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ORIGINAL PAPER

Studying Complexes Between PPI Dendrimers and Mant-ATP

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Abstract The aim of this study was to investigate the interactions between poly(propylene imine) (PPI) dendrimers and 2′-/3′-O-(N′-methylanthraniloyl)-ATP (Mant-ATP). Mant-ATP was used as a model molecule. Purine and pyrimidine nucleoside analogues are antimetabolites commonly used in therapy for cancer. Drug molecules can complex with dendrimers in two ways: therapeutic agents may be attached in dendrimer interior or bind to functional groups on the surface. Drugs attached to nanoparticles are characterized by improved solubility, pharmacokinetics and stability. Here, we have used poly(propylene imine) dendrimers of the 4th and 5th generations (PPI G4 and PPI G5) with primary amino surface groups partially modified with maltose (PPI-m) or without modification (PPI). We assessed the efficiency of complex formation in relation to dendrimer generation, pH of solution and the type of dendrimer used. A double fluorimetric titration method was used to estimate the binding constant (K_b) and the number of binding centers per molecule of the binding agent (n).

Keywords Dendrimer · Nucleoside analogues · Complexation · Fluorimetric titration · Mant-ATP

Introduction

Dendrimers are a promising class of macromolecules owing to their many potential applications in pharmacy and biomedicine, such as drug delivery, gene transfection and imaging [1–4]. They are nanoscaled, highly branched, monodisperse macromolecules with a well-defined size and shape [3, 5, 6]. Dendrimers are characterized by a unique structure. They comprise a central core and branches that are composed of repeated monomeric units. They possess internal cavities filled with solvent and numerous functional end groups on the surface [5]. They also possess functional groups inside the molecule. Thanks to the presence of the peripheral functional groups, dendrimers are highly reactive and extremely soluble. Therefore, this structure makes them suitable for drug carrier systems. Drug molecules can be complexed with dendrimers in two ways: therapeutic agents can be encapsulated within the dendrimer interior, or can bind to functional groups on the surface [1, 7, 8]. The functional groups that bind drug molecules in the interiors of dendrimers are called endoreceptors, while the surface groups are called exoreceptors [1].

Dendrimers are used in cancer therapies to improve the solubility, pharmacokinetics, bioavailability and targetability of drugs and to ensure the controlled release of those drugs at their target tumor tissue [6, 9, 10]. However, such nanocarriers should be non-toxic and non-immunogenic [1]. Their toxicity depends strongly upon the nature of the surface groups. Dendrimers with a positive charge on the surface are considerably more cytotoxic and hemolytic than anionic or neutral molecules; this is associated with the binding of cationic dendrimers to negatively charged cell membranes [3, 7]. Cationic macromolecules (e.g. those with peripheral amino groups) cause damage to the cell membranes, whereas anionic or neutral dendrimers are more biocompatible [7, 11]. To decrease dendrimer toxicity, neutral molecules such as oligosaccharides [11, 12], polyethylene glycol (PEG) [3, 7, 8], fatty acids [3], and acetyl and glycidol groups [7] can be attached to the peripheral groups. Modifying the surface of the dendrimer can cause additional beneficial effects: dendrimers with PEG chains attached to

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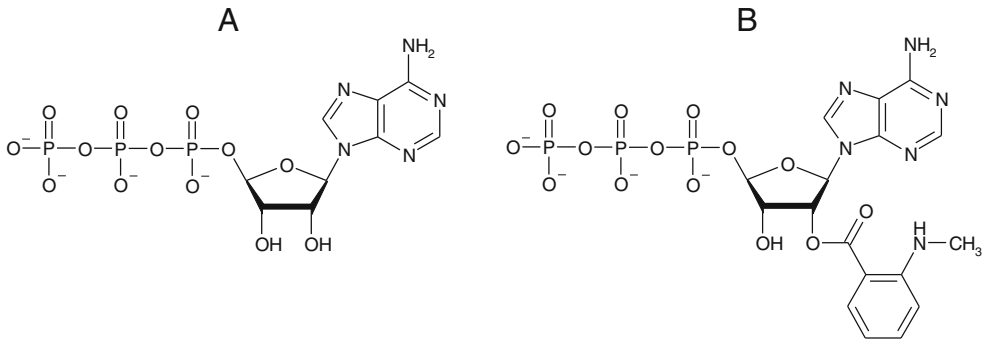

