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Interaction study between maltose-modified PPI dendrimers and lipidic model membranes

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ABSTRACT

The influence of maltose-modified poly(propylene imine) (PPI) dendrimers on dimyristoylphosphatidylcholine (DMPC) or dimyristoylphosphatidylcholine/dimyristoylphosphatidylglycerol (DMPC/DMPG) (3%) liposomes was studied. Fourth generation (G4) PPI dendrimers with primary amino surface groups were partially (open shell glycodendrimers — OS) or completely (dense shell glycodendrimers — DS) modified with maltose residues. As a model membrane, two types of 100 nm diameter liposomes were used to observe differences in the interactions between neutral DMPC and negatively charged DMPC/DMPG bilayers. Interactions were studied using fluorescence spectroscopy to evaluate the membrane fluidity of both the hydrophobic and hydrophilic parts of the lipid bilayer and using differential scanning calorimetry to investigate thermodynamic parameter changes. Pulsed-filed gradient NMR experiments were carried out to evaluate common diffusion coefficient of DMPG and DS PPI in D_2O when using below critical micelle concentration of DMPG. Both OS and DS PPI G4 dendrimers show interactions with liposomes. Neutral DS dendrimers exhibit stronger changes in membrane fluidity compared to OS dendrimers. The bilayer structure seems more rigid in the case of anionic DMPC/DMPG liposomes in comparison to pure and neutral DMPC liposomes. Generally, interactions of dendrimers with anionic DMPC/DMPG and neutral DMPC liposomes were at the same level. Higher concentrations of positively charged OS dendrimers induced the aggregation process with negatively charged liposomes. For all types of experiments, the presence of NaCl decreased the strength of the interactions between glycodendrimers and liposomes. Based on NMR diffusion experiments we suggest that apart from electrostatic interactions for OS PPI hydrogen bonds play a major role in maltose-modified PPI dendrimer interactions with anionic and neutral model membranes where a contact surface is needed for undergoing multiple H-bond interactions between maltose shell of glycodendrimers and surface membrane of liposome.

1. Introduction

Many new drugs have been recently developed that need to be effectively delivered to target organs, tissues or cells by a drug delivery system (DDS). The usage of drug carriers is motivated by many advantages, such as the protection of cargo from inactivation by light or enzymes, the reduction in toxicity or the increase in solubility. Good DDSs can enhance biodistribution and direct the drug to the location where it should act. Moreover, DDSs can provide long-term release of the pharmacotherapeutic at the target location and reduce its undesirable side effects [1–3]. Thus, beneficial DDSs are those that spend enough time in the blood stream and are tailored to the desired tissue. In addition, they should not accumulate in body tissues and need to exhibit highly biocompatible properties in *in vitro* and *in vivo* conditions.

Dendrimers are promising groups of nanoparticles that have been widely investigated in the field of DDSs. The history of these globular polymers dates back to the end of the 1970s; however, the number of varieties is still growing, and the area of modification and bioapplication is still being developed. Dendrimers generate interest because of their perfectly branched molecular structure, which is susceptible to external surface group modification to enhance both their complexation properties to drugs and their biocompatibility in biological systems.

Despite the many advantages of these potential DDSs, there is a problem with the high toxicity of positively charged polyamine dendrimers. It has been shown that cytotoxicity is a concern with dendrimer generation and a growing number of cationic amino surface groups that are prone to destabilize cell membranes and cause lysis of the cell [4–6]. As mentioned before, an ideal dendritic candidate for a

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DDS is preferentially non-toxic. Thus, much effort has been invested to achieve new properties of cationic polyamine dendrimers through various surface modifications [5,7–9].

For the successful use of sugar-decorated poly(propylene imine) (PPI) dendrimers as a DDS [10–12] and a biological [13–15] and stabilizing [16] agent in (bio-)medical applications, one has to not only search for those potential application fields but also understand the cellular uptake and cellular trafficking in various cells. Moreover, a fundamental understanding of their molecular interaction properties against a biological cell membrane and model lipid membranes is mostly unknown. Efforts have been made to evaluate the complex cellular uptake in normal and cancer melanoma cells [17]. In this context, open and dense shell PPI glycodendrimers (Fig. 1) are generally used to determine their potential use in the bio-application, while until now, no answer has been given concerning whether neutral or cationic PPI glycodendrimer (Fig. 1) undergoes stronger interactions with biological membranes. In this study, we describe the fundamental interaction features of cationic and neutral PPI glycodendrimers against neutral and anionic model lipid membranes. These results may help us to better understand their general interaction features against supramolecular entities such as vesicles in biological systems. A large number of protonated amine groups in parental PPI dendrimers at higher generations that are present at a physiological relevant pH 7.4 have resulted in high toxicity of the parental 3rd and 4th generation PPI dendrimers in previous investigations of PPI dendrimer toxicity *in vitro* and *in vivo* [8,18]. Previous research has demonstrated the high negative impact of unmodified PPI dendrimers on cells as well as animals. The results lead to the conclusion that a number of cationic surface groups have an influence on the compound toxicity. The higher amount of positively charged amino groups causes higher cytotoxicity. The results also

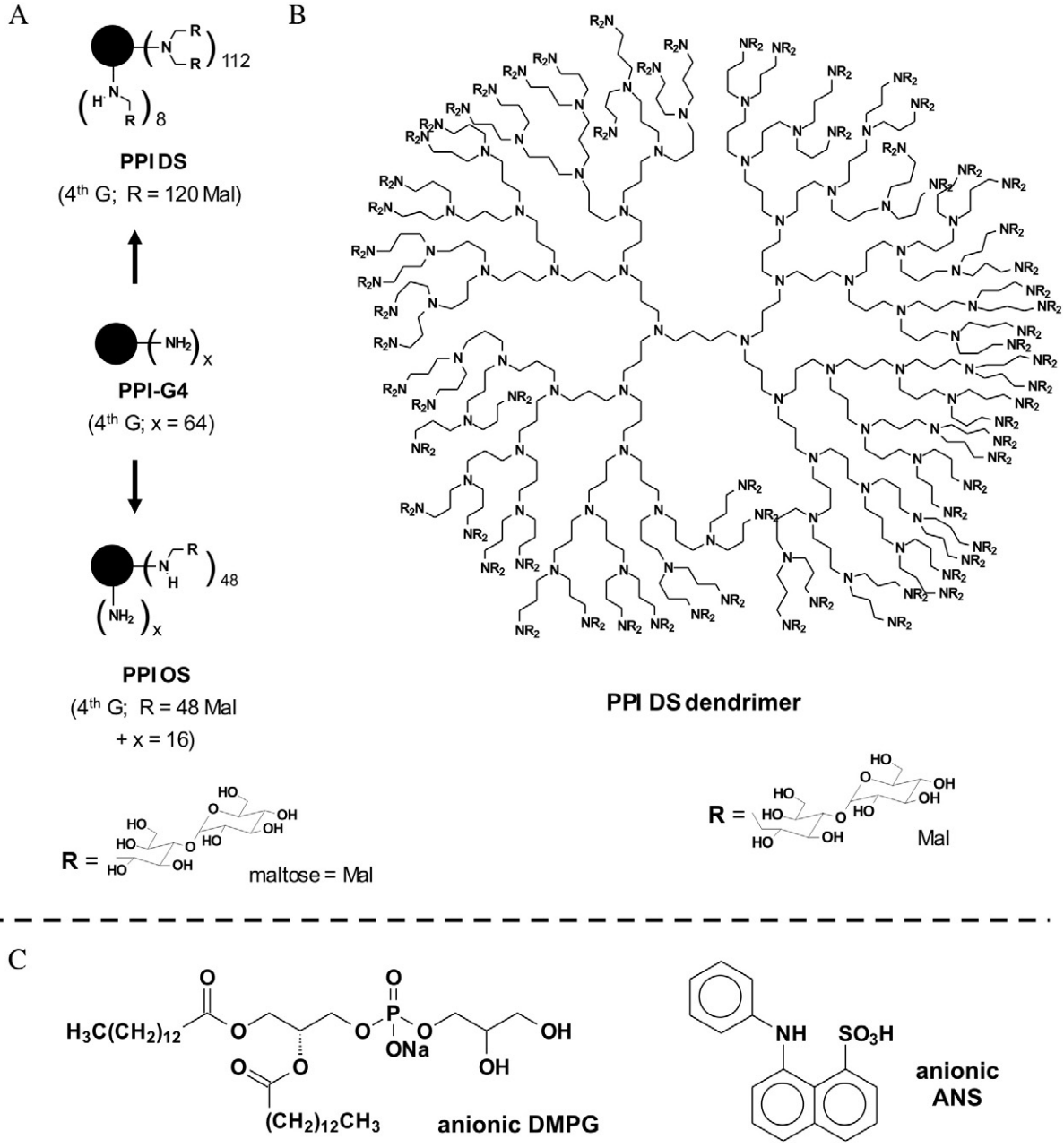

Fig. 1. (A) Synthetic pathway of 4th-generation PPI OS and DS dendrimers using the parental PPI dendrimer. (B) Idealized chemical structure of PPI DS dendrimer. Molecular masses of both dendrimers are presented in the experimental section. (C) Molecular structures of anionic dimyristoylphosphatidylglycerol (DMPG) and anionic 8-anilino-1-napthalene sulfonic acid (ANS) both used in PFG NMR experiments.

indicate that the PPI dendrimers' toxicity is associated with the interaction with components of biological membranes such as proteins or negatively charged lipids. It is therefore not surprising that surface group modifications, especially the sugar decoration of PPI dendrimers, have been made to decrease the toxicity of cationic PPI dendrimers [8–10,18,19].

