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DSC studies on interactions between low molecular mass peptide dendrimers and model lipid membranes

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Abstract

It has recently been shown that a newly synthesized peptide dendrimers possess antimicrobial activity against Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria as well as against fungal pathogens (*Candida albicans*) [Klajnert, B., Janiszewska, J., Urbanczyk-Lipkowska, Z., Bryszewska, M., Shcharbin, D., Labieniec, M., 2006. Biological properties of low molecular mass peptide dendrimers. Int. J. Pharm. 309, 208–217]. To extend our knowledge about their impact on biological systems, interactions between a group of low molecular mass lysine based dendrimers and model lipid bilayers were examined by differential scanning calorimetry (DSC). Conformational stability of dendrimers in 5–85 °C temperature range was confirmed by circular dichroism measurements (CD). The dendrimer structure has been shown to play an important role in interactions with the membranes. A two-step mechanism of dendrimer–bilayer interactions was proposed. The first step involves electrostatic attractions between dendrimers and polar lipid heads, while the second one is a result of hydrophobic interactions between acyl chains and arms of dendrimers. While one dendrimer did not interact with the membrane, another with long hydrophobic arms significantly perturbed the membrane. Nevertheless, for all tested dendrimers the main transition in DSC scans was retained that indicates that these compounds at the tested concentrations did not cause the loss of membrane integrity.

Keywords: Dendrimer; Peptide; DSC; CD; Liposome; Vesicle; MLV; Bilayer

1. Introduction

Generally, it is established that the structure of dendrimers consists of a core and several layers of branches, enabling location of a well-defined number of active elements on the surface. However, in the case of peptide dendrimers, it is based on a branched structure that resembles a tree. Lysine is the most common amino acid branching unit in peptide dendrimer chemistry (Kim et al., 1999; Sadler and Tam, 2002). Peptide dendrimers have recently attracted a considerable interest due to their potential biomedical applications. Poly(L-lysine) dendrimers can serve as carriers of genetic materials during transfection (Männistö et al., 2002; Ohsaki et al., 2002). Peptide dendrimers, as other dendrimers, can be applied as drug transport systems by attaching active compounds to their functional groups (Chen and Cooper, 2002). Antiviral and antimicrobial properties are also interesting features of peptide dendrimers. Poly(L-lysine) dendrimers have been found to block herpes simplex virus attachment to a cell membrane (Bourne et al., 2000). Our compounds have recently been shown to possess an antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli* and antifungal activity against *Candida albicans* (Janiszewska et al., 2003; Klajnert et al., 2006). This property may be due to the branched structure that not only ensures the multiplicity of active elements, but it also provides a spatial distribution of cationic and aromatic groups that is essential for interactions with bacterial membranes.

The possibility of using peptide dendrimers in medicine is a motivation for basic scientific investigations of their properties. It is obvious that if a clinical application of these compounds

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Table 1
Characteristics of dendrimers

Dendrimer	Structure	Molecular weight (Da)
R-121	Z-Lys(HCl) | Z-Lys(HCl)-Lys-TyrNH$_2$	832.4
R-124	Z-Lys(HCl) | Z-Lys(HCl)-Orn-PheNH$_2$	802.9
R-155	HClLys(2-Cl-Z) | HClLys(2-Cl-Z)-Lys-Pro-NH-CH$_2$C$_6$H$_4$	923.8
R-169	HClLys(2-Cl-Z)-Pro | HClLys(2-Cl-Z)-Pro-Lys-NH-CH$_2$C$_6$H$_4$	1022.9

is considered, the lack of systemic toxicity is as important as a broad spectrum of antimicrobial activity. Therefore, we previously studied cytotoxicity, hematotoxicity and genotoxicity of the peptide dendrimers (Klajnert et al., 2006). Studies on interactions between dendrimers and erythrocytes showed that the tested compounds caused membrane alterations. To continue these studies and to understand the nature of interactions, we employed liposomes as models of biological membranes. We used four structurally different peptide dendrimers shown in Table 1: R-121, R-124, R-155, and R-169 to determine how their topology determines interactions with liposomes. Structure of the studied dendrimers is characterized by two symmetrical branches of variable lengths terminated with aromatic residues, and one aromatic side group (Tyr, Phe or benzylamine) located at the C-end of the lysine core. Two of the tested dendrimers (R-155 and R-169) previously showed a strong hemolytic effect and the rest did not cause a loss of the membrane integrity.

2. Materials and methods

2.1. Materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-*sn*-glycero-3-phospho-1-glycerol (DMPG) were obtained from Avanti Polar Lipids (Alabaster, AL) and used without further purification. PIPES buffer (pH 8.0 and 6.5) used to hydrate films contained: 20 mM Pipes, 0.14 M NaCl, 1 mM EDTA and 20 mg/L NaN_3. All of these chemicals were of analytical grade. Water used to prepare solutions was double-distilled and deionised.