Fig. 1 Chemical structure of **a** ATP, **b** Mant-ATP

the surface have a larger size and molecular mass, which leads to a prolongation of their blood circulation time and affects their biodistribution [1, 7, 8, 13, 14]. Moreover, this modification increases the loading capacity of the carriers and delays drug release [1, 8, 13, 14]. In this area of research, there are only a few examples investigating complex formation with oligosaccharide-modified poly(propylene imine) (PPI-m) dendrimers [15, 16]. To date, it has not been completely clear whether drugs are bound more in the interior or on the exterior of such PPI glycodendrimers, so we were interested in exploring this in more detail. Our previous studies, using similar structures, have identified the desired interaction features for hyperbranched poly(ethylene imine) (PEI) with an open maltose shell (PEI-Mal), beginning with complex formation with ATP inside the dendritic PEI scaffold, followed by the formation of an ATP complex on the outer shell of the dendritic glycopolymer [17].

The objective of this study was to evaluate the interactions between different generations and types of poly(propylene imine) dendrimers (PPI) and 2′-/3′-O-(N′-methylanthraniloyl)-ATP (Mant-ATP). Mant-ATP was used as a model molecule. The fluorescence intensity of Mant-ATP increases markedly in nonpolar solvents or upon binding to most proteins [18], making it an ideal molecule with which to study the binding efficiency of anticancer drugs belonging to the group of nucleoside analogues (Fig. 1).

Nucleoside analogues are antimetabolites that are widely used in the treatment of hematological disorders and solid tumors owing to their cytotoxic influence on cancer cells. One of these therapeutic analogues is fludarabine, an analogue of deoxyadenosine that is used in the treatment of chronic lymphocytic leukemia (CLL) [19, 20]. All nucleoside analogues have a similar mechanism of activity: they interfere with nucleic acids during synthesis and disrupt the enzymes responsible for physiological nucleotide metabolism. This action ultimately leads to cellular apoptosis [19, 21–23]. Nucleoside analogues are administered as prodrugs, which need to go through a sequence of metabolic processes to form the cytotoxic metabolite—nucleoside triphosphate—to exert their anticancer activity. Therefore, anti-tumor therapies based on nucleoside analogues frequently have limited efficacy owing to several resistance mechanisms such as fast metabolism, low solubility, unfavorable biodistribution, or low specificity of interaction with the cancer cells [21, 24, 25]. Thus, there is a need for efficient drug carrier systems that can improve the efficiency and specificity of anticancer drugs [3]. The use of dendrimers as drug delivery devices may help to overcome the metabolic limitation of chemotherapeutics by delivering activated nucleosides directly to cancer cells.

Materials and Methods

2′-/3′-O-(N′-methylanthraniloyl)-ATP (Mant-ATP) was purchased from Jena Bioscience (Germany). Poly(propylene imine) dendrimers of the 4th generation (PPI G4, MW 3513.87 g/mol) and poly(propylene imine) dendrimers of the 5th generation (PPI G5, MW 7167.97 g/mol) were obtained from SyMO-Chem (Eindhoven, The Netherlands). PPI dendrimers with primary amino surface groups partially modified with maltose (open shell glycodendrimers) were used (PPI-m). Poly(propylene imine) glycodendrimers of the 4th generation (PPI-mG4, MW 10,040 g/mol) and poly(propylene imine) dendrimers of the 5th generation (PPI-mG5, MW 19,890 g/mol) were synthesized and characterized as previously described [26]. The molecular weights of both PPI glycodendrimers were determined by an established ^{1}H NMR approach [12]. For fluorescence measurements, phosphate-buffered saline (PBS: 137 mmol/L NaCl, 2.7 mmol/L KCl, pH 7.4) and phosphate buffer (137 mmol/

Table 1 Parameters of spectrofluorimetric emission spectra of Mant-ATP (c=1 μmol/L) in the presence of dendrimers (c=20 μmol/L). F_{max}- fluorescence intensity for λ_{max}, $\Delta\lambda$- the shift of λ_{max}

Type of dendrimer	pH	the 4th generation (G4)		the 5th generation (G5)	
		F_{max} [a.u.]	$\Delta\lambda$ [nm]	F_{max} [a.u.]	$\Delta\lambda$ [nm]
PPI	7.4	181.6	2.8	249.5	4.4
PPI	5.8	225.1	4.2	272.8	5.0
PPI-m	7.4	280.4	6.3	321.1	7.5

