic PAMAM G5-NH_2 dendrimer compared with its hydroxyl equivalent. The small screening off effect observed in the spectra of cyclocreatine-dendrimer mixture indicates that the interactions are also stronger than in the case of flutamide.

To sum up, one may state that the spectroscopic examinations made show that the macromolecules of cationic PAMAM G5-NH_2 dendrimer stronger interact with the molecules of the two antitumor drugs selected for investigations: flutamide (Flt) and cyclocreatine (Crt) compared with hydroxyl PAMAM G5-OH dendrimer. The analysis of the ^{1}H NMR spectroscopic measurement results suggests that the flutamide molecules bound are placed on the surface of cationic PAMAM G5-NH_2 dendrimer. Probably the interaction of hydroxyl PAMAM G5-OH dendrimer with the drugs (Flt, Crt) proceeds through hydrogen bonds. Cationic PAMAM G5-NH_2 dendrimer interacts with neutral (non-ionizing in solution) molecules of drugs probably only through hydrogen bonds (flutamide) or through hydrogen bonds as well as electrostatic interactions with a character of ionic pairs between protonated amine groups of dendrimer and deprotonated drug molecules (cyclocreatine).

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