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A DSC and Raman spectroscopy study on the effect of PAMAM dendrimer on DPPC model lipid membranes

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Abstract

The interaction between PAMAM (polyamidoamine) dendrimer generation 4 (G4) and 3,5 (G3,5) with model lipid membranes composed of dipalmytoylphosphatidylcholine (DPPC) has been investigated. Differential scanning calorimetry (DSC) and Raman spectroscopy were applied to assess the thermodynamic changes caused by PAMAM G4 and G3,5 and to specify the exact location of these dendrimers into the DPPC lipid bilayer. DSC thermograms indicated that the maximum percentages of PAMAM G4 and of G3,5 that can be incorporated in the DPPC membrane without deranging its integrity were 5% and 3%, respectively. The Raman intensity ratios $I_{2935/2880}$, $I_{2844/2880}$ and $I_{1090/1130}$ cm^{-1} showed the degree of the fluidity of the lipid bilayer, while the absorption at 715 cm^{-1} showed a strong interaction of PAMAM G4 and G3,5 with the polar head group of phospholipid. The results showed that the incorporation of the PAMAM G4 and G3,5 dendrimers in DPPC bilayers causes a concentration dependent increase of the membrane fluidity and that the bilayers interact strongly with both the lipophilic part and the polar head group of the phospholipids. Due to the current weak knowledge relating to the mechanism(s) under which dendrimers interact with lipidic membranes and transport through cells, these results may justify the tendency of dendrimers to disrupt biological membranes. The findings from this study could also prove helpful to rationally design new liposomal drug carriers for bioactive molecules by combining dendrimeric and liposomal technologies.

Keywords: Lipid membrane; Dendrimer; PAMAM; DSC; Raman spectroscopy

1. Introduction

Drug delivery systems are of major importance and their successful applicability is based on their efficiency to alter the pharmakokinetic properties and tissue distribution of bioactive molecules. A large number of drugs have not been effective due to their inability to reach to the target tissue. Diseases like cancer, need effective drug carriers which can retain their physicochemical characteristics within the biological media. It is obvious that the design and development of drug carriers is a difficult issue because they have to behave as biocolloidal systems after administration. Liposomes and recently dendrimers are considered as effective drug delivery systems (Jevprasesphant et al., 2004). Liposomes are non-toxic carrier system mainly for intravenous delivery of drugs and they can modulate their in vivo behavior (Allen and Stuart, 1999; Drummond et al., 1999). Dendrimers, are considered as highly branched macromolecules; they are small in size, while their low polydispersity can contribute to the reproducibility of their pharmacokinetic behavior (Cloninger, 2002; Aulenta et al., 2003). An ideal dendrimer as drug delivery system must be non-toxic, non-immunogenic and biodegradable (Aulenta et al., 2003). The first complete dendrimer family which has been synthesized, characterized and commercialized is the Poly(amidoamine) (PAMAM) dendrimers. They are characterized as safe and non-immunogenic and they are used in drug delivery, delivery of antisense nucleotides and gene therapy, both in vitro and in vivo (Eichman et al., 2001).

The use of dendrimers to increase entrapment of drug in the liposomes and to modulate their release seems promising, as it is possible to modulate the release of drug from the dendrimeric liposomal formulations (Khopade et al., 2002). The development of a Modulatory Liposomal Controlled Release System

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(MLCRS) based on a combination of liposomes and PAMAM dendrimer attached to liposomes has recently been published (Papagiannaros et al., 2005). It can modulate the release of drugs from dendrimeric liposomal formulations while the final liposomal formulation consisting of liposomes and a drug–dendrimer complex could be able to increase the therapeutic index of the incorporated drug and reduce its cytotoxicity (Papagiannaros et al., 2005).