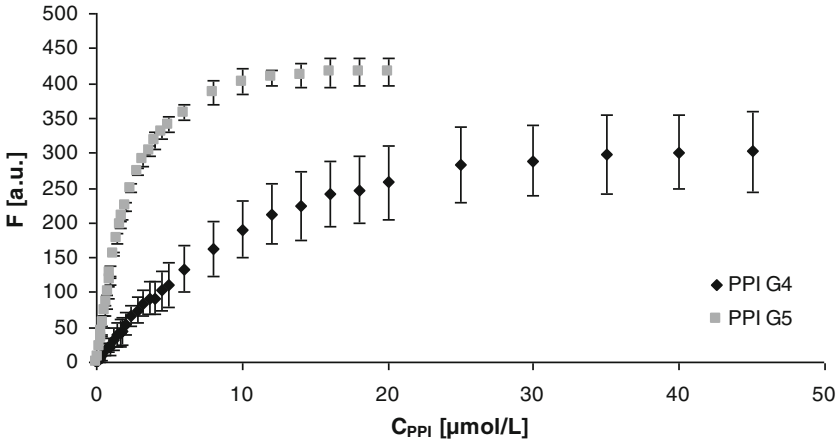

Fig. 2 The dependence of Mant-ATP fluorescence intensity on concentration of PPI G4 dendrimer (*black graph*) and PPI G5 dendrimer (*grey graph*) at pH 7.4. $C_{Mant\text{-}ATP}$=1 µmol/L; λ_{exc}=355 nm

L NaCl, 2.7 mmol/L KCl, pH 5.8) were used. Generally, PPI dendrimers and Mant-ATP were dissolved in PBS. Phosphate buffer with a different pH was used to study the effect of pH on dendrimers and Mant-ATP complex formation. The water used to prepare solutions was double-distilled.

Fluorescence spectra were measured using a Perkin-Elmer LS 55 spectrofluorimeter. The excitation wavelength was set at 355 nm. The emission spectra were recorded from 380 to 540 nm. The dendrimers were not excited by 355 nm and did not emit fluorescence in this range. The excitation and emission slit widths were set to 5 and 5 nm, respectively. Samples were measured in 1-cm path length quartz cuvettes and were continuously stirred and maintained at 37 °C.

A Scatchard double fluorimetric titration method was used to estimate the binding constant (K_b) and the number of binding centers per dendrimer molecule (n) [14, 27–29]. In the first step of this method, the binding agents—dendrimers—were added in increasing concentrations to a solution of the ligand—Mant-ATP. Changes in the fluorescence intensity (F_{max}) of Mant-ATP at the emission maximum (440 nm) were recorded. When the fluorescence intensity reached maximum, all molecules of the ligand were complexed with binding agents. Next, the maximum fluorescence intensity of the ligand was recalculated to the specific fluorescence intensity for the bound probe as follows:

$$F_{sp} = \frac{F_{\max}}{C_{Mant-ATP}} \tag{1}$$

$C_{Mant\text{-}ATP}$- Mant-ATP concentration during the first fluorimetric titration.

In the second step of this method the situation was reversed. The ligand—Mant-ATP—was added at increasing concentrations to a solution of a binding agent—the dendrimer. The fluorescence intensity (F) was recorded. The fluorescence intensity was then recalculated for the concentration of ligands bound to the binding agent and the concentrations of bound and free ligands were calculated:

$$C^{bound}_{Mant-ATP} = \frac{F}{F_{sp}} \tag{2}$$

$$C^{free}_{Mant-ATP} = C_{Mant-ATP} - C^{bound}_{Mant-ATP} \tag{3}$$

The binding constant (K_b) and the number of binding centers in the solution (N) can be determined from a linear plot of $\frac{1}{C^{bound}_{Mant-ATP}}$ on the ordinate versus $\frac{1}{C^{free}_{Mant-ATP}}$ on the

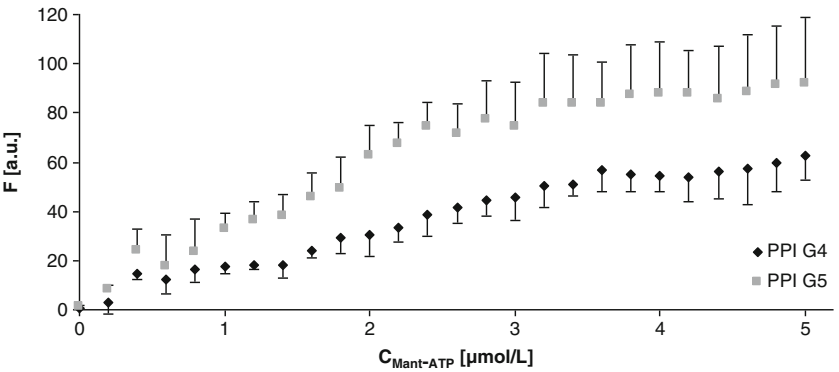

Fig. 3 The dependence of Mant-ATP fluorescence intensity on its concentration in the presence of 0.75 µmol/L PPI G4 dendrimer (*black graph*) and 0.5 µmol/L PPI G5 dendrimer (*grey graph*) at pH 7.4; λ_{exc}=355 nm