2.2. Dendrimer synthesis

All tested dendrimers were synthesised using either Boc- or Fmoc-chemistry in the solid phase, as described previously (Klajnert et al., 2006). The peptides were converted into an amide form by treating with ammonia in MeOH overnight. Crude peptide dendrimers were purified using gel filtration on Sephadex LH-20 in methanol, followed by preparative HPLC in a water/ethyl acetate system. The fraction containing pure peptide was lyophilized twice and purity was confirmed by mass spectrometry. All dendrimers were confirmed to have the correct molecular weight, structure, and purity by an analytical HPLC and ESI mass spectrometry, analyzing both the molecular peak and fragmentation ions.

2.3. Preparation of MLVs

The appropriate amounts of lipids were dissolved in chloroform–methanol (2:1, v/v). The solvent was evaporated under a stream of nitrogen and then samples were placed under vacuum for 2 h to remove any traces of residual solvent. PIPES buffer was then added to hydrate the dry film, giving a final lipid concentration of 2.7 mM. Multilamellar vesicles (MLVs) were obtained by shaking a tube on a vortex mixer for approximately 2 min (sufficient time for the lipid on the wall to become suspended in buffer) and then the sample was subjected to five freeze-thaw cycles in which dispersions were frozen in liquid nitrogen and then thawed at 50 °C in a water bath. Between the each cycle the sample was vortexed for 15 s at a temperature above the main transition temperature of the pure lipid.

After obtaining multilamellar liposomes, a solution of dendrimer in buffer was added and the sample was vortexed to ensure mixing.

2.4. DSC measurements

All samples were degassed before the measurement. The DSC measurements were performed using MicroCal VP-DSC calorimeter. A 0.5 ml liposome suspension was placed in the sample cell. All thermograms were run using the same volume of PIPES buffer as a reference. Samples were heated and cooled repeatedly five or six times at a rate 30°/h between 15 and 65 °C in the case of DPPC MLVs, and between 5 and 45 °C in the case of DMPC and DMPG MLVs. There was a delay of 5 min between sequential scans to allow for thermal equilibration.

2.5. Circular dichroism spectroscopy

The CD spectra of dendrimers at 25 and 65 °C were recorded using a AVIV Model 215 instrument equipped with a programmable thermoelectrically controlled sample compartment. The dendrimer solution at a concentration of 0.5 mg/ml in PIPES was contained in a 0.5 mm path-length quartz cell. The thermal stability of dendrimers was measured by heating and cooling samples at a rate 0.5 °C/min. The CD signal at 220 nm for R-124, R-155 and R-169 and at 226 nm for R-121 was recorded at 3 °C intervals.

3. Results

Differential scanning calorimetry (DSC) was used to study the thermotropic behavior of DPPC multilamellar vesicles (MLVs) upon addition of dendrimers. Dendrimers were added to liposomes at two different concentrations, 1 and 10 mol%, after liposomes were formed. Therefore, dendrimers could only

Table 2
Calorimetric data for DPPC MLVs obtained upon addition of dendrimers after five cycles of heating and cooling (values correspond to equilibrium state)

	Heating						Cooling					
	Main transition			Pretransition			Main transition			Pretransition		
	ΔH (kcal/mol)	T_m (°C)	$T_{1/2}$ (°)	ΔH (kcal/mol)	T_m (°C)	$T_{1/2}$ (°)	ΔH (kcal/mol)	T_m (°C)	$T_{1/2}$ (°)	ΔH (kcal/mol)	T_m (°C)	$T_{1/2}$ (°)
DPPC	7.5	41.17	0.208	1.0	34.44	1.929	−6.6	40.78	0.249	−0.4	30.18	2.095
R-155 (1 mol%)	7.6	41.07	1.128	–	–	–	−7.0	40.47	0.922	–	–	–
R-155 (10 mol%)	7.8	40.42	1.666	–	–	–	−7.1	40.04	1.677	–	–	–
R-169 (1 mol%)	8.8	41.08	0.252	0.6	31.90	3.093	−8.3	40.72	0.245	−2.8	28.32	2.477
R-169 (10 mol%)	8.9	40.34	0.748	–	–	–	−9.1	39.81	0.706	–	–	–
R-124 (1 mol%)	8.5	41.09	0.251	0.8	33.84	2.138	−7.7	40.76	0.248	−5.1	29.69	2.512
R-124 (10 mol%)	9.0	40.82	0.463	–	–	–	−9.0	40.43	0.248	–	–	–
R-121 (1 mol%)	7.6	41.12	0.206	0.7	33.96	1.973	−7.2	40.76	0.249	−2.5	30.09	1.843
R-121 (10 mol%)	8.4	41.00	0.335	0.4	32.77	2.800	−8.0	40.67	0.331	−2.3	28.39	2.819

interact with the external surface of the MLV. During heating, DPPC liposomes exhibit two endothermic transitions: a broad pretransition with a low enthalpy and a sharp main transition. For both transitions an enthalpy, a maximal temperature and a width of a peak at a half-height were determined. The exothermic calorimetric plots measured during cooling cycles usually confirmed the results obtained during heating cycles but were of opposite sign. The summary of calorimetric data obtained upon the addition of dendrimers to DPPC MLVs is shown in Table 2. In pure DPPC liposomes, the values obtained for the main transition and for the pretransition were in a good agreement with those reported in the literature (Huang and Li, 1999).