Differential scanning calorimetry (DSC) has been proved as a valuable tool for studying the interaction of bioactive compounds with model lipid bilayers such as DPPC (Mavromoustakos et al., 1996). It has been considered as a sensitive tool in the exploration of the thermodynamic lipid phase transition. DSC measures thermal changes on the lipid bilayers and has been extensively used in studies of the molecular interactions of additives with model lipid bilayers (Hata et al., 2000; Bae et al., 1989; Ambrosini et al., 1998). Changes on thermotropic properties [(ΔH (transition enthalpy), T_m (transition temperature), $T_{1/2}$ (temperature at which the transition is half completed)], of lipid bilayers could be used for designing liposomes (Matsingou et al., 2005) and for evaluating the percentage of bioactive compounds, which can be incorporated in liposomes composed of DPPC. Raman spectroscopy helps to specify the location of the bioactive compound in the DPPC lipid bilayer (Gardikis et al., 2006). Taking into account the bibliography (Hata et al., 2000; Bae et al., 1989; Ambrosini et al., 1998) concerning the interactions of additives with model lipid bilayers, we can assume that the incorporation of molecules, such as dendrimers, into the lipid bilayers could provoke changes on their thermotropic behavior as well as of their conformation properties. These effects were taking into account in the design of liposomal formulations (conventional or MLCRS liposomal formulations) as drug controlled release delivery systems.

In this work we studied the effect of PAMAM G4 and G3,5 dendrimers on lipid bilayers composed of DPPC using DSC and Raman spectroscopy in order to contribute to the knowledge of designing new drug delivery systems that consist of dendrimers incorporating bioactive molecules attached to liposomes. The interaction between DPPC lipid bilayers and PAMAM G4 and G3,5 dendrimers, is of interest in the understanding of the interaction of dendrimers with native cell membranes. According to the recent literature (Jevprasesphant et al., 2004), dendrimers have been shown to cross cell barriers by endocytosis, and our results complement these studies in terms of the dendrimer concentration which should be available to pass through and not to disrupt the biological membrane.

This is the first report combining DSC and Raman spectroscopy studies on dendrimer–DPPC lipid membranes interaction and the results could also provide useful information for liposomal formulation of anticancer molecules by combining dendrimeric and liposomal technologies (Papagiannaros et al., 2005).

2. Materials and methods

PAMAM G4 and G3,5 dendrimers were purchased from Aldrich. The dendrimers' solutions were 10% w/w in methyl alcohol and were dried under reduced pressure. Then they were dissolved in pure methyl alcohol in order to obtain 10% w/v solutions. DPPC was purchased from Avanti Polar Lipids. Mixtures of DPPC and PAMAM G4 and G3,5 dendrimers, were prepared at 3%, 5%, 10%, 15%, 20% and 30% molar ratio. The mixture was dissolved in pure chloroform and dried under vacuum. HPLC grade deionized water filtered through Millipore Filters (pore size 200 nm) was added (1:2 w/w) in the dried mixtures.

2.1. Differential scanning calorimetry

Samples containing mixtures of DPPC and PAMAM G4 and G3,5 dendrimers at 3%, 5%, 10%, 15%, 20% and 30% molar ratio, were prepared in 40 μl aluminium crucibles hermetically sealed with lid. Prior to DSC scanning, the samples underwent a quick heating and a quick cooling cycle (scanning rate of 10 and 20 °C/min, respectively) to ensure equilibration and exemption of the thermal history of the specimens. All samples were scanned three to four times from 10 to 60 °C until identical thermograms were obtained using a scanning rate of 2 °C/min. As a reference sample an empty pan was used and the temperature scale of the calorimeter was calibrated using indium (T_m = 156.60 °C). A Mettler Toledo DSC822^e heat-flux DSC was used. A quantity of 4–7 mg (total weight) was used for the DSC measurements.