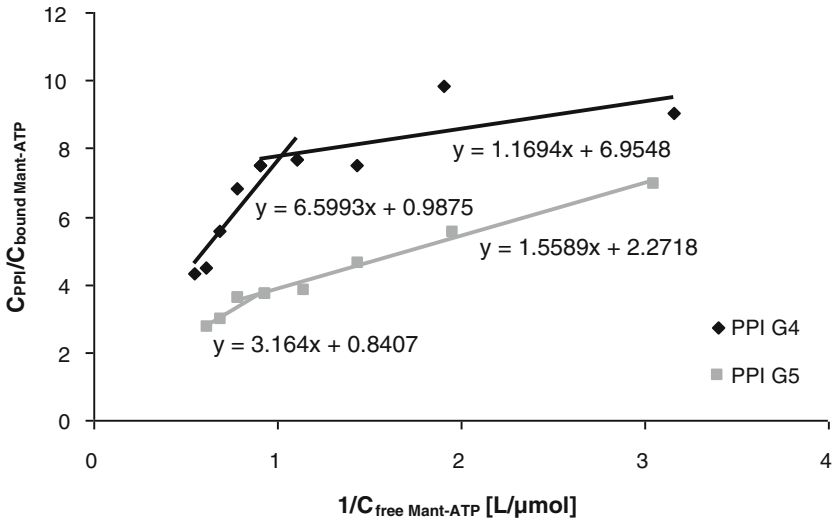

Fig. 4 The determination of K_b and n for PPI G4 dendrimer (*black graph*) and PPI G5 dendrimer (*grey graph*) at pH 7.4 in the double inverse coordinates

abscissa according to the equation:

$$\frac{1}{C^{bound}_{Mant-ATP}} = \frac{1}{K_b \cdot N \cdot C^{free}_{Mant-ATP}} + \frac{1}{N} \tag{4}$$

The number of ligand binding centers in the solution (N) can be recalculated to the number of ligand binding centers per one binding agent molecule (n).

$$n = \frac{N}{C_{PPI}} \tag{5}$$

C_{PPI}—molar concentration of the binding agent. Thus the final version of Eq. (4) is [28, 29]:

$$\frac{C_{PPI}}{C^{bound}_{Mant-ATP}} = \frac{1}{K_b \cdot n \cdot C^{free}_{Mant-ATP}} + \frac{1}{n} \tag{6}$$

Results

The fluorescence yield of Mant-ATP is significantly altered by environmental conditions. Pure Mant-ATP in aqueous solution emits weak fluorescence in the range of 380–540 nm with a maximum at 440 nm. The fluorescence intensity increases considerably in nonpolar solvents or upon binding to most proteins [18]. We observed a similar behavior when Mant-ATP and dendrimers were mixed. There was a significant increase in the intensity of the Mant-ATP fluorescence spectrum upon addition of the dendrimers. This was observed for all types of dendrimer. The effect was strongest for modified PPI-m dendrimers, less marked for unmodified PPI dendrimers in an acidic environment, and weakest for unmodified PPI dendrimers at pH 7.4 (Table 1).

Additionally, the shift in the maximum emission wavelength was monitored to study the environment of the Mant-ATP chromophore. The position of the emission maximum of Mant-ATP changed after the addition of successive doses of dendrimers and this corresponded to changes in the polarity around the chromophore. Adding PPI dendrimers caused a blue shift of the emission spectrum. This indicated that the Mant-ATP probe was located in a more hydrophobic environment owing to Mant-ATP binding to the PPI dendrimers (Table 1).