Thus, maltose surface modification of PPI dendrimers results in promisingly high biocompatibility [18]. On the other hand, these cationic (open shell) and neutral (dense shell) PPI glycodendrimers are still able to interact with cationic and anionic proteins [8,20,21]. From these studies two tendencies can be concluded: First cationic open shell glycodendrimers have stronger binding strengths against biomolecules than neutral dense shell glycodendrimers. On the other hand the largest generation (4th G) within the series of dense shell PPI glycodendrimers has the higher binding strengths against HSA biomolecules than the 3rd generation [8], while smaller 1st and 2nd generation of dense shell glycodendrimers do not interact with HSA molecules at all (unpublished results). These results are promising for their future biomedical use, unlike the neutral hydroxyl-terminated PAMAM dendrimers, which did not show affinity to biomolecules [8,22]. Despite the useful properties of these PPI-based nanomaterials for the development of an efficient DDS or biological agents, the important question that has not yet been answered is whether the maltose-modified PPI dendrimers possess an affinity to biological membranes and, if so, what the nature of this interaction is. To clarify the results presented in this article related to the interaction mechanism of 4th-generation maltose-modified PPI dendrimers (Fig. 1) with model lipid membranes, a very short introduction of dendrimer physical and chemical properties follows. An open shell 4th-generation PPI glycodendrimer contains a low degree of maltose substitution on the peripheral primary amino groups (Fig. 1: 37.5%). The low maltose substitution of a 4th-generation PPI dendrimer is generally accompanied by the presence of cationic charge density. This always results in stronger interaction properties with isolated protein solutions in comparison to those of neutral dense shell PPI glycodendrimers [20,21]. Furthermore, ionic interactions are mainly favored when cationic open shell PPI glycodendrimers are involved in biological interaction studies [13,15,20,21]. In addition, a dense shell 4th-generation PPI glycodendrimer (Fig. 1: degree of maltose substitution for peripheral primary amino groups: >90%) can be considered as an amphiphilic macromolecule possessing a cationic PPI core and a neutral maltose shell [13,23]. Neutral charge density of dense shell glycodendrimers [13,23] is characterized by the non-charge compensation of cationic PPI core when anionic polyelectrolyte is added to dense shell glycodendrimer solutions for carrying out polyelectrolyte titration experiments. This means that dense maltose shell hampers penetration of anionic polyelectrolyte to compensate cationic charge of PPI core in dense shell PPI glycodendrimers [13,23]. As shown in previous studies, the interaction features of dense shell PPI glycodendrimers against peptides and proteins are preferentially tailored by H-bonds [8,13,20,21,23].

Instead of biological membranes, simpler lipid models (e.g., lipid bilayers) are used to reflect the influence of new chemicals on the lipid part of membranes. The use of liposomes not only eliminates the influence of membrane proteins but also has other advantages, such as high time stability and simple preparation. Model lipid membranes, consisting of commercially available lipids, can simulate the composition of every type of cell membrane. Moreover, they can be created with lipid extracted from cell membranes. Model membrane studies are sources of information about the phenomena happening at the bilayer domains, membrane fusion and permeability [24–26].

Here, we report our investigation of the influence of two types (neutral and cationic) of maltose-modified 4th-generation PPI dendrimers on neutral DMPC and negatively charged DMPC/DMPG liposomes. The data presented in this article help to clarify (I) the nature of maltose-modified PPI dendrimer interactions with lipid bilayers and (II) the phenomena that may occur in biological membranes.

2. Materials and methods

2.1. Materials

Lipids:1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-*sn*-glycero-3-phospho-rac-(1-glycerol) sodium salt (DMPG) and fluorescent probes: 1,6-diphenyl-1,3,5-hexatriene (DPH); N,N,N-trimethyl-4-(6-phenyl-1,3,5-hexatrien-1-yl) phenylammonium p-toluenesulfonate (TMA-DPH); 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES buffer); and anionic anionic 8-anilino-1-napthalene sulfonic acid (ANS) were purchased from Sigma Chemical Company. Maltose monohydrate, borane-pyridine complex (8 M in THF) and sodium borate were purchased from Fluka. Fifth generation poly(propylene imine) (PPI) dendrimer was supplied by SyMO-Chem (Eindhoven, Netherlands). Fifth generation supplied by SyMO-Chem does not correspond to the general rules for describing polyamine dendrimers [24]. Thus 4th generation PPI dendrimer is used here in this study. Moreover, all mentioned generations of PPI dendrimers follow the recommending nomenclature of polyamine dendrimers as suggested in a recent review [27]. Maltose-modified 4th-generation PPI dendrimers (Fig. 1) were synthesized and characterized as described in previous papers [8,13]. Molecular mass is 22,670 g/mol for an open shell glycodendrimer, 46,000 g/mol for a dense shell glycodendrimer and 7168 g/mol for an unmodified PPI dendrimer G4.

2.2. Liposome preparation

Phospholipids were dissolved in organic solvents. Appropriate amounts of lipid solution with or without fluorescent probes were placed in a flask under a stream of nitrogen to evaporate the solvent. The resulting lipid film was hydrated with an appropriate volume of buffer and mixed. The lipid mixture was incubated at a temperature above the lipid phase-transition point. Subsequently, the suspension was forced to pass >15 times through a polycarbonate membrane of 100 nm porosity (Nuclepore, T-E) mounted in a mini-extruder (Avanti Polar Lipids) fitted with two 1000-μl Hamilton gastight syringes. After preparation, the suspension of liposomes was incubated in a water bath at 37 °C for 10 min. For fluorescence experiments, the final lipid concentration was 100 μM, and the phospholipid/fluorescent probe molar ratio was 500:1 [28,29].

2.3. Characterization of research models and aggregation process

The size of particles was measured using the dynamic light scattering (DLS) method in a Zetasizer Nano-ZS instrument (ZEN3600, Malvern Instruments, UK) [30]. The refraction factor was assumed to be 1.33, while the detection angle was 173° and the wavelength of red laser light was 633 nm. Samples in 10 mM Hepes, pH 7.4, were placed in the glass cell (PCS8501, Malvern) and measured at 37 °C. The data were analyzed using the Malvern software.

The particle charge measurements were conducted with a Zetasizer Nano-ZS using a combination of two measurement techniques: electrophoresis and laser Doppler velocimetry. The electrophoretic mobility of particles was measured in an applied electric field using Malvern capillary plastic cells (DTS1061) with a cuprum electrode covered with gold. Samples were prepared and measured at 37 °C in 10 mM Hepes, pH 7.4. The zeta potential value was calculated by Malvern software from the Helmholtz–Smoluchowski equation [31]. To characterize lipid model membranes and dendrimers, concentrations of 200 μM and 50 μM, respectively, were used. During the liposome titration by dendrimers, the concentration of dendrimers increased from 0.5 μM to 120 μM.

2.4. Fluorescence spectroscopy

Fluorescence anisotropy measurements were carried out with a FluoroMax-4 spectrofluorimeter (Horiba Scientific, France). To monitor

membrane fluidity in a whole bilayer, two fluorescent probes were used. The first, DPH, an apolar molecule, was incorporated into the hydrophobic region of the liposome bilayer, and the second, TMA-DPH, was anchored on the surface of the liposome bilayer and exposed to a hydrophilic environment due to its positively charged amino groups. The excitation and emission wavelengths were 340 nm and 428 nm, respectively. The slit width of the excitation monochromator was 3 nm for both labels, whereas the slit width of the emission monochromator was 2.5 nm for DPH and 3.5 nm for TMA-DPH [32–34]. The temperature of 37 °C was stable and controlled during all experiments. All dendrimers were dissolved in 10 mM Hepes with or without NaCl buffers, pH 7.4, and added to the sample to reach the appropriate concentration. The liposome concentration was 200 μM.