2.2. Raman spectroscopy

High-frequency Raman spectra were recorded with a Perkin-Elmer GX Fourier transform spectrometer. A diode pumped Nd:YAG laser at 1064 nm was used as excitation source. The spectra were obtained at 4 cm^{-1} resolution from 3500 to 500 cm^{-1} with interval 2 cm^{-1}. The laser power was controlled to be constant at 400 mW during the experiments. Analysis of the spectra was carried out using GRAMS/32 data analysis software. A quantity of 10–12 mg (total weight) was used for the Raman spectroscopy. Prior to Raman spectroscopy the hydrated mixture was left in the spectrometric cell for 16–24 h to ensure equilibration. Experiments at all concentrations (3%, 5%, 10%, 15%, 20% and 30% molar ratio) were held at 25 °C in order to assess the change of the interaction between DPPC and PAMAM G4 and G3,5 at increasing concentrations while concentrations of 3% and 10%, were further investigated from 30 to 50 °C.

3. Results and discussion

3.1. Differential scanning calorimetry

DPPC bilayers undergo phase transition upon increasing the temperature. DPPC in aqueous environment exists in two totally different mesomorphic phases known as L_α and $L_{\beta'}$. The $L_{\beta'}$ crystalline form of DPPC corresponds to the *gel phase* while the L_α crystalline form corresponds to the *liquid crystalline phase*. The transition from the gel phase to the liquid–crystalline phase can be done by temperature rising. In that case the DPPC molecules move more rapidly increasing the lateral and rota-

Table 1
Interaction of PAMAM G4 dendrimer with DPPC lipid bilayers

Sample (x = mol%)	T_{onset} (°C)	S.D.	T_m (°C)	S.D.	$T_{1/2}$ (°C)	S.D.	ΔH (kJ/mol)	S.D.
DPPC	41.12	0.01	41.43	0.02	41.51	0.02	35.61	1.01
DPPC/PAMAM G4 (x = 3)	39.36	0.01	40.49	0.03	40.61	0.02	13.39	0.32
DPPC/PAMAM G4 (x = 5)	40.02	0.02	40.58	0.02	40.57	0.01	11.43	0.07
DPPC/PAMAM G4 (x = 10)	39.90	0.01	41.50	0.06	41.51	0.05	6.32	0.04
DPPC/PAMAM G4 (x = 15)	40.42	0.01	41.54	0.02	41.54	0.02	4.90	0.14
DPPC/PAMAM G4 (x = 20)	39.93	0.01	40.43	0.02	40.63	0.02	4.07	0.03
DPPC/PAMAM G4 (x = 30)	39.95	0.03	40.39	0.03	40.50	0.03	4.46	0.38

Calorimetric parameters. T_{onset}: temperature at which the thermal effect starts; T_m: temperature at which heat capacity (ΔC_p) at constant pressure, is maximum; $T_{1/2}$: temperature at which the transition is half completed; ΔH: transition enthalpy normalized per mol of DPPC.

tional diffusion among them, while the intermolecular motion around C–C bonds increases and, thus, kink forms are created. The *all-trans* conformation of DPPC in the gel phase can be leaded through kink conformations to a *gauche* form (120° rotation of the simple C–C bond) and formation of a *gauche-trans-gauche* conformation of the lipid chain (Goniotaki et al., 2004; Kyrikou et al., 2005).

Phosphatidylcholines with the choline polar head (as DPPC) demonstrate an endothermic transition that happens prior to the main gel to liquid–crystalline transition. This thermotropic phenomenon is called *pre-transition* and creates bilayers with the lipid chains fully stretched and tilted ($L_{\beta'}$ phase). The tilt angle of the lipid chain is temperature dependable and is minimized at the pre-transition temperature. Then the crystalline phase is called ripple phase ($P_{\beta'}$). The exact knowledge and control of the thermodynamic properties of the lipid bilayers [(ΔH (transition enthalpy), T_m (transition temperature), $T_{1/2}$ (temperature at which the transition is half completed), $\Delta T_{1/2}$ (cooperatively of the bilayer)], is of importance in the development of lipidic controlled release systems (i.e. liposomes) because the encapsulation, stability and release of biologically active molecules depend on them (Matsingou et al., 2005).