Table 2 Binding constants (K_b) and number of binding centers (n) for binding between Mant-ATP and PPI dendrimers

The type of dendrimer; buffer pH	the 4th generation (G4)				the 5th generation (G5)			
	Partial incorporation		Surface binding		Partial incorporation		Surface binding	
	n (for 10 molecules of PPI)	K_b [L/mol]	n (for 10 molecules of PPI)	K_b [L/mol]	n (for 10 molecules of PPI)	K_b [L/mol]	n (for 10 molecules of PPI)	K_b [L/mol]
PPI; pH 7.4	1.4±0.3	5.95×10^6	10.1±0.6	1.5×10^5	4.4±0.3	1.46×10^6	11.9±0.8	2.66×10^5
PPI; pH 5.8	–	–	11.9±2.0	3.39×10^5	–	–	18.6±0.4	4.44×10^5
PPI-m; pH7.4	1.4±0.2	3.45×10^6	13±0.7	1.1×10^5	–	–	19.1±1.8	2.08×10^5

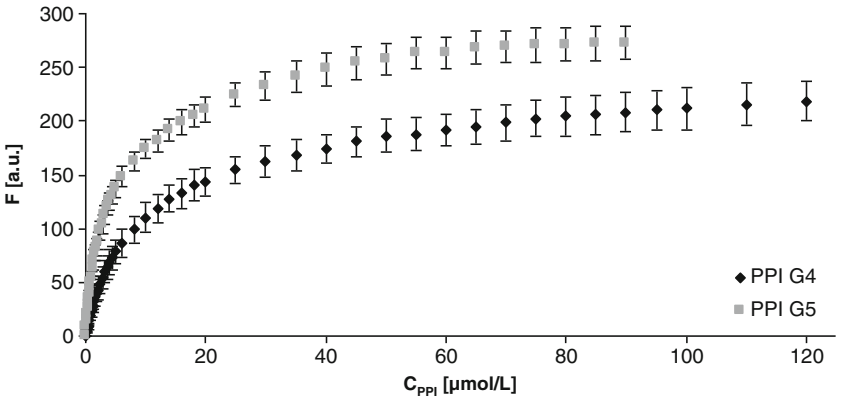

Fig. 5 The dependence of Mant-ATP fluorescence intensity on concentration of PPI G4 dendrimer (*black graph*) and PPI G5 dendrimer (*grey graph*) at pH 5.8. $C_{Mant\text{-}ATP}$=1 μmol/L; λ_{exc}=355 nm

This significant environmental sensitivity of the chromophore makes Mant-ATP useful for investigating the nucleoside analogue-dendrimer interactions. The binding constant (K_b) and the number of binding centers per dendrimer molecule (n) for the fourth and the fifth generations of unmodified PPI dendrimers were estimated by a double fluorimetric titration approach using the Scatchard method. The exact value of F_{max} was read from Fig. 2. Values of K_b and n were calculated from the initial linear region of the dependence of Mant-ATP fluorescence intensity on its concentration in the presence of 0.75 μmol/L for PPI G4 and 0.5 μmol/L for PPI G5 (Fig. 3). In Fig. 3 the linear dependences are presented for Mant-ATP concentrations in the ranges 0.4–3.6 μmol/L and 0.2–2.4 μmol/L, respectively. Plots of $C_{dendrimer}/C_{bound\ Mant\text{-}ATP}$ versus $1/C_{free\ Mant\text{-}ATP}$ over these concentration ranges for the fourth and the fifth generations of unmodified PPI dendrimer are shown in Fig. 4. Values of K_b and n obtained from Fig. 4 are summarized in Table 2.

As in the case of unmodified PPI dendrimers at pH 7.4, an increase in Mant-ATP fluorescence intensity and a blue shift of the emission maximum were observed for unmodified PPI dendrimers at pH 5.8. However, the increase in fluorescence intensity and the blue shift were significantly more pronounced at lower pHs (Table 1). Values of K_b and n for unmodified dendrimers at pH 5.8 were calculated as for PPI dendrimers at a physiological pH. The value of F_{max} for Mant-ATP was estimated from Fig. 5. K_b and n were calculated from the initial linear region of the dependence of Mant-ATP fluorescence intensity on its concentrations in the presence of 0.75 μmol/L for the fourth generation PPI and 0.5 μmol/L for the fifth generation PPI (Fig. 6). In Fig. 6, linear dependences were observed for Mant-ATP concentrations between 0.2 and 1.0 μmol/L for PPI G4 and between 0.2 and 1.2 μmol/L for PPI G5. Plots of $C_{dendrimer}/C_{bound\ Mant\text{-}ATP}$ versus $1/C_{free\ Mant\text{-}ATP}$ over these concentration ranges for the fourth and the fifth generations of unmodified PPI dendrimer are shown in Fig. 8. Values of K_b and n obtained from Fig. 7 are presented in Table 2.