For dendrimers R-121, R-124, and R-169 at concentration 1 mol%, the main phase transition was not significantly altered (Fig. 1A). In the case of R-169 there were changes in the pretransition. It was broadened, slightly shifted towards lower temperatures and its enthalpy was lower. For R-155 dendrimers alterations were the biggest and they affected both the pretransition and the main transition. The disappearance of the pretransition was accompanied by broadening of the main transition peak. When dendrimer concentration increased ten times, the loss of the pretransition was a common phenomenon observed for all dendrimers except for R-121. But even for R-121 the pretransition was smaller. The shape of the main transition peak was significantly changed in case of R-124, R-169, and R-155 and the maximum temperature was slightly shifted towards lower values (Fig. 1B). The most pronounced alterations were observed for R-155 dendrimer.

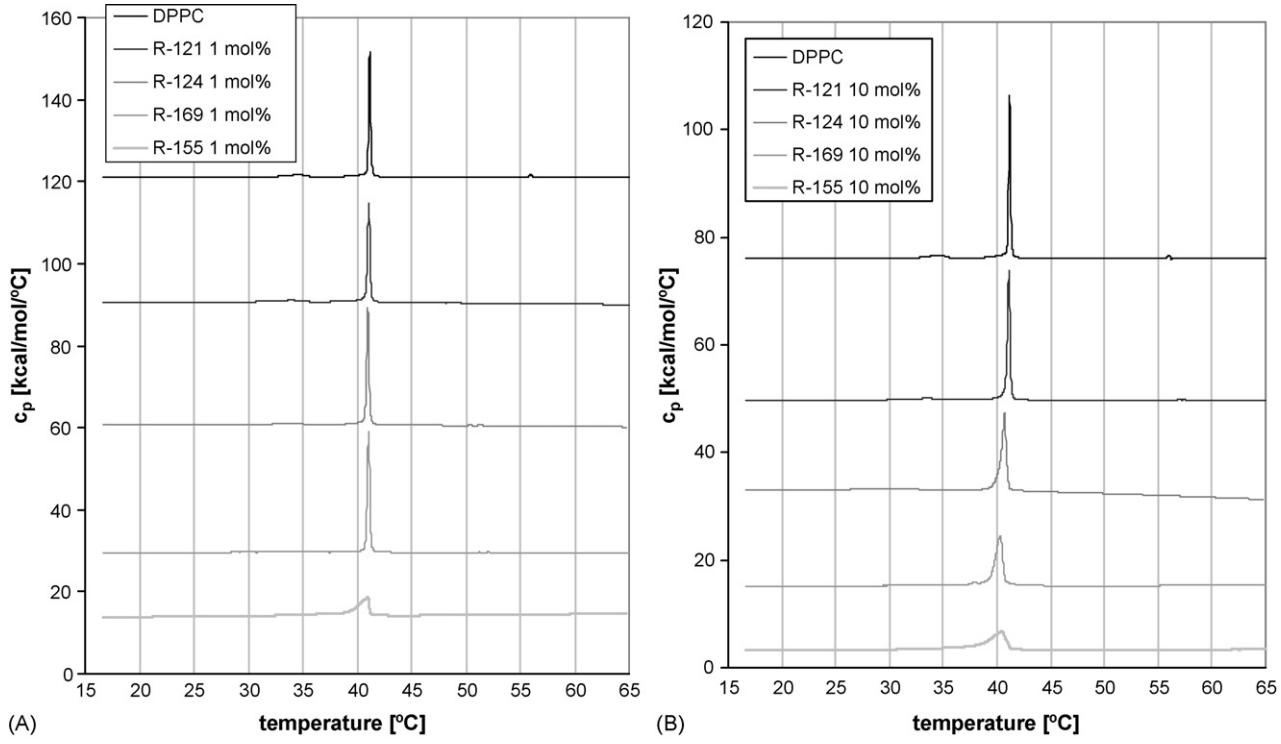

Fig. 1. DPPC MLVs scans upon the addition of 1 mol% (A) and 10 mol% (B) of dendrimer. All scans are after five cycles of heating and cooling and they correspond to equilibrium state.