The changes of the enthalpy as a function of phase transitions when dendrimer is incorporated into lipid bilayers are correlated to the design of drug carriers. The modifications on the ordered lipid structure of DPPC bilayers, probably due to the structural conformation and to the surface charge of dendrimer, which can strongly interact either with the lipophilic part of DPPC and thus resulting greater thermotropic changes in T_m, or with the polar group of DPPC resulting to an abolition of pre-transition peak at 36.14 °C.

In this study DSC was applied in order to assess the thermodynamic changes in the lipid bilayers caused by the incorporated dendrimer (i.e. PAMAM G4 and G3,5) and thus further understand dendrimers' interaction with DPPC lipid bilayers (Koynova and Caffrey, 1998). Thermal analysis results are based on T_{onset}, T_m, $T_{1/2}$ and ΔH. Fully hydrated DPPC bilayers incorporating PAMAM G4 dendrimer showed thermograms consisting of broad enthalpy transitions, abolition of the pre-transition and reduction of the enthalpy change of the gel to liquid–crystalline phase transition of DPPC bilayers as shown in Table 1 and Fig. 1. The results for the mixture of DPPC and PAMAM G3,5 were similar and are shown in Table 2 and Fig. 2. At concentrations 10% PAMAM G4 dendrimer, the homogeneity of the transition is lost, a fact that can be attributed to the apparition of 'domains' of PAMAM G4 high concentrations in the lipid bilayer. The 'domains' are already apparent at 5% concentration of PAMAM 3,5. The pre-transition was abolished at all concentrations of PAMAM G4 and at concentrations of PAMAM G3,5 above 10%. The pretransition for the mixtures of PAMAM G3,5 is apparent, though diminished at 3% concentration and almost abolished at 5% when the thermograms were zoomed in at 0.1 W/g. The abolition of this low enthalpy transition may be attributed to the interactions between the PAMAM polar groups and the DPPC head groups.

The reduction of ΔH (Table 1) is great at concentrations up to 15% and is greater at 20 and 30 mol% concentrations. This is attributed to the disorganization of the lipid bilayer that caused

Table 2
Interaction of PAMAM G3,5 dendrimer with DPPC lipid bilayers

Sample (x = mol%)	T_{onset} (°C)	S.D.	T_m (°C)	S.D.	$T_{1/2}$ (°C)	S.D.	ΔH (kJ/mol)	S.D.
DPPC	41.12	0.01	41.43	0.02	41.51	0.02	35.61	1.01
DPPC/PAMAM G3,5 (x = 3)	40.46	0.01	41.25	0.04	41.33	0.03	19.87	0.10
DPPC/PAMAM G3,5 (x = 5)	39.56	0.06	41.33	0.03	41.29	0.05	18.83	0.18
DPPC/PAMAM G3,5 (x = 10)	40.04	0.05	41.61	0.07	41.62	0.08	14.36	0.18
DPPC/PAMAM G3,5 (x = 15)	40.05	0.06	41.74	0.05	41.66	0.16	5.89	0.04
DPPC/PAMAM G3,5 (x = 20)	40.07	0.11	42.08	0.21	42.12	0.25	5.85	0.06
DPPC/PAMAM G3,5 (x = 30)	39.64	0.15	40.59	1.01	41.63	0.06	4.41	0.05

Calorimetric parameters. T_{onset}: temperature at which the thermal effect starts; T_m: temperature at which heat capacity (ΔC_p) at constant pressure, is maximum; $T_{1/2}$: temperature at which the transition is half completed; ΔH: transition enthalpy normalized per mol of DPPC.