We carried out similar studies to compare the loading and binding capacities of unmodified PPI dendrimers with maltose-modified PPI-m dendrimers. As previously, the increase in Mant-ATP fluorescence intensity and a blue shift of the Mant-ATP emission maximum were observed for the modified PPI dendrimers (Table 1). These results are shown in Figs. 8 and 9. In Fig. 9 the initial linear regions of the dependence of Mant-ATP fluorescence intensity on its concentrations in the presence of 0.75 μmol/L for the fourth generation of PPI-m dendrimer and 0.5 μmol/L for the fifth generation of PPI-m dendrimer were observed for Mant-ATP concentrations in the ranges 0.2–2.0 μmol/L and 0.2–

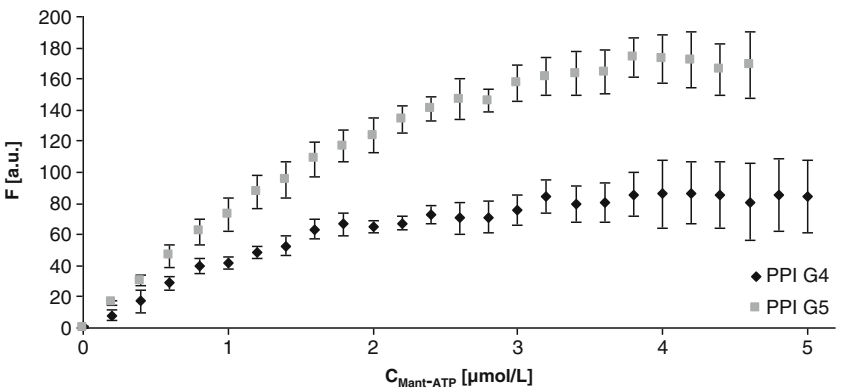

Fig. 6 The dependence of Mant-ATP fluorescence intensity on its concentration in the presence of 0.75 μmol/L PPI G4 dendrimer (*black graph*) and 0.5 μmol/L PPI G5 dendrimer (*grey graph*) at pH 5.8; λ_{exc}=355 nm

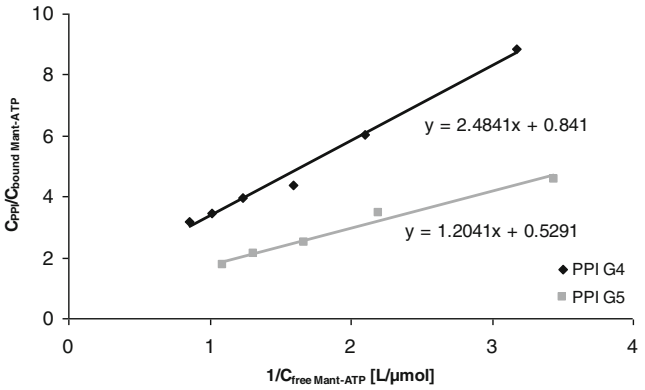

Fig. 7 The determination of K_b and n for PPI G4 dendrimer (*black graph*) and PPI G5 dendrimer (*grey graph*) at pH 5.8 in the double inverse coordinates

4.0 μmol/L, respectively. To estimate K_b and n the Scatchard method was used (Fig. 10) and the data obtained are presented in Table 2. These results clearly demonstrate that PPI dendrimers modified with maltose residues also efficiently bind molecules of Mant-ATP.

Discussion

Mant-ATP is a spectrofluorimetric dye. The shape of the Mant-ATP fluorimetric spectrum strongly depends upon its microenvironment. This feature can be utilized for studies of complex formation. When dendrimers were added to a solution of Mant-ATP, the intensity of the fluorescence emission increased significantly. This change was accompanied by a blue-shift in the position of the emission maximum. Blue-shift indicates that a dye is in a less polar environment. The extent of these alterations depended on the type of dendrimer. Generally, the bigger the increase in fluorescence intensity observed, the more pronounced a blue-shift was recorded.

In our study the 5th generation of all types of dendrimers caused larger changes in the shape of the Mant-ATP spectrum than the 4th generation PPIs. PPI G5 possess 64 surface groups, whereas PPI G4 only possess 32. Therefore, the surface area available for nucleotide molecules increases for higher generations [14]. Unmodified PPI dendrimers have amino groups on the surface. These groups are partly ionized at pH 7.4, so we predicted that interactions between the negatively charged Mant-ATP and cationic PPI dendrimers would be driven by electrostatic forces. This was confirmed when the experiment was repeated in a buffer at pH 5.8. At pH 5.8 the degree of protonation of amino groups is greater than at pH 7.4. Stronger electrostatic interactions between PPI and Mant-ATP resulted in bigger changes in the spectrum shape. To check the influence of a surface modification on the ability to form complexes, PPI dendrimers were tested that had been partially modified by attaching maltose to the surface. Even though the number of charged amino groups decreased after such a modification, these dendrimers caused bigger changes in the spectrofluorimetric spectra.