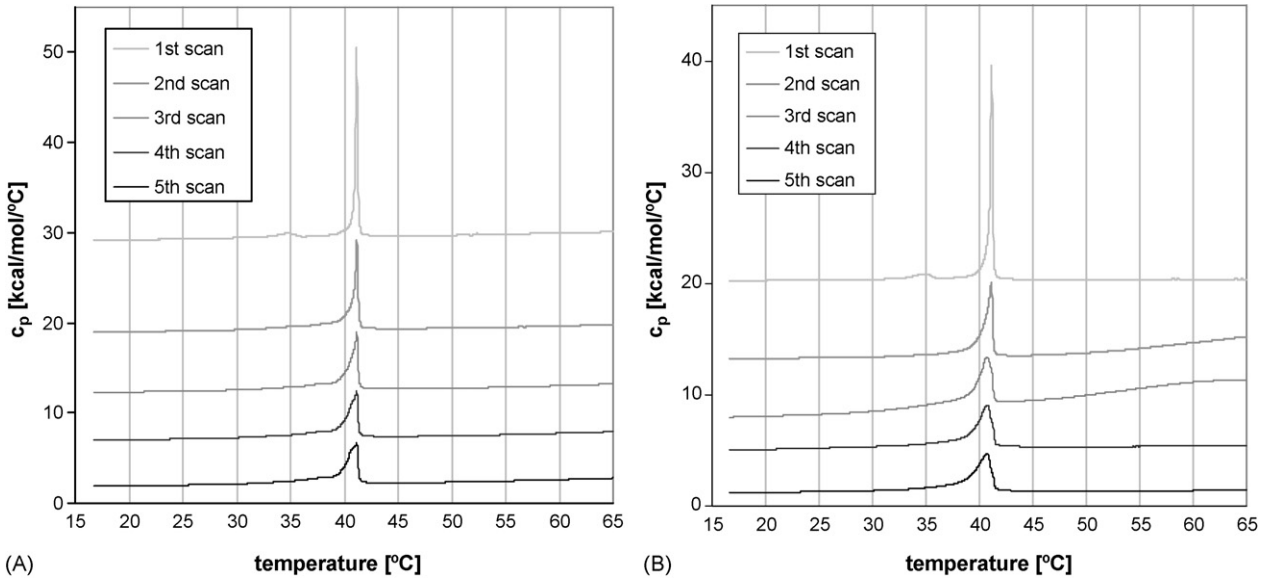

Fig. 2. Heating scans for DPPC MLVs in the presence of 1 mol% (A) and 10 mol% (B) of R-155 dendrimer.

Interestingly for R-155, regardless of concentration, we observed progressive changes after each heating and cooling cycle (Fig. 2). However, the tendency to reach equilibrium was indicated by the observation that there were no significant differences in the main transition width at half-height after the fourth heating scan (Fig. 3). For the rest of dendrimers sequential scans showed good reproducibility. To determine if heating the sample to 65 °C played an important role in this phenomenon, we studied interactions between the R-155 dendrimer and DMPC MLVs. Dipalmitoyl phosphatidylcholine (DPPC) possesses sixteen carbons in each acyl chain while dimyristoyl phosphatidylcholine (DMPC) has only fourteen. The shorter the acyl chains, the lower the temperature of the main transition. We found that the impact of R-155 dendrimer on a thermal behavior of DMPC MLVs was much smaller and at a concentration of 1 mol% even the pretransition was not lost (Fig. 4A). Moreover, we did not observe progressive changes with a scan number and an equilibrium state was reached in the second scan.

Because generally the R-155 dendrimer had the biggest impact on membrane properties, we decided to study this dendrimer in another system: with DPPG MLVs. Dimyristoyl phosphoglycerol (DMPG) similarly to dimyristoyl phos-

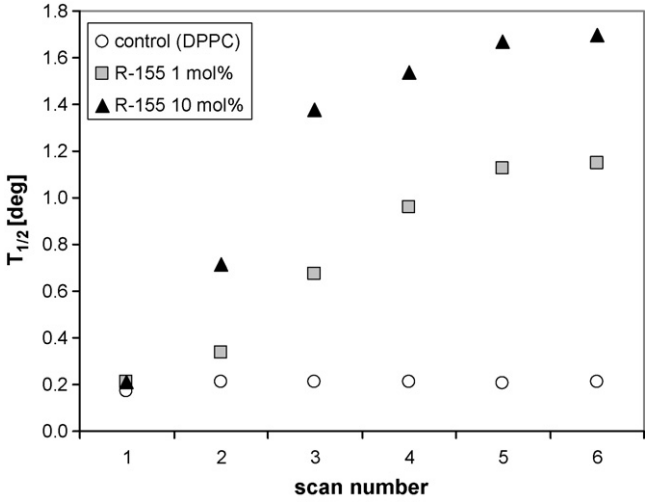

Fig. 3. Changes in the main transition width at a half-height with a heating scan number.

Table 3
Calorimetric data for DMPC and DMPG MLVs obtained upon addition of 1 mol% of R-155 dendrimer

	Heating-main transition			Heating-pretransition		
	ΔH (kcal/mol)	T_m (°C)	$T_{1/2}$ (°)	ΔH (kcal/mol)	T_m (°C)	$T_{1/2}$ (°)
DMPC						
Control	7.1	23.82	0.168	1.0	13.95	1.551
R-155 (pH 6.5)	7.5	23.73	0.209	0.8	13.16	2.052
R-155 (pH 8.0)	5.4	23.48	0.252	0.3	12.15	2.553
DMPG						
Control	6.3	22.56	0.587	0.3	10.91	2.227
R-155 (pH 6.5)	6.1	22.47	0.590	–	–	–
R-155 (pH 8.0)	5.9	22.24	0.878	–	–	–

Data were collected after five cycles of heating and cooling (values correspond to equilibrium state).