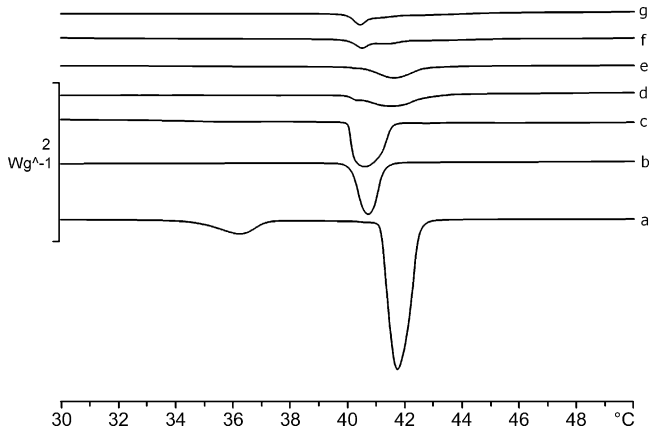

Fig. 1. DSC thermograms of fully hydrated bilayers of DPPC with varying amounts (a: 0 mol%; b: 3 mol%; c: 5 mol%; d: 10 mol%; e: 15 mol%; f: 20 mol%; g: 30 mol%) of PAMAM G4 dendrimer at 25 °C.

a 'phase segregation' (Bonora et al., 2003) due to the increase of the incorporated PAMAM.

The weakening of the van der Waals forces between the alkyl chains, due to the incorporated PAMAM molecules, gives rise to more fluid bilayers. Because these weak van der Waals interactions dictate the structure of the membrane we can conclude that at high-incorporated PAMAM concentrations (very low ΔH values) the lipid bilayer becomes heterogeneous. The maximum percentage of PAMAM G4 dendrimer that can be incorporated in the DPPC bilayers is 5% and the respective percentage of PAMAM G3,5 is 3%, as shown in the thermograms (Figs. 1 and 2).

3.2. Raman spectroscopy

Raman spectroscopy was used in order to specify the exact location of the compound-perturbed in the lipid bilayer. Experiments at all concentrations were held at 25 °C in order to assess the change of the interaction between DPPC and PAMAM G4 and G3,5 at increasing concentrations. The peak height intensity ratios $I_{2935/2880}$, $I_{2844/2880}$ and $I_{1090/1130}$ provided us with information about the conformation of the alkyl chain of DPPC at different concentrations and temperatures (Huang and Levin, 1983; O'Leary et al., 1984; Xynogalas et al., 2005). The spectral changes in the hydrocarbon chain C–H stretching mode region, 2800–3100 cm^{-1}, gives us information about the interactions involved between the alkyl chains of DPPC and thus about their conformation. The intensities and the frequencies of that spectral region are susceptive to the changes that occur to the conformation of the lipid chains due to the transition of the solid to the liquid–crystalline phase. The incorporation of molecules, such as dendrimers, into the lipid bilayers provokes changes of their conformation and as a consequence, of the intensity and frequencies of this region. The bands we studied appear at 2844 and 2880 cm^{-1} and are attributed to the symmetric and asymmetric stretching vibration of the C–H bond of the methylene groups and the band at 2935 cm^{-1} is attributed in part to a Fermi resonance component of the alkyl chain terminal methyl C–H symmetric stretching mode (Hill and Levin, 1979). The peak height intensity ratios of the bands at 2935 and 2880 cm^{-1}, or 2884 and 2880 cm^{-1} have been demonstrated to be a sensitive measure of both interchain and intrachain order–disorder processes in the bilayer alkyl chains. In addition, at the spectral region 1000–1200 cm^{-1}, the C–C stretching mode region, reflects intramolecular *trans*/*gauche* conformational changes within the alkyl chains of the phospholipids. The band at 1130 cm^{-1} is attributed to the stretching vibration of the C–C bond for the *trans* conformations of the alkyl chains, while the band at 1090 cm^{-1} is attributed to the stretching vibration of the C–C bond for the *gauche* conformations of the alkyl chains. Thus, the peak height intensity ratio of these bands can also give us information about the proportion between disorder and order that exists in the conformation of the alkyl chain (O'Leary et al., 1984). At the first place, experiments at all concentrations were held at 25 °C. The results of the intensity ratios $I_{2935/2880}$ and $I_{1090/1130}$ appear in Tables 3 and 4, for mixtures of DPPC with PAMAM 4 and 3,5, respectively.