A simple analysis of spectrum alterations only allowed general conclusions to be drawn about whether a certain type of dendrimer binds to Mant-ATP. For more detailed analysis, a Scatchard method was employed. In this approach, it is possible to calculate the number of bound Mant-ATP molecules and a binding constant. Moreover, the analysis of a Scatchard plot allows the researcher to distinguish between two situations: when Mant-ATP binds to the dendrimer surface and when it partly incorporates inside the dendrimer.

In all of the cases studied, more Mant-ATP molecules were bound to the 5th generation than the 4th generation PPI. Interestingly, in the case of unmodified PPI dendrimers at pH 7.4, the number of Mant-ATP molecules attached to the surface was only slightly higher for the 5th generation than for the 4th. In contrast, approximately three times more molecules were incorporated into the dendrimer interior in the higher generation.

The interior of the maltose-modified (PPI-m) dendrimers incorporated Mant-ATP molecules only in the 4th

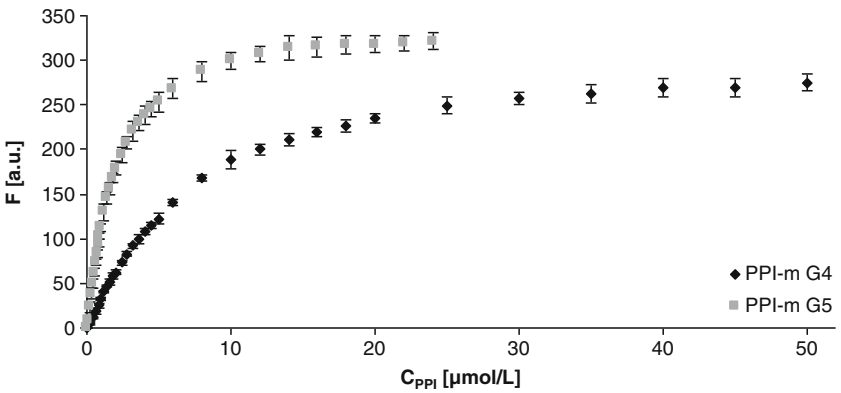

Fig. 8 The dependence of Mant-ATP fluorescence intensity on the concentration of modified PPI-mG4 dendrimer (*black graph*) and modified PPI-mG5 dendrimer (*grey graph*) at pH 7.4. $C_{Mant\text{-}ATP}$=1 μmol/L; λ_{exc}=355 nm

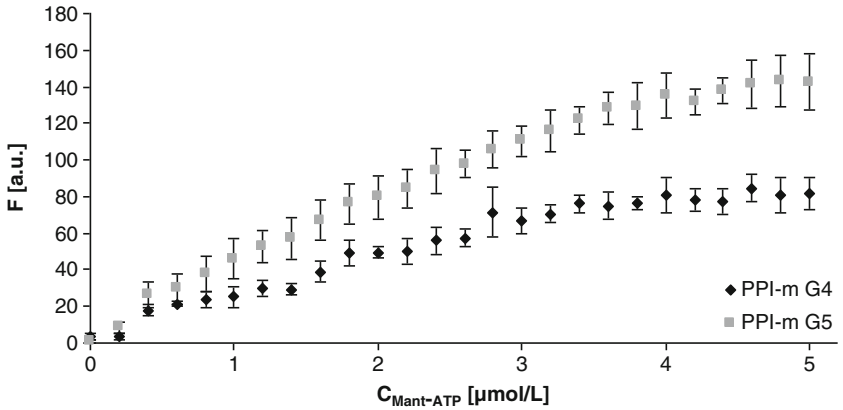

Fig. 9 The dependence of Mant-ATP fluorescence intensity on its concentration in the presence of 0.75 µmol/L modified PPI-mG4 dendrimer (*black graph*) and 0.5 µmol/L modified PPI-mG5 dendrimer at pH 7.4; λ_{exc}=355 nm

generation. This indicates that the structure was open and flexible enough to be able to partially incorporates the guest molecules. In the case of the 5th generation no Mant-ATP molecules were incorporated inside. This could be the result of a densely packed surface on these dendrimers (Fig. 11).