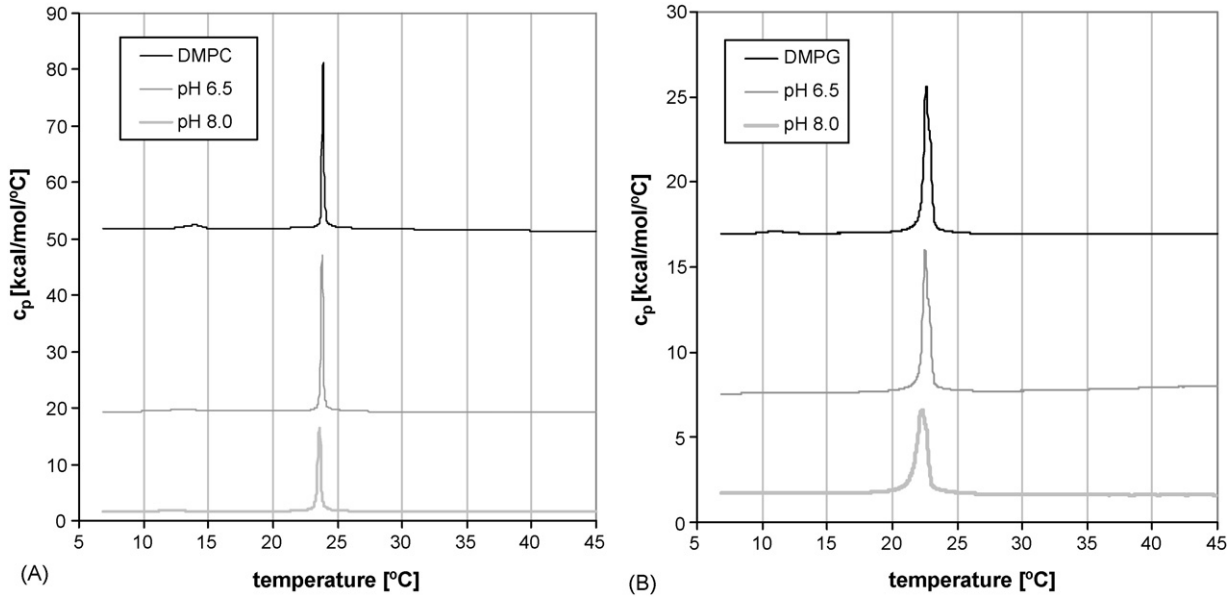

Fig. 4. DMPC (A) and DMPG (B) MLVs scans upon the addition of 1 mol% of R-155 dendrimer. All scans are after five cycles of heating and cooling and they correspond to equilibrium state.

phatidylcholine (DMPC) possesses fourteen carbons in each acyl chain but its net charge is negative, contrary to DMPC, which is a zwitterionic lipid. To estimate the importance of electrostatic interactions we conducted experiments in two different pH values: 8.0 and 6.5 and compared results to those obtained for DMPC (Table 3). The R-155 dendrimer strongly affected DMPG MLVs. A concentration of 1 mol% dendrimer was enough to diminish the pretransition. At pH 8.0 this loss was accompanied by broadening of the main transition peak (Fig. 4B). At pH 6.5 the dendrimer had less effect. This pH effect was also observed in the case of DMPC.