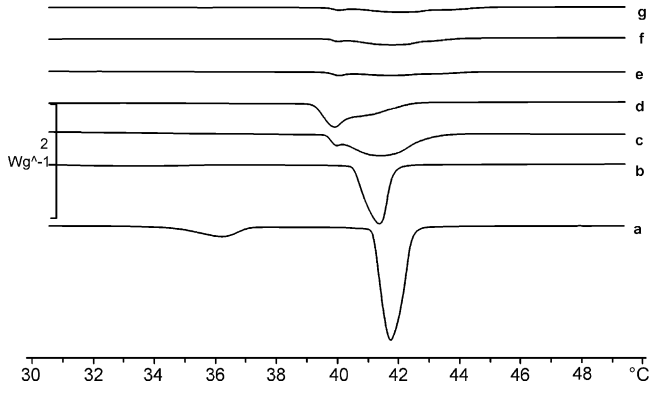

Fig. 2. DSC thermograms of fully hydrated bilayers of DPPC with varying amounts (a: 0 mol%; b: 3 mol%; c: 5 mol%; d: 10 mol%; e: 15 mol%; f: 20 mol%; g: 30 mol%) of PAMAM G3,5 dendrimer at 25 °C.

Table 3
Interaction of PAMAM G4 dendrimer with DPPC lipid membrane

Sample (x = mol%)	$I_{2935/2880}$	$I_{1090/1130}$
DPPC	0.48	0.76
DPPC/PAMAM G4 (x = 3)	0.56	0.82
DPPC/PAMAM G4 (x = 5)	0.71	0.93
DPPC/PAMAM G4 (x = 10)	1.10	0.99
DPPC/PAMAM G4 (x = 15)	0.95	0.99
DPPC/PAMAM G4 (x = 20)	1.33	1.00
DPPC/PAMAM G4 (x = 30)	1.25	1.08

The peak height intensity ratios $I_{2935/2880}$ and $I_{1090/1130}$ correspond to the disorder degree of the alkyl chain and of the terminal methyl group of DPPC lipid bilayers at 25 °C.

An increase of the perturbation of the carbon-chain and the terminal methyl group of DPPC was noticed at all incorporated concentrations as concluded by the increase of the peak height intensity ratios $I_{2935/2880}$ and $I_{1090/1130}$. These *gauche*/*trans* ratios that can be interpreted as disorder/order ratio demonstrate that at a 3% concentration the alkyl chain remains organized and that above 10% (PAMAM G4), and 5% (PAMAM G3,5) the *gauche* conformation is more intense, so the alkyl chain bends leading to its liquidation and its degradation.

Table 4
Interaction of PAMAM G3,5 dendrimer with DPPC lipidic membrane

Sample (x = mol%)	$I_{2935/2880}$	$I_{1090/1130}$
DPPC	0.48	0.76
DPPC/PAMAM G3,5 (x = 3)	0.53	0.74
DPPC/PAMAM G3,5 (x = 5)	0.75	0.74
DPPC/PAMAM G3,5 (x = 10)	0.93	1.00
DPPC/PAMAM G3,5 (x = 15)	1.09	0.77
DPPC/PAMAM G3,5 (x = 20)	1.21	0.92
DPPC/PAMAM G3,5 (x = 30)	1.37	0.95

The peak height intensity ratios $I_{2935/2880}$ and $I_{1090/1130}$ correspond to the disorder degree of the alkyl chain and of the terminal methyl group of DPPC lipid bilayers at 25 °C.