The polarization values (r) of the samples were calculated by the fluorescence data manager program (FluorEssence software) using the following equation:

$$r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH})$$

where I_{VV} and I_{VH} are the vertical and horizontal fluorescence intensities, respectively, to the vertical polarization of the excitation light beam. The factor $G = I_{HV}/I_{HH}$ (grating factor) corrects the wavelength response to the polarization of the emission optics and detectors.

2.5. Differential scanning calorimetry

The differential scanning calorimetry (DSC) method was used for the study of dendrimer interactions with both types of neutral and negatively charged liposomes. Liposome/dendrimer buffered suspension samples were loaded in the measurement cell of a DSC III microcalorimeter (Setaram, France). Buffer was used as a reference for the reference cell. The typical lipid concentration was 2.3 mM, and a 10:1 lipid: dendrimer molar ratio was used. For both types of liposomes, two heating/cooling cycles at a range of 5–45 °C were performed at a 0.5 °C/min scan rate [28,35].

2.6. NMR diffusion experiments

To probe a possible interaction between the DPMG and the dendrimer, pulsed-field gradient (PFG) NMR [36] has been performed. Details of the experimental set-up are described in former studies [37,38]. Experiments had been optimized to observe the diffusion of the surfactant with a gradient duration of 0.7 ms, a diffusion time of 7 ms 64 gradient steps between 0.2 T/m and 7 T/m. This optimization step is forced by the concentration of DMPG below the critical micelle concentration (DMPG: 0.0044 mM (cmc = 0.011 mM); DS PPI: 0.022 mM). The low concentration solution of DMPG in PFG NMR experiments provides a low sensitivity over the experimental setup.

2.7. Statistical analysis

Statistical analysis and exponential curve fitting were performed using Statistica software. Results are expressed as mean ± standard error of the mean (SEM).

3. Results

3.1. Size and zeta potential

Hydrodynamic diameters and the zeta potential of nanoparticles were measured to characterize dendrimers, model membranes and their interaction. The results show the differences between both types of used dendrimers. The PPI DS dendrimer was slightly negatively charged and has a diameter 2 nm larger than the PPI OS dendrimer. The zeta potential value measured for the open shell PPI dendrimer reflected the positively charged surface of this molecule. All data are shown in Table 1.

Liposomes consisting of a single dimyristoylphosphatidylcholine (DMPC) or binary mixtures of DMPC and 3% dimyristoylphosphatidylglycerol (DMPG) were used. The only difference between those two types of lipids is the structure of the polar part. Both are made of a nitrogenous base bonded with a choline (DMPC) or glycerol (DMPG) unit. The addition of DMPG was done to achieve a negative charge on the liposome surface. As was expected, the resulting zeta potential value of these two types of liposomes was different. In the case of the DMPC liposome, the value was −2.0 mV, whereas liposomes made of the lipid mixture DMPC/DMPG had a zeta potential with a very low negative value (Table 1). The size of both types of liposomes was similar, but the hydrodynamic diameter of negatively charged liposomes was approximately 17 nm larger.

During the experiments with titration liposomes by dendrimers, there were no hydrodynamic diameter changes for all neutral (DMPC) liposomes and negatively charged liposomes (DMPC/DMPG) with a dense shell dendrimer (Table 2). The increase in the diameter was only observed when model membranes were negatively charged and titrated by positively charged open shell dendrimers. There were differences between samples suspended in a buffer with or without the addition of NaCl. The aggregation process happened much slower in a buffer with salt.

3.2. Fluorescent anisotropy measurements

The thermodynamic properties of the DMPC and DMPG lipids are similar, so the structure of a bilayer made of the DMPC/DMPG mixture would be the same as for pure DMPC liposomes at a temperature above the transition point. Fluorescent anisotropy experiments were carried out at 37 °C, a temperature above the main transition points for both lipids, so that the structure of the bilayer was in a liquid crystal phase for all used types of liposomes.

Unmodified PPI G4 and both types of maltose modified PPI dendrimers significantly influence the values of fluorescence anisotropy of the probes present in all types of liposomes (DMPC as well as the mixture of DMPC/DMPG). The fact that liposomal membranes become more rigid in the presence of dendrimers is reflected by the increase in the fluorescence anisotropy value (Figs. 2, 3 and 4). The strength of interaction was the lowest for unmodified PPI (Fig. 2).

Experiments were carried out in two types of environments in a buffer with or without the 150 mM concentration of NaCl. Changes in anisotropy were observed for all variants used during the experiments. By using two types of fluorescent probes, it was possible to monitor interactions with dendrimers between external and internal regions of the membrane. In all cases, dendrimers influenced both the hydrophobic and the hydrophilic part of the DMPC bilayers, while fluidity decreased. The significant changes in anisotropy were observed for neutral liposomes above 10 μM in the dendrimer concentration (Figs. 3A, B and 4A, B). The influence of the PPI DS (Fig. 4) dendrimers on neutral and negatively charged liposomes was stronger than that

Table 1
Characteristics of the dendrimers and liposomes used in the experiments.

	Hydrodynamic diameter d_h [nm]	Zeta potential [mV]	Polydispersity index (PdI)
Dendrimers			
PPI OS	3 ± 1	4.0 ± 0.7	0.381
PPI DS	5 ± 1	−5.2 ± 1.1	0.373
PPI-G4	3 ± 2	24.0 ± 0.5	0.210
Liposomes			
DMPC	120 ± 2	−2.0 ± 3.0	0.063
DMPC/DMPG	137 ± 3	−23.4 ± 1.3	0.040

Table 2
Changes in hydrodynamic diameter during liposome titration with dendrimers.

Dendrimer concentration [μM]	0	0.5	5	10	20	30	60
Hydrodynamic diameter [nm]							
DMPC + OS	120 ± 2	121 ± 1	121 ± 1	121 ± 1	121 ± 1	120 ± 1	121 ± 1
DMPC + OS[a]	118 ± 1	119 ± 2	120 ± 1	120 ± 1	121 ± 1	119 ± 1	122 ± 1
DMPC + DS	120 ± 2	120 ± 1	121 ± 1	121 ± 2	121 ± 2	121 ± 2	121 ± 3
DMPC + DS[a]	118 ± 1	119 ± 1	120 ± 1	121 ± 1	119 ± 1	120 ± 2	121 ± 1
DMPC + PPI	122 ± 1	126 ± 1	126 ± 2	128 ± 1	128 ± 2	127 ± 1	127 ± 2
DMPC + PPI[a]	118 ± 2	123 ± 2	124 ± 1	126 ± 3	125 ± 1	127 ± 2	128 ± 2
DMPC/DMPG + OS	137 ± 3	145 ± 6	166 ± 5	281 ± 57	260 ± 43	252 ± 48	1306 ± 172
DMPC/DMPG + OS[a]	132 ± 2	131 ± 2	136 ± 5	136 ± 10	134 ± 5	135 ± 7	148 ± 17
DMPC/DMPG + DS	137 ± 3	136 ± 3	135 ± 3	135 ± 2	134 ± 2	134 ± 1	132 ± 1
DMPC/DMPG + DS[a]	132 ± 2	133 ± 1	134 ± 2	135 ± 2	133 ± 1	135 ± 2	134 ± 2
DMPC/DMPG + PPI	136 ± 1	164 ± 8	275 ± 42	685 ± 71	1120 ± 129	-	-
DMPC/DMPG + PPI[a]	131 ± 2	130 ± 1	132 ± 2	132 ± 1	133 ± 2	136 ± 2	138 ± 1

[a] Samples in a buffer with 150 mM NaCl.

of the PPI OS (Fig. 3) and unmodified PPI (Fig. 2) dendrimers. PPI DS dendrimers made the structure of the bilayer more rigid. The PPI OS dendrimer had a strong impact on the hydrophilic part of negatively charged liposomes. Anisotropic changes in the DMPC/DMPG liposomes due to interaction with the PPI OS and PPI dendrimers were made only to the 60 μM (PPI OS) or 20 μM (PPI) dendrimer concentrations because of the precipitation that occurred in the sample. All types of dendrimers affected the lipid order packing of DMPC and DMPC/DMPG liposomes, but the fluidity changes were weaker when the experiments were carried out in a buffer with 150 mM NaCl. These observations shed light on the mechanism of lipid–dendrimer interactions and were revealed using the DSC technique.