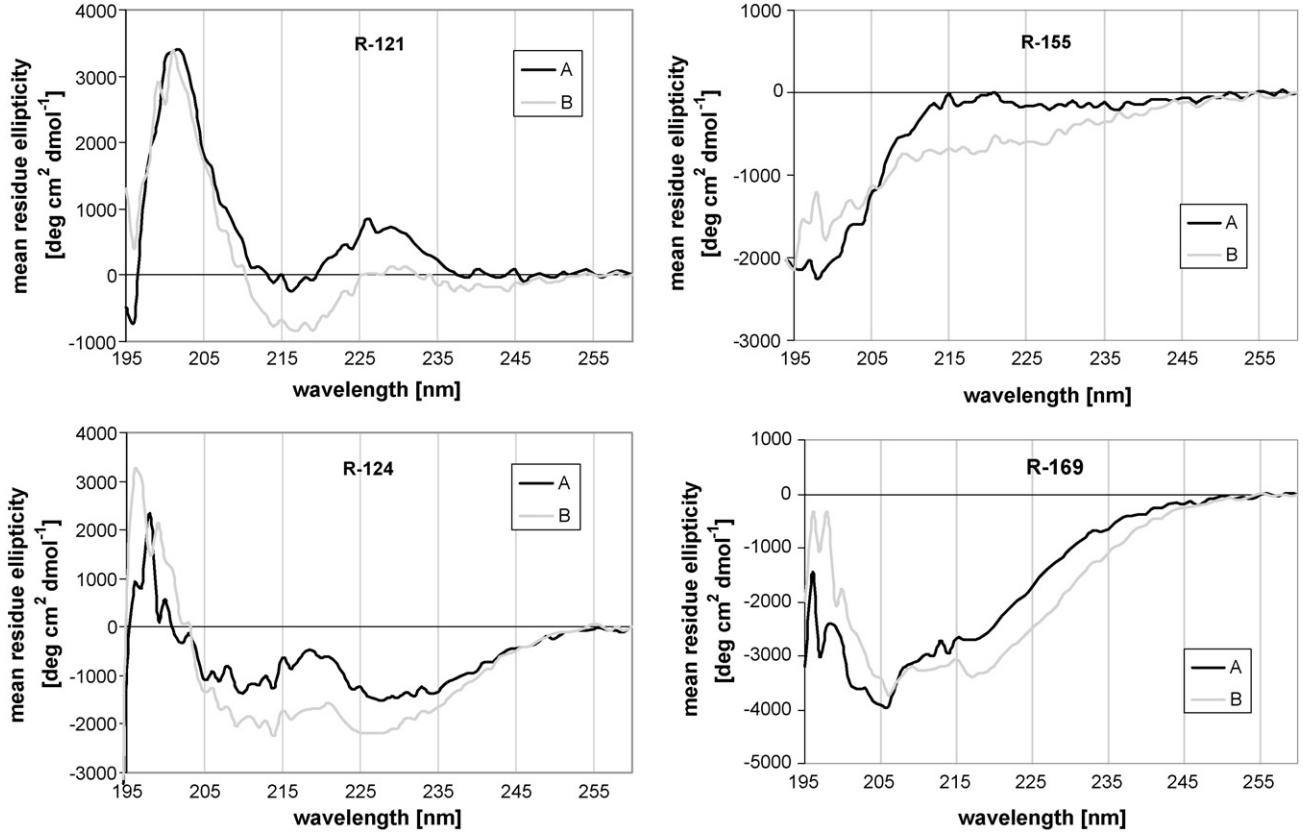

Fig. 5. CD spectra of dendrimers at 25 °C (A) and 65 °C (B).

All peptide dendrimers are characterized by a CD spectrum (Fig. 5). Because our compounds are built from 4-5 amino acids, we cannot analyze the CD spectra in terms of a secondary structure. Nevertheless, we can conclude that each dendrimer is distinguished by a different spectrum. Its shape depends on temperature, therefore we assessed a dendrimer thermal stability by monitoring a CD signal. A single wavelength was chosen (the one that corresponded to the biggest changes in a CD spectrum upon heating) and the CD signal was recorded continuously as the temperature was raised. In the same way the signal was recorded when the sample was cooled. The curves for heating and cooling almost superimposed each other (Fig. 6).