The absorption at 715 cm^{-1} is assigned to the totally symmetric stretching mode of vibration of the C–N bond of the head-group choline of DPPC. The results show a concentration dependent interaction of PAMAM G4 and G3,5 with the polar head of DPPC, fact that is in accordance with the exemption of the pre-transition peak shown by DSC.

Experiments at indicative concentrations 3% and 10% of PAMAM G4 and G3,5 were held at increasing temperatures: 30, 33, 36, 38, 39, 40, 41, 42, 43, 44, 45, 47 and 50 °C. These experiments took place in order to understand in details the molecular interactions between DPPC and PAMAM G4 and G3,5 at concentration in which pre-transition peak (ripple phase of DPPC) was abolished (3%) and phase separation and possible domains formation have been occurred at concentrations of 10% (Figs. 1 and 2). Graphs of $I284_{4/2880}$ or $I_{1090/1130}$ versus temperature are shown in Figs. 3–6. The results show transition onsets and maxima that are very close to those derived from DSC. The $I_{2844/2880}$ and $I_{1090/1130}$ ratios remain practically constant until the temperature of 39 °C. Then, there is an abrupt increase at 40–41 °C and finally remain again constant at temperatures above 42 °C. As already mentioned, these ratios indicate the interaction among the alkyl chains and more specifically, they correspond to the ratio disorder/order among the chains. This is a verification of the fact that the increase of disorder phase takes

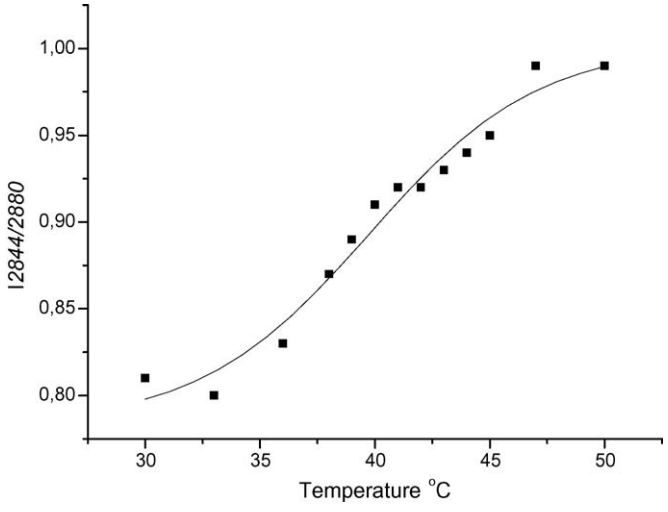

Fig. 3. $I_{2844/2880}$ vs. temperature graph for DPPC incorporating PAMAM G4 at 3% concentration.

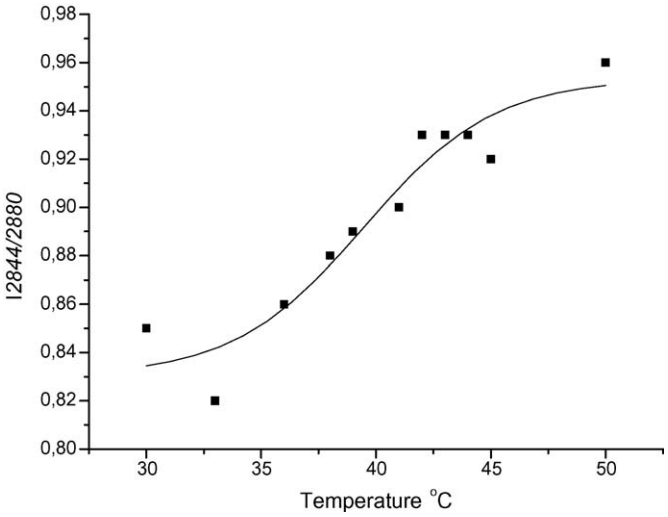

Fig. 4. $I_{2844/2880}$ vs. temperature graph for DPPC incorporating PAMAM G4 at 10% concentration.