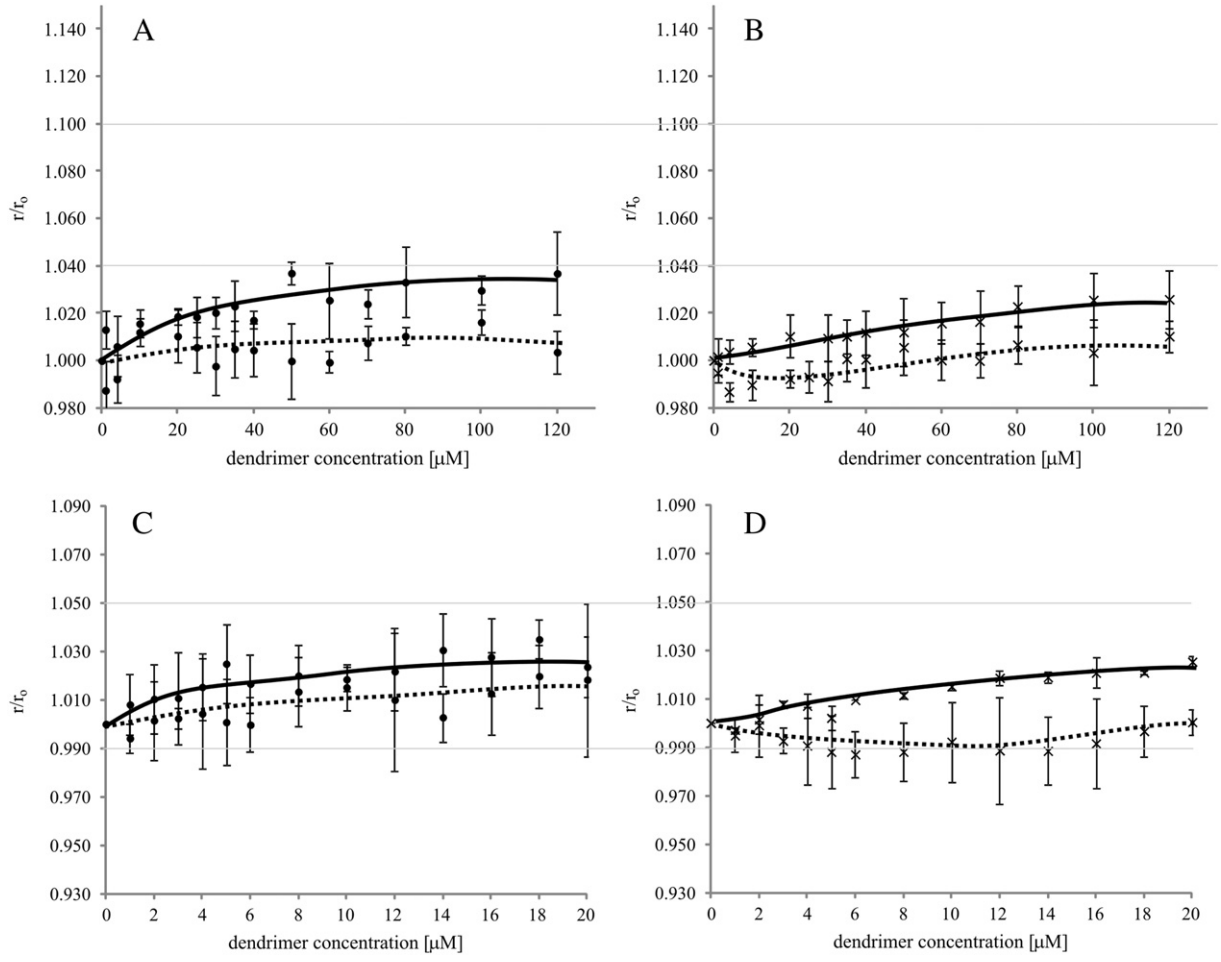

Fig. 2. Fluorescence anisotropy of DPH (A, C) or TMA-DPH (B, D) probes in DMPC (A, B) or DMPC/DMPG (C, D) liposome/PPI dendrimer mixtures. –, buffer without NaCl; …, buffer with 150 mM NaCl; r, sample fluorescence anisotropy value; r_0, control fluorescence anisotropy value in the absence of dendrimers. Values of control fluorescence anisotropy: A – 0.07358 ± 0.0005; A_{NaCl} – 0.0741 ± 0.0011; B – 0.1884 ± 0.0013; B_{NaCl} – 0.1836 ± 0.0027; C – 0.0725 ± 0.0021; C_{NaCl} – 0.0735 ± 0.0024; D – 0.1742 ± 0.0028; D_{NaCl} – 0.1823 ± 0.0010. All data are expressed as the mean ± SEM. $n = 3$; $p < 0.05$ for each point vs. control.

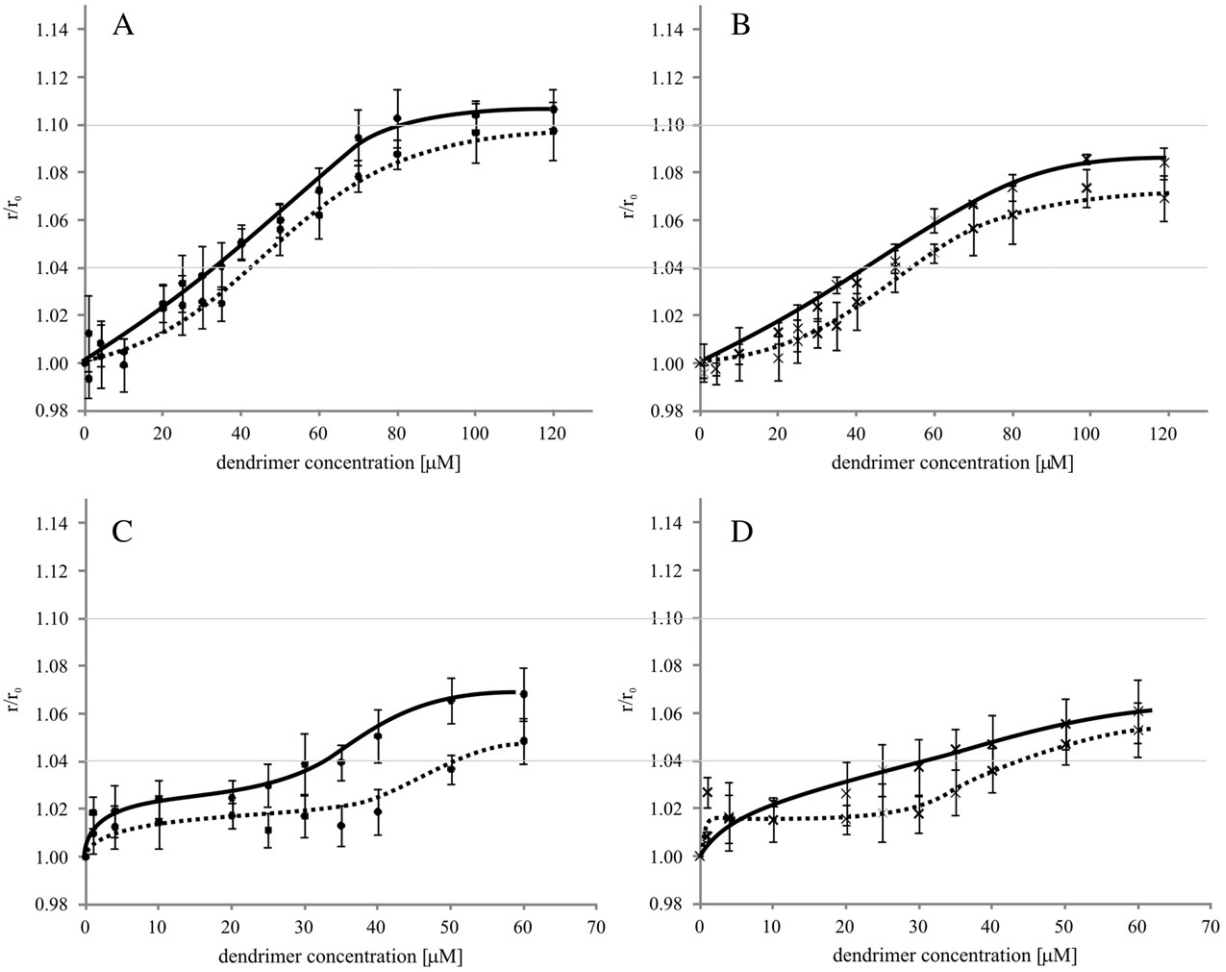

Fig. 3. Fluorescence anisotropy of DPH (A, C) or TMA-DPH (B, D) probes in DMPC (A, B) or DMPC/DMPG (C, D) liposome/PPI OS dendrimer mixtures. –, buffer without NaCl; …, buffer with 150 mM NaCl; r, sample fluorescence anisotropy value; r_0, control fluorescence anisotropy value in the absence of dendrimers. Values of control fluorescence anisotropy: A – 0.0645 ± 0.0007; A_{NaCl} – 0.0620 ± 0.0012; B – 0.1634 ± 0.0024; B_{NaCl} – 0.1643 ± 0.0010; C – 0.0633 ± 0.0025; C_{NaCl} – 0.0705 ± 0.0029; D – 0.1634 ± 0.0016; D_{NaCl} – 0.1632 ± 0.0042. All data are expressed as the mean ± SEM. $n = 3$; $p < 0.05$ for each point vs. control.