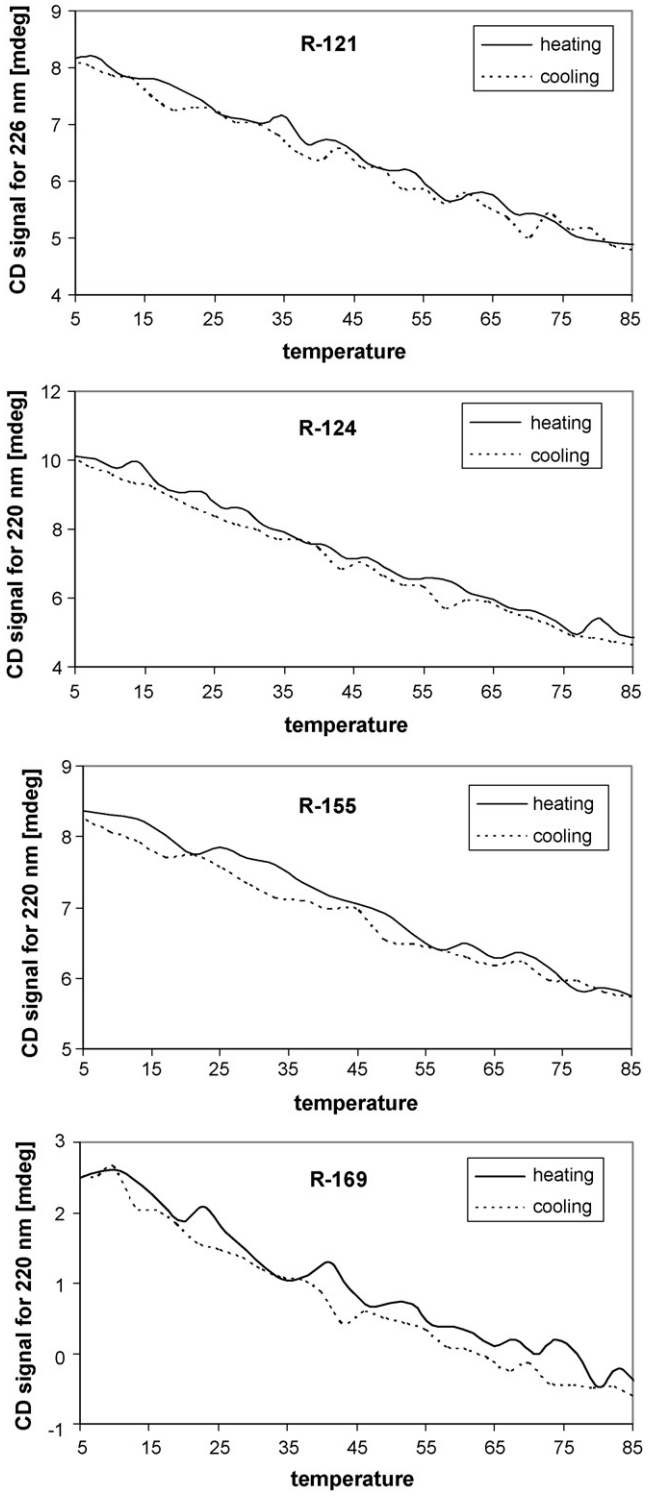

Fig. 6. Thermal stability graphs for dendrimers.

4. Discussion

Many naturally occurring or synthetic peptides interact with lipid bilayers. They can cause a variety of changes in membrane properties, such as fluidity or affect a gel–liquid crystal phase transition (Lasch et al., 1998; Bonev et al., 2000). They are even able to perforate lipid bilayers by forming membrane pores (Zemel et al., 2005). This activity is a result of hydrophobic interactions with the hydrocarbon chain region of the membrane or, when the peptides are charged, electrostatic attractions with oppositely charged lipids. Very similar behavior was found for dendrimers. Dendrimers are able to destabilize the membrane by creating small holes in a bilayer (Hong et al., 2004). The strength of the dendrimer activity depends on its size and the charge. Protonated dendrimers showed more effective dendrimer–vesicle interactions and higher generation dendrimers created more disturbances (Ottaviani et al., 1998, 1999). Peptide dendrimers can combine properties of both peptides and dendrimers. Therefore studying their interactions with membranes seems crucial, especially since they are considered as potential antimicrobial agents.

The aim of the study was to broaden our knowledge about the systemic toxicity of peptide dendrimers; that is why most studies were performed with liposomes composed of phosphatidylcholine, a lipid commonly found in mammalian plasma membranes. Based on previous studies we chose four compounds (R-121, R-124, R-155, and R-169). Two of them showed a strong hemolytic activity (R-155 and R-169), and the others (R-121 and R-124) did not cause a loss of membrane integrity (Klajnert et al., 2006).

Generally, DSC studies confirmed results obtained from hemolysis tests. R-121 dendrimer did not cause any significant changes in the thermal behavior of DPPC MLVs. The loss of the pretransition was observed upon addition of R-124 dendrimer at a concentration 10 mol%. However, it does not indicate either serious disturbances in the membrane, or a deep dendrimer penetration into the bilayer. The pretransition corresponds to the conversion of a lamellar gel phase to a rippled gel phase and is very sensitive to the presence of exogenous substances. The pretransition can disappear when only a very small amount of material is incorporated into the membrane (Bonora et al., 2002). The loss of the pretransition can be a consequence of interactions with phospholipid headgroups which lead to the increase in the spacing between them, thus eliminate the driving force for the formation of a rippled phase.

In the case the of R-169 dendrimer, increasing the concentration to 10 mol% caused the disappearance of the pretransition but it also affected the characteristics of the main transition which became broader. The main transition reflects the conversion of a gel phase to a liquid crystal phase. The width of the peak gives information about the co-operativity of this conversion. The narrower the peak, the higher the co-operativity. High co-operativity

is observed in homogenous systems, therefore the change in the width of the transition is an evident result of incorporation of dendrimers into the hydrophobic core of the bilayer.

The biggest disturbances were observed for the R-155 dendrimer which even at a low concentration of 1 mol% caused the disappearance of the pretransition and broadening of the main transition. Interestingly, only for this dendrimer we observed progressive alterations with a scan number. For R-121, R-124, and R-169 dendrimers a reproducible and reversible pattern was already established in the second scan. A similar phenomenon was earlier shown in the case of interactions between DMPC liposomes and poloxamers and between DPPC liposomes and polyamidoamine dendrimers (Castile et al., 1999; Klajnert and Epand, 2005). It seems that the several crossings of the phase transition temperature during heating and cooling, as well as incubating the sample for a while at a relatively high temperature (around 65 °C), allows greater penetration of the dendrimer into the bilayer. It is likely that when the R-155 dendrimer incorporates into the bilayer, it creates defects in the membrane structure that are enhanced during heating, enabling other R-155 molecules to also partition into the membrane (Fig. 7). To study how heating to 65 °C influenced interactions between R-155 dendrimer and the membrane, we employed another model:

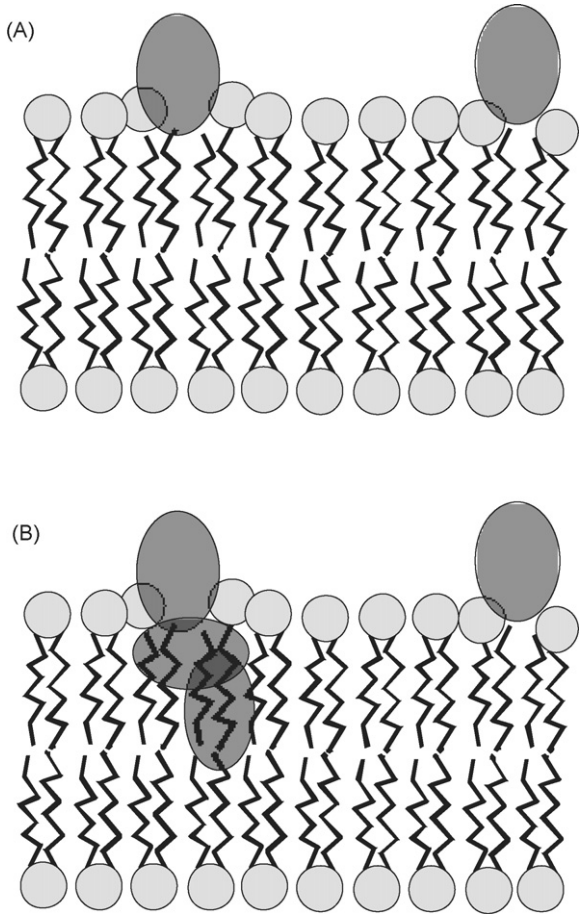

Fig. 7. A model of interactions between R-155 dendrimer and a bilayer. During first scans few dendrimer molecules interact with the membrane (A). When heating and cooling of the sample is continued next dendrimer molecules incorporate into the membrane (B).

DMPC liposomes, which are characterized by a much lower temperature of the main transition. As expected, the extent of membrane disturbances was much smaller and for a concentration of 1 mol% even the pretransition was preserved. We excluded that the observed difference was due to permanent changes in dendrimer structure upon heating to 65 °C, since CD experiments showed that all dendrimers were stable at high temperatures and changes in a dendrimer structure upon heating were completely reversible under applied conditions. However, transient changes in the dendrimer structure upon heating could influence the depth of dendrimer incorporation into the bilayer. It is possible that the dendrimer molecule becomes more flexible at higher temperatures and this would facilitate their insertion. The second probable explanation is related to the difference between DPPC and DMPC. It is likely that longer acyl chains can better accommodate dendrimers. It was previously shown that the incorporation of peptides into the bilayer was dependent on the acyl chain length of the phospholipid (Aranda et al., 2003). Peptides were better incorporated into distearoyl phospatidylcholine (DSPC) or dipalmitoyl phospatidylcholine (DPPC) than into dimyristoyl phosphatidylcholine (DMPC). It means that a peptide could be better accommodated into the longer C-16 and C-18 chains than in C-14 chains.

In order to investigate the influence of electrostatic effects on the interactions of R-155 dendrimers with lipid membranes we performed experiments on DMPG liposomes. As expected, interactions with anionic DMPG liposomes were stronger than with DMPC liposomes. Although attractions between polar head groups and cationic dendrimers may play some role, they cannot explain differences observed for different compounds since all dendrimers have a similar number of protonated lysine amino groups, and therefore a similar charge. The explanation could be found in structural dissimilarities. It was previously shown that the secondary structure of peptides is one of the determinants of the strength of their interactions with membranes, e.g. beta-sheet peptides were less disruptive than their alpha-helical counterparts, particularly towards membranes containing phosphatidylcholine (Jin et al., 2005). In the case of our dendrimers, the secondary structure is not known, but CD spectra of dendrimers showed that there are structural differences among them. It allows us to conclude that the structure determines the effect of dendrimers on the membrane and this effect follows the order: R-121 > R-124 > R-169 > R-155.

A simplified mechanism of dendrimer–bilayer interactions can be regarded as two-step process: step 1 involves electrostatic interactions between positively charged $–NH_3^+$ groups of lysine and negatively charged or polar head groups located on the hydrated surface of bilayer. Step 2 involves entropy assisted hydrophobic interactions between terminal aromatic rings and intra-layer aliphatic chains. All dendrimers possess +2 charge located at the protonated α- or ε-amine groups of the first generation lysines. Several amide groups located around the lysine core supports docking of the dendrimer molecules to the bilayer via hydrogen bond formation. Compensation of unfavorable interactions between hydrophilic surface and hydrophobic/aromatic terminal side groups of the dendrimer, can proceed by insertion of these residues into mobile structure of the bilayer. At

this point the length of lipid chain and character of dendrimer's arms is essential. Among the four dendrimers, R-155 and R-169 having long arms terminated with 2-chloro-benzyloxycarbonyl residues of approximately seem to have right structure for diffusion inside the bilayer. The observation that R-155 significantly reduces the pretransition state is in accord with its larger flexibility. The diffusion process is enhanced by raising temperature, when molecules constituting the membrane and dendrimers have more degrees of freedom.

Finally, it is worth emphasizing that although we observed membrane disturbances (especially for R-169 and R-155 dendrimers), for all compounds the main phase transition was retained, indicating that dendrimers at the tested concentrations did not cause the loss of the membrane integrity.

Acknowledgement

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