No encapsulation was observed for unmodified PPI dendrimers at pH 5.8. At this pH all amino groups on the surface of the dendrimer were protonated and electrostatic interactions between cationic dendrimers and negatively charged Mant-ATP molecules were stronger than at pH 7.4, at the same time decreasing the affinity between Mant-ATP and the interior. The primary amine groups on the dendrimer surface were more basic than the tertiary amine groups inside the dendrimer structure [30–33]. The calculated binding constant at pH 5.8 was higher than at pH 7.4. Additionally, the structure of the dendrimer depends upon pH, and in an acidic pH it is more packed.

In summary: first, our experiments have shown that complexing of PPI dendrimers with nucleoside analogues is generation-dependent. This process is more efficient for higher generations of dendrimers. Secondly, lower pH causes stronger Mant-ATP binding to the dendrimers. This is because electrostatic interactions are enhanced under these conditions. Thirdly, the most efficient nucleotide binding occurs for modified dendrimers because of their increased size and surface area, where the maltose shell supports nucleotide binding. Nucleotide-dendrimer complexes are stabilized by non-covalent interactions such as electrostatic and non-specific hydrogen bonds due to the presence of the maltose shell in the glycodendrimer. Thus, it can be concluded that surface-modified cationic dendrimers are good candidates for a drug delivery system.

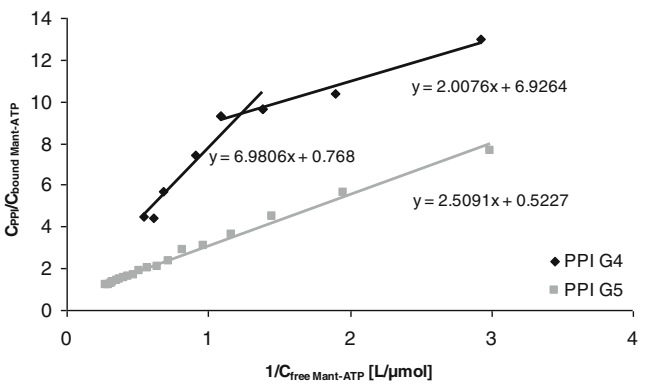

Fig. 10 The determination of K_b and n for modified PPI-mG4 dendrimer (*black graph*) and modified PPI-mG5 dendrimer (*grey graph*) at pH 7.4 in the double inverse coordinates

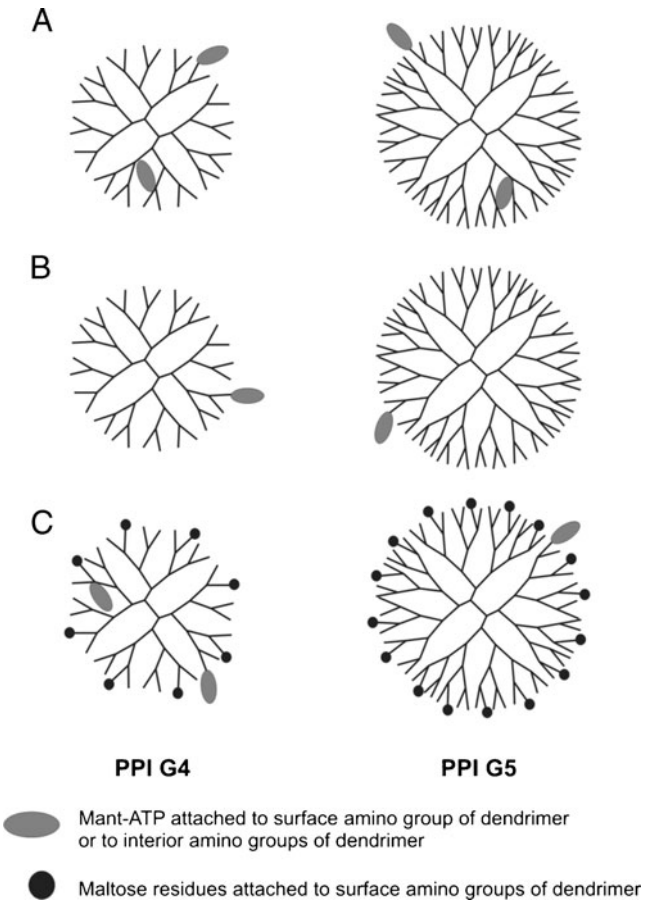

Fig. 11 Two hypothetical ways of binding of nucleotide molecules by **a** unmodified PPI dendrimers at pH 7.4; **b** unmodified PPI dendrimers at pH 5.8; **c** modified PPI dendrimers with maltose residues at pH 7.4

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