3.3. Differential scanning calorimetry

The increasing temperature allows lipids to change their mesomorphic phases from the gel phase to the liquid-crystalline phase. At temperatures above the transition point, lipid molecules have higher energy and move faster and more easily. Two peaks can usually be observed in the thermograms. The first one, which appears at a lower temperature, is connected to pretransitions, and the other one, which appears at a higher temperature, is a main transition point. For lipids used in the experiment, the pretransition point should be observed at temperatures near 13 °C (DMPC) and 12 °C (DMPG), and the main transition should be observed at 24 °C (DMPC) and 23 °C (DMPG).

Fig. 5 shows a series of heating scans for pure DMPC (curve A) and the DMPC/DMPG mixture (curves B and C). The thermotropic parameters of thermograms are given in Table 3. It was not possible to observe a good curve for the pretransition point for all samples. The main transition for DMPC occurred at 24.40 °C, and the addition of DMPG to the mixture slightly increased the lipids' transition temperature to 24.50 °C. Experiments with additional NaCl were carried out only for DMPC/DMPG liposomes because more significant changes could be observed in the thermodynamic parameters. In the presence of 150 mM of NaCl, the main transition temperature of DMPC/DMPG increased by 0.1 °C. The enthalpy values for all samples did not change.

The interactions between DMPC or DMPC/DMPG liposomes and unmodified PPI G4 (Fig. 6), PPI G4 DS (Fig. 7) and PPI G4 OS (Fig. 8) dendrimers were examined. The same lipid:dendrimer ratio of 10:1 was investigated in all experiments. Isotherms of interactions between liposomes and PPI OS dendrimers are shown in Fig. 6. The main transition temperature of the lipid increased after the dendrimer addition. Those changes are observed for all types of liposomes used in the experiments. The comparison of transition temperatures between samples with and without NaCl showed that the temperatures of transition points had lower values when the environment of interaction was enriched with salt (Table 3). An interesting phenomenon occurred during the experiment with DMPC/DMPG liposomes and PPI OS dendrimers in 10 mM Hepes. The change in the shape of the transition curve can be related with a phase separation in the beginning of the phase changes. This phenomenon was not noticed in the experiment with the buffer that consisted of 10 mM Hepes with 150 mM NaCl.

A series of heating scans for liposomes and their interactions with PPI DS dendrimers are shown in Fig. 7. As it was for the open shell dendrimer, the main transition temperature of the lipid increased after the dendrimer addition. As before, when the environment of interaction was enriched with salt, the transition temperature decreased. The temperature of the liposome transition was higher when vesicles interacted with the PPI DS dendrimer (Table 3). The dense shell dendrimer changed the temperature of transition more than the open shell dendrimer. The temperature of the main transition point increased more in the case of the dense shell dendrimer.

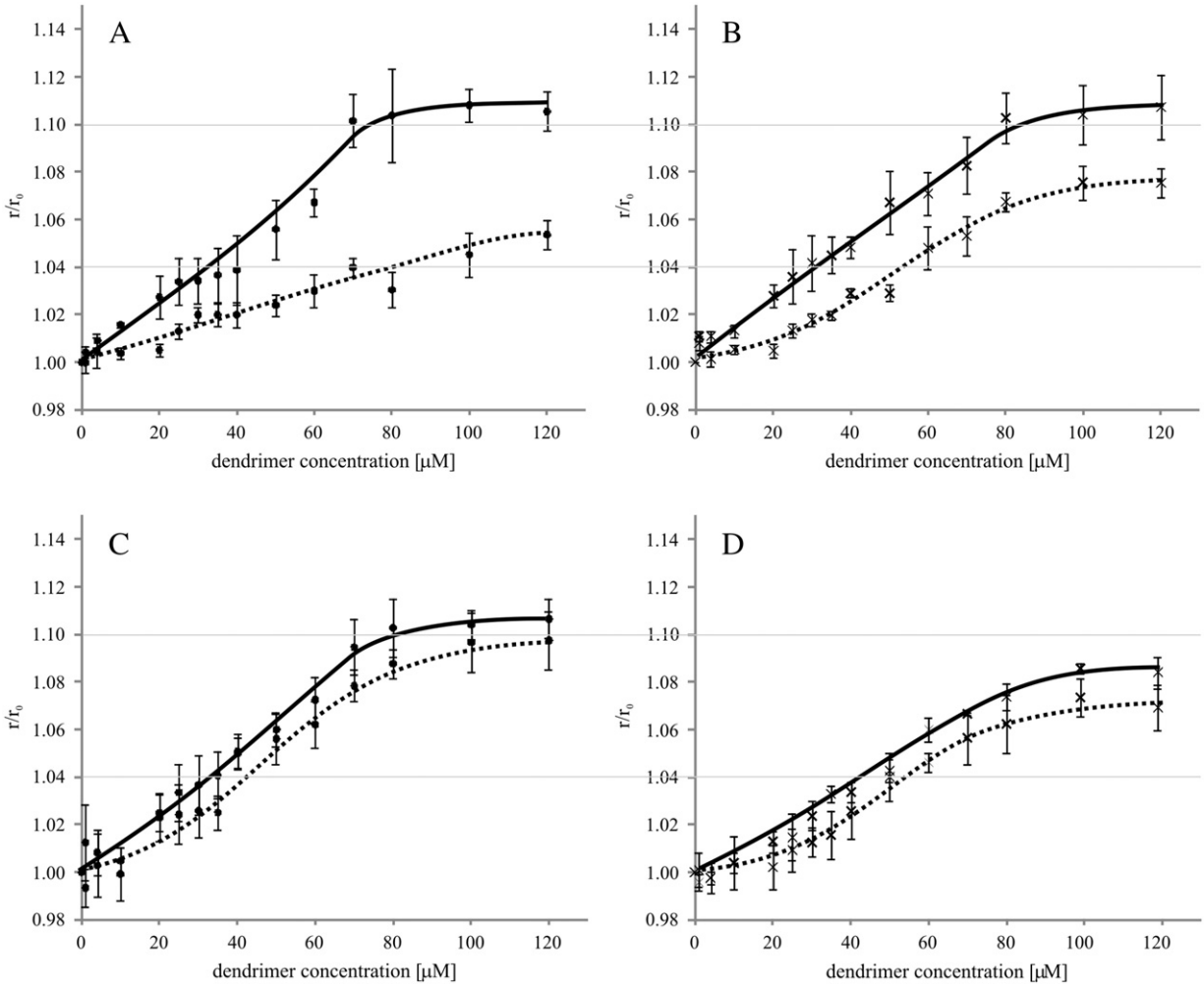

Fig. 4. Fluorescence anisotropy of DPH (A, C) or TMA-DPH (B, D) probes in DMPC (A, B) or DMPC/DMPG (C, D) liposome/PPI DS dendrimer mixtures.–, buffer without NaCl; …, buffer with 150 mM NaCl; r, sample fluorescence anisotropy value; r_0, control fluorescence anisotropy value in the absence of dendrimers. Values of control fluorescence anisotropy: A – 0.0645 ± 0.0007; A_{NaCl} – 0.0620 ± 0.0012; B – 0.1634 ± 0.0024; B_{NaCl} – 0.1643 ± 0.0010; C – 0.0633 ± 0.0025; C_{NaCl} – 0.0705 ± 0.0029; D – 0.1634 ± 0.0016; D_{NaCl} – 0.1632 ± 0.0042. All data are expressed as the mean ± SEM. $n = 3$; $p < 0.05$ for each point vs. control.

3.4. NMR diffusion experiments

Interaction of anionic DMPG against neutral dense shell glycodendrimer was investigated by using several NMR methods and concentration of DMPG below critical micelle concentration (cmc). The use of DMPG below cmc is directed to following point: does the non-assembled anionic phospholipid DMPG undergo any non-covalent interactions with DS PPI? The reason for this is that postulated H-bond-driven interactions between anionic vesicles and neutral DS PPI cannot be studied directly by any NMR methods. This is explainable by the huge difference in molecular weight of large phospholipid vesicles against smaller DS PPI macromolecules which exhibits low intensity of NMR signals for smaller DS PPI macromolecules. With this pre-consideration in mind it is reasonable to perform NMR interaction study by down-sizing the huge mass difference in this manner to use non-assembled phospholipid DMPG below cmc and DS PPI. Thus, only pulsed-field gradient (PFG) NMR experiments were the only choice to obtain some information about the interaction properties between non-assembled phospholipid DMPG molecules and excess DS PPI macromolecules.