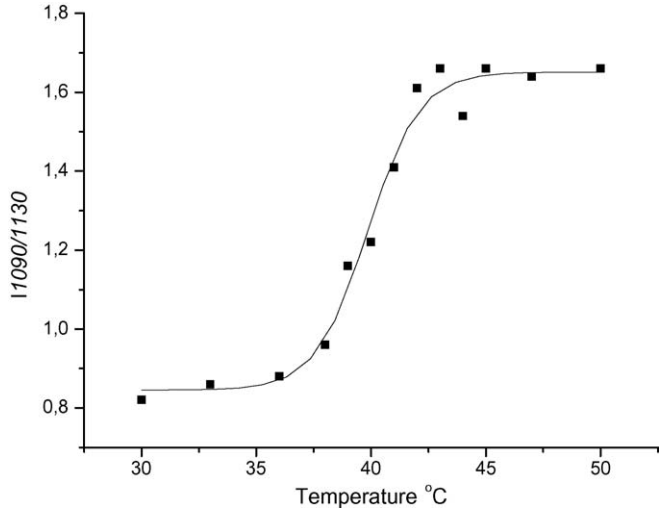

Fig. 5. $I_{1090/21130}$ vs. temperature graph for DPPC incorporating PAMAM G3,5 at 3% concentration.

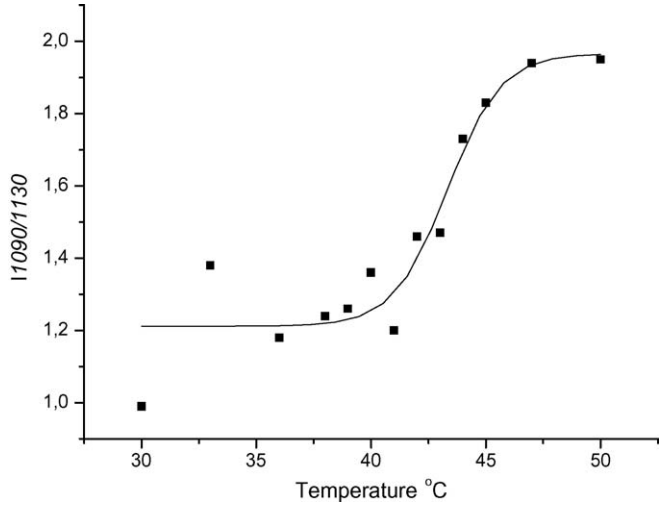

Fig. 6. $I_{1090/21130}$ vs. temperature graph for DPPC incorporating PAMAM G3,5 at 10% concentration.

place very close to the temperature where the transition from the well-organized solid phase of the DPPC bilayer to the less organized liquid–crystalline phase occurs (Figs. 3–6).

4. Conclusion

The results of this study show that the incorporation of PAMAM G4 and G3,5 dendrimer in DPPC bilayers causes (a) abolition of the pre-transition peak of DPPC at all concentrations of PAMAM G4 and at concentrations of PAMAM G3,5 above 10% and (b) concentration dependent lowering of ΔH while little change of the T_m was observed in DSC thermograms; G4 seems to interact more intensely with DPPC but can incorporated in greater percentage (5%) than that of G3,5 (3%) as thermograms indicated. The above observations support the strong interaction of dendrimers even in low concentrations with the DPPC bilayers. The intensity ratios and the changes of frequencies of the spectra areas which have been studied by Raman spectroscopy claim that the conformational properties of the alkyl chains have changed due to the incorporation of PAMAM G4 and G3,5 dendrimer into the lipid bilayers.

These results complement studies, which have concluded that dendrimers are able to undergo endocytosis through cell membranes. This mechanism depends on the dendrimer concentration, which should be available to pass through and not to disrupt the biological membrane. Finally, these results could prove informative for the rational design of effective drug carriers, consisting of liposomes and dendrimers which can deliver bioactive molecules to the target tissues and maintain continuous drug release.

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