Results from PFG NMR experiments are presented in Figs. 9 and 10. While the diffusion coefficient of DS PPI in a separate experiment is $5.5 * 10^{-11}$ m^2/s, for the phospholipid DMPG the same mono-exponential decay is observable with a diffusion coefficient between $3.5 * 10^{-10}$ m^2/s and $3.9 * 10^{-10}$ m^2/s in the absence and presence of DS PPI (Fig. 9). This clearly implies that no non-covalently-driven interactions (H-bonds and/or ionic interaction) exist between isolated, non-assembled anionic phospholipid DMPG and neutral DS PPI. In opposite to this weak interaction of anionic ANS against neutral DS PPI is determinable by PFG NMR experiments under comparable conditions (Fig. 10). This is in accordance with previously reported results that a low complexation capacity of DS PPI against anionic ANS exists [8]. Overall, the sensitivity is low because of using cmc of DMPG (DMPG: 0.0044 mM in D_2O (cmc = 0.011 mM); DS PPI: 0.022 mM).

4. Discussion

The common problems with substances that have potential use as new biopharmaceutics are their insolubility in aqueous solutions, cytotoxicity and sensitivity to environment factors such as light, enzymes or pH. It is not surprising that significant effort has been spent to create molecular systems that will be able to not only protect and make new biopharmaceutics more biocompatible but also transport them to the target location and release them in a time-dependent manner. Promising molecules in this area are dendrimers. Perfectly branched structures and easy surface chemical modifications predispose them as highly biocompatible materials that can become very efficient drugs or nucleic acid carriers. However, many reports are available describing the

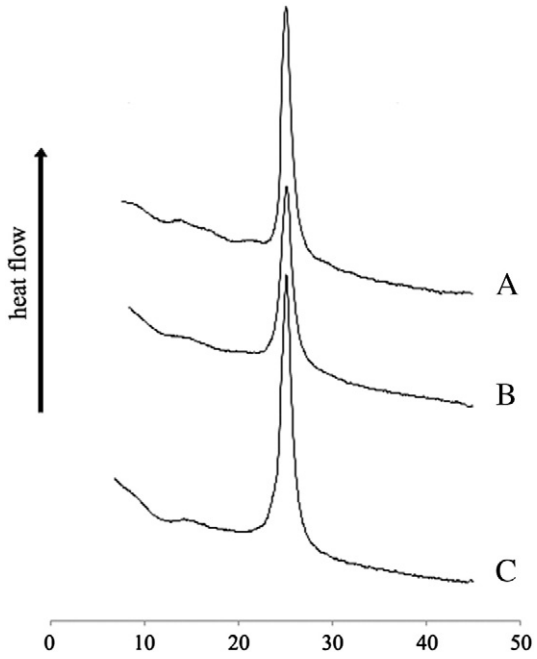

Fig. 5. DSC heating scans of model DMPC (A – in 10 mM Hepes) or DMPC/DMPG (B – in 10 mM Hepes, C – 10 mM Hepes + 150 mM NaCl) liposomes.

relationship between the number of positive groups and toxic properties of these molecules. Apparently, increasing the number of positively charged groups on dendrimers increases their toxicity [8,9,18,19].

Several possible models of the interactions between dendrimers and model membranes have already been proposed. Positively charged dendrimers are highly toxic to the cells as a result of increasing permeability and destroy the integrity of the cell membrane [8,9,18]. The destabilization of the lipidic part of biological membranes has been investigated in several models of liposomes. Some dendrimers are able to remove lipids and create nanoholes in the structure of the lipid bilayer [39–41]. It has been shown that dendrimers change the properties of the lipid bilayer not only on the surface but also in the hydrophobic region [28,29,42,43]. Several authors have also focused their attention on the role of functional groups and the size of molecules in the investigated interactions [26,44]. The number of surface groups, their charge and the shapes of molecules affect the strength of interaction. Higher generations of dendrimers affect the structure of the lipid bilayer triggered by number of cationic surface groups [27,45,46].

Table 3
DSC parameters of the DMPC and DMPC/DMPG liposome interaction with 4th-generation maltose-modified PPI dendrimers.

		10 mM Hepes		10 mM Hepes + 150 mM NaCl	
		T_m [°C]	H_n/H_c	T_m [°C]	H_n/H_c
Pure Lipids	DMPC	24.40	1.00	–	–
	DMPC/DMPG	24.50	1.00	24.61	1.00
PPI	DMPC	24.33	0.98	24.43	1.02
PPI	DMPC/DMPG	24.59	0.96	24.47	0.97
PPI OS	DMPC	24.54	1.03	–	–
	DMPC/DMPG	24.80	0.95	24.65	0.97
PPI DS	DMPC	24.63	1.00	–	–
	DMPC/DMPG	24.97	0.99	24.81	0.92

Calorimetric parameters: T_m — temperature at which heat capacity at constant pressure is maximum; H_n/H_c — ratio between transition enthalpies of sample with dendrimer (H_n) and plane lipids (H_c).

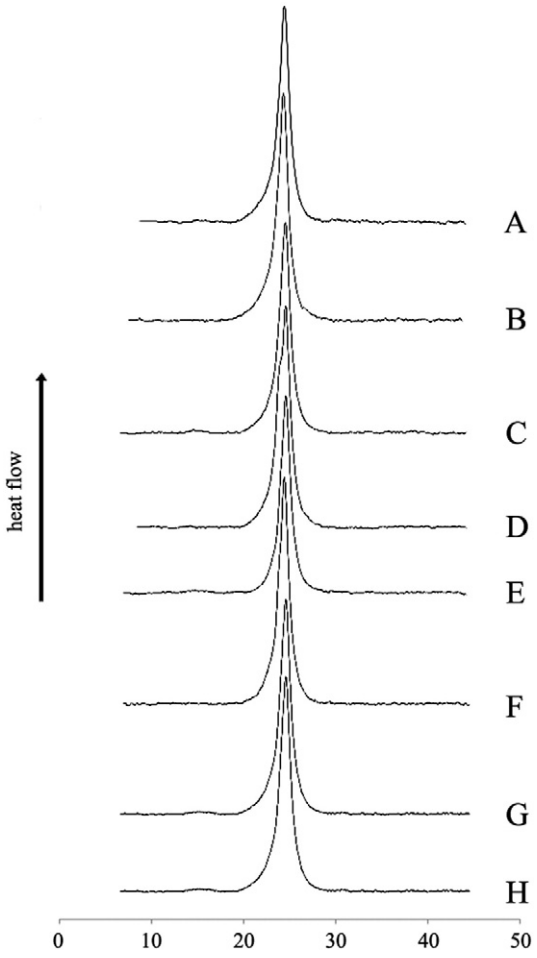

Fig. 6. DSC heating scans of DMPC (A, B, E, F) or DMPC/DMPG (C, D, G, H) liposomes in 10 mM Hepes (A, B, C, D) and in 10 mM Hepes with 150 mM NaCl (E, F,G, H) in the presence of unmodified PPI dendrimer generation 4 (B, D, F, H). The molar ratio lipid: dendrimer was 10:1.

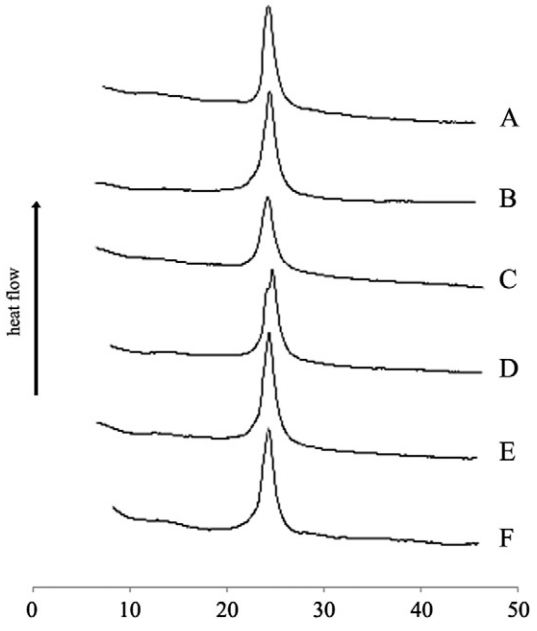

Fig. 7. DSC heating scans of DMPC (A, B) or DMPC/DMPG (C, D, E, F) liposomes in 10 mM Hepes (A, B, C, D) and in 10 mM Hepes with 150 mM NaCl (E, F) in the presence of PPI OS dendrimer generation 4 (B, D, F). The molar ratio lipid:dendrimer was 10:1.

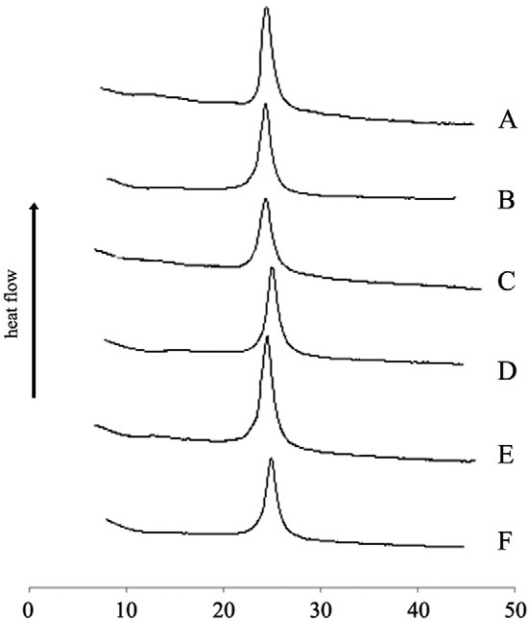

Fig. 8. DSC heating scans of DMPC (A, B) or DMPC/DMPG (C, D, E, F) liposomes in 10 mM Hepes (A, B, C, D) and in 10 mM Hepes with 150 mM NaCl (E, F) in the presence of PPI DS dendrimer generation 4 (B, D, F). The molar ratio lipid:dendrimer was 10:1.

In the present work, we used cationic unmodified PPI dendrimer and cationic open shell PPI glycodendrimer, but also a neutral dense shell glycodendrimer to investigate their interaction with model lipid membranes. PPI dendrimers without any modifications possess amine surface groups, which are cationic at a neutral pH. Because of their positive charge, they are very toxic for cells and are not suitable for biomedical applications without additional surface modifications. Reducing the amount of surface charge decreases their toxicity but may also decrease the affinity to the membrane as well. The neutral and slightly positively charged glycodendrimers exhibit differences in their interactions with neutral DMPC and negatively charged DMPC/DMPG liposomes in the presence and absence of NaCl. The various experiments should reflect the influence of sodium chloride at physiological concentrations on investigated systems.

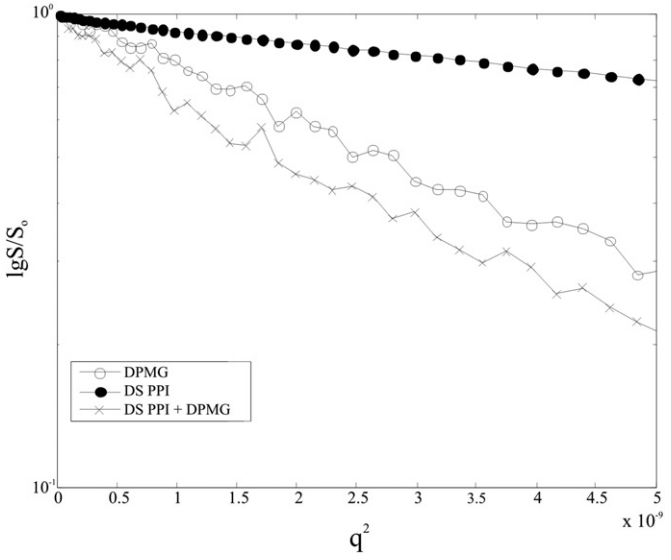

Fig. 9. Decay of diffusion for phospholipid DMPG (Fig. 1c) in the presence and absence of glycodendrimer DS PPI presented by the Steijskal–Tanner plot in solvent D_2O.

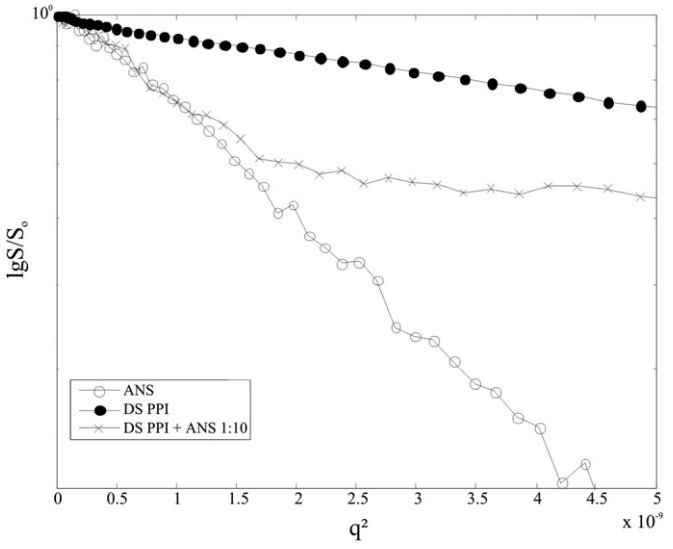

Fig. 10. Decay of diffusion for the dye ANS (Fig. 1c) in the presence and absence of glycodendrimer DS PPI presented by the Steijskal–Tanner plot in solvent D_2O.

Thus, the number of modified surface groups determines the surface charge of dendrimers, and as a consequence, unmodified PPI and PPI OS dendrimers were positively charged, while PPI DS dendrimers were slightly negatively charged in Hepes buffer at pH 7.4. In this context, previous studies have shown that PPI DS dendrimers also exhibit negative zeta potential in PBS buffer at pH 7.4, while the charge density of PPI DS dendrimers, determined by polyelectrolyte titration experiments, is neutral [8] when the real parameter of charged and non-charged macromolecules is considered. This means that the surface charge determined by zeta potential measurements has some limitations in its priority when macromolecules are additionally functionalized with neutral molecules, as occurred here in this study. On the other hand, there is a good correlation between surface charge, determined by zeta potential, and charge density, determined by polyelectrolyte titration experiments, when evaluating the (surface) charge of parental PPI dendrimers [8]. Modifications changed not only the charge of molecules but also the molecular weight and size. The PPI OS dendrimer has the half of the molecular mass of the PPI DS dendrimer, and similarly, the hydrodynamic diameter is much lower compared to PPI DS [PPI $d_h = 3 \pm 2$ nm; PPI OS $d_h = 3 \pm 1$ nm; PPI DS $d_h = 5 \pm 1$ nm (Table 1)].

Light scattering experiments evaluated the interaction between negatively charged model membranes and positively charged open shell glycodendrimer and pure PPI dendrimer (Table 2). In those samples, the aggregation process was observed, and the increasing concentration of both dendrimers caused the growth of the aggregates. Even at low 0.5 μM PPI OS dendrimer concentration influences the diameter of negatively charged liposomes, and the size changes from 137 nm to 147 nm. The highest concentration of dendrimer used was 60 μM (PPI OS) and 20 μM (PPI) and it was also the highest concentration for the spectrofluorimetry measurements. Above this concentration, no measurements were performed because of the large aggregates that occurred in the sample. Changes in the liposomes' diameter suggest an aggregation process between negatively charged liposomes and positively charged dendrimers based on electrostatic interactions. Those results were not surprising because similar observations have been made for positively charged phosphorus-containing dendrimers [28,47] as well as for cationic carbosilane dendrimers [29]. The authors suggested that the mechanism of interaction was mainly based on the electrostatic binding between dendrimers and lipid vesicles.

Considering the interaction properties of dense shell glycodendrimer with liposomes observed by DLS study (Table 2), it is well known that the composition of the environment can influence the shape and dimension of liposomes [48–51]. This is in accordance

with our DLS results where sizes of liposomes become a bit smaller in the presence of NaCl. The influence of NaCl presence on a hydrodynamic diameter of liposomes is not very significant for neutral DMPC liposomes, but it is more pronounced for negatively charged DMPC/DMPG liposomes. Thus, anionic liposomes in the absence of NaCl are approximately 5 nm larger than those prepared in a salty environment (Table 2). Similar to other interaction studies of dendrimer against liposomes [28, 45, 46], we observed that the presence of various PPIs in solution influences the hydrodynamic diameter of liposomes. Hydrodynamic diameter of neutral DMPC liposomes increases after the addition of pure PPI to the liposome solution (Table 2), while no (pure Hepes buffer) or nominal (Hepes buffer with NaCl) increase of neutral liposomes' diameter in the presence of OS PPI and DS PPI is recognizable. This can be explained by the interaction of PPIs with the surface of lipid bilayers as observed also by other techniques (change of membrane fluidity). Here, the hydrodynamic diameter of neutral liposomes is (slightly) increased probably due to captured dendrimers protruding out of the lipid bilayer. Contrary to this, the hydrodynamic diameter of anionic DMPC/DMPG liposomes in 10 mM Hepes buffer decreases in a concentration dependent manner when DS PPI was added to the solution, reaching at highest 60 μM concentration of DS PPI. In this context a similar value of hydrodynamic diameter of anionic liposome in 10 mM Hepes buffer with 150 mM NaCl is available. A closer view on lipid membrane properties from literature [51] reveals following point that increasing concentration of monovalent ions (as Na^+, Cl^-) can induce changes of lipid membrane properties (e.g., dehydration, change of polarization, lipid diffusion or phase separation). Such structural changes can mirror in the change of hydrodynamic diameter of liposomes [51]. This is mainly present in the case of negatively charged DMPC/DMPG liposomes where significantly smaller diameter of liposomes prepared in a buffer with 150 mM NaCl was observed as compared to liposomes prepared in NaCl-free buffer. Similarly to this, we hypothesize that the observed decrease of anionic DMPC/DMPG liposome diameters in the presence of DS PPI in Hepes buffer without NaCl can be related to the outlined processes of membrane structural changes [51] due to the local presence of (few) dendrimers on liposomes' surface. This local interaction on liposomes' surface is also responsible for the changes in membrane fluidity observed (Figs. 2 and 3). Instead of growing size dimension for describing the interaction as observed between cationic OS PPI and anionic liposomes it is possible to detect decrease of liposomes' diameter in the presence of neutral DS PPI. Overall the DLS study revealed that ionic interactions exist between cationic OS PPI and anionic liposomes for all experimental conditions. Molecular interactions of neutral DS PPI, preferentially triggered by H-bonds, can be postulated in the presence of anionic liposomes, while molecular interactions for OS PPI and DS PPI in the presence of neutral liposomes are only marginally recognizable in Hepes buffer with NaCl.

When other methods (fluorescence and DSC) have been used, stronger indications are given that open and dense shell PPI dendrimers are involved in the interactions with neutral and negatively charged liposomes as well. This observation leads to the conclusion that the interactions were not related only to dendrimer charge. Moreover, the results indicate that the DS dendrimer induced higher perturbations in the lipid bilayers than the OS and unmodified dendrimer at the same concentration. This was observed with both measurement methods used (DSC and spectrofluorimetry). Stronger perturbations shown by DS PPI can be influenced by the higher surface group density of the PPI DS dendrimer where the peripheral amino groups of PPI core are preferentially modified with maltose units in DS PPI dendrimer. Our results suggested that a macromolecule with a higher surface group density produces a higher perturbation when it interacts with the lipid bilayer. It has been shown that maltose-modified dendrimers have less flexible structures than unmodified ones [13], so the number of maltose groups influences not only the physico-chemical (charge change) but also the physical properties (size, molecular weight and molecule flexibility). The observed differences in the interactions might be due to the different numbers of maltose groups attached to the dendrimer surface. Because the OS PPI dendrimer has fewer maltose groups, its structure could be more flexible that hampers an aimed interactions against neutral and anionic liposomes. This also means that it is easier for the membrane to keep its original properties. These data suggested that the nature of interactions is mostly entropic and depends on the exclusion volume of the dendrimers. PPI that is 100% maltose modified has a larger diameter than OS PPI and produces more significant changes in the structure of the lipid bilayer. It can be clearly seen that the PPI DS dendrimer could interact even more through non-covalent interactions (H-bonds) than the slightly positively charged OS PPI dendrimer through not only hydrogen bonds but also ionic interactions. The influence of ionic interactions can also be decreased by the backfolding properties of maltose units in open shell glycodendrimers [13]. Similar behavior has already been observed for phosphorus-containing dendrimers. Those cationic macromolecules interacted with neutral DMPC or DPPC liposomes according to the temperature and the concentration of dendrimers inside the sample. The strength of interaction depended on the generation of the dendrimer as well as on the thermodynamic state of the lipid bilayer. The higher generation dendrimer caused larger disturbances. Interactions did not occur when experiments were carried out below the main transition temperature of lipids [28]. The mechanism of interaction through the hydrogen bonding of maltose-modified PPI dendrimers has already been observed. Klajnert et al. have shown that maltose-modified dendrimers can efficiently interact with proteins (human serum albumin) as non-modified PPI dendrimers by nonspecific hydrogen bonding [8]. During our experiments, precipitation occurred when the DMPC/DMPG liposomes were incubated with a high concentration (60 μM) of PPI OS and (20 μM) PPI dendrimers. This leads to the conclusion that the ionic interaction also appears between positively charged dendrimers and negatively charged liposomes. Our results suggested that these interactions are less important in the process of membrane penetration by both dendrimers. Finally, results from DSC and fluorescence study confirm our working hypothesis concluded from DLS that marginal changes in size dimension give first indication on weakly non-covalently driven interactions of neutral dense shell glycodendrimers against neutral and anionic liposomes.

Explanation for the marginal changes in size dimension, especially, in the case of DS PPI determined by DLS study can be as postulated in former interaction studies between dense shell glycodendrimers and various charged proteins [8,20,21]. H-bond-driven interaction of DS PPI on the surface of liposomes is only available when *larger contact surfaces* are present for the interaction between DS PPI and neutral or anionic liposomes. This implies that for the non-covalently driven interaction between two differently large entities the smaller DS PPI has to adapt to the larger molecular surface of liposomes, fabricated by assembled neutral or neutral/anionic phospholipids. This possible molecular interaction process is reasonable where DS PPI can preferentially undergo H-bond-driven interaction with the H-bond-active surface of neutral and anionic liposomes. H-bond-active surface of liposomes is provided by the presence of anionic phosphate groups (from phospholipid DMPG and DMPC), hydroxy groups (from anionic DMPG) and cationic amino groups (from neutral DMPC). The molecular adaptation of liposome's surface by DS PPI is one requirement to undergo multiple H-bond interactions with liposome's surface. Consequently, it results in the highest perturbation of membrane fluidity compared to the molecular interaction of OS PPI against liposomes, while the ionic interaction properties of cationic OS PPI may play a minor role in this multiple interaction process with liposome surface to disturb the fluidity of membranes in liposomes. Moreover, results from PFG NMR experiments support our working hypothesis of multiple H-bonds between DS PPI and liposome surface that non-assembled DMPG molecules below cmc are not able to undergo any non-covalently driven interaction with DS PPI. This impressively implies that the individual anionic and H-bond-active head group in DMPG molecules does not allow any defined non-covalent

interaction with the maltose shell in DS PPI and with the cationic PPI core [8,15] in DS PPI. It also exhibits that individual anionic DMPG molecules cannot penetrate dense shell to undergo ionic interaction with cationic core as found in PFG NMR experiment for anionic ANS molecules. Finally, it is also noteworthy to mention that generation G4 used in DS PPI outlines a much stronger capacity on H-bond active groups in the outer shell as available in the case of G3. This discrepancy was nicely shown in former studies where a higher amount of G3 dense shell PPI glycodendrimer is required compared to those of G4 to suppress the formation of fibrils fabricated by amyloidogenic peptides [15, cited reference in 15]. Therefore, only the largest generation, G4, within the PPI dendrimer family was used in our study to guarantee the high capacity on H-bond-active surface in dense shell glycodendrimers for the interaction with H-bond-active liposome's surface.

Another aspect revealed during the experiments was the influence of the physiological concentration of sodium chloride on dendrimer–lipid interactions. It has been revealed that the addition of cations to the environment surrounding the liposomes influences their properties. The highest membrane fluidity was observed without ions, whereas the supplementation of ions reduced membrane flexibility. This observation was associated with the type of ion, its presence in the interference region of the bilayer structure and the dehydration of the outer region of the bilayer structure. It has been reported by others that sodium chloride reduced membrane fluidity more than potassium or calcium ions and caused a high amount of lipid dehydration [52]. Our experiments were conducted in environments with and without the addition of NaCl to investigate the influence of salt on dendrimer/lipid bilayer interactions. For all experiments and methods used, it was observed that the addition of NaCl to the experimental environment decreased the strength of interactions between dendrimers and liposomes. We hypothesize that the membrane became more rigid because of the influence of NaCl on the bilayer structure and less accessible for interaction with other compounds, such as dendrimers.

5. Conclusions

The influence of G4 PPI dendrimers with primary amino surface groups partially (open shell glycodendrimers – OS) or completely (dense shell glycodendrimers – DS) modified with maltose residues on DMPC or DMPC/DMPG liposomes was studied. Both types of dendrimers showed interactions with neutral as well as with negatively charged liposomes. The results suggest that the interactions were mostly related to hydrogen bonding between surface grafted maltose and lipid molecules and partially related to ionic interactions but more investigation in this area is necessary. This situation resulted in the phenomenon where neutral DS PPI dendrimers interacted more strongly with model lipidic membranes than with cationic OS dendrimers. The nature of interactions seems to be mostly entropic and depends on the exclusion volume of the dendrimers. At the same concentration, the DS dendrimer induced higher perturbations in both types of lipid bilayers than the OS dendrimer. The addition of NaCl to the experiment environment reduced the strength of interactions between dendrimers and liposomes. It can be hypothesized that maltose-modified dendrimers are able to interact with model membranes irrespectively of the surface charge by hydrogen bonding. The main factors involved in the investigated interactions were the concentration, size and density of surface groups of the dendrimer molecule. The properties of these maltose-modified PPI dendrimers highlight their potential use as a new type of drug delivery system.

The results presented in this article lay the foundation for future investigations that will help to understand the interaction between PPI glycodendrimers and liposomes.

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