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# A fluorescence investigation of the effects of polylysine on dipalmitoylphosphatidylglycerol bilayers

Danielle Carrier [a], Jean Dufourcq [b], Jean-F. Faucon [b] and Michel Pézolet [a]

[a] *Département de Chimie, Université Laval, Québec G1K 7P4 (Canada) and* [b] *Centre de Recherche Paul Pascal, CNRS, Domaine Universitaire, 33405 Talence Cédex (France)*

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**Fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene was used to study the effects of polylysine on the thermotropic behavior of dipalmitoylphosphatidylglycerol (DPPG) bilayers. The molecular weight of the polypeptide was shown to be a key parameter. Long polylysine (mol. wt. $\geqslant$ 60 000) induces a lateral phase separation in three domains for lipid to lysyl residue ratios ($R_i$) greater than unity and a shift of +4.0 Cdeg of the single gel-fluid transition is observed when there is an excess of lysine. With short polylysine (mol. wt. approx. 4000), no domain formation was observed and the transition temperature shift was much smaller. Polylysine with mol. wt. 17 000 yielded a very complex lipidic behavior, with a triphasic transition for $R_i > 1$, another triphasic transition for $1 > R_i > 0.1$, and finally, a 4.0 Cdeg positively shifted one-step transition at higher lysine concentration. These divergencies in effects for different degrees of polymerization of the polypeptide are believed to be related to structural parameters. Multilamellar liposomes and small unilamellar vesicles react in the same way to polylysine, whatever its degree of polymerization. However, the use of the latter has permitted the observation of the gradual destruction of unilamellar structure when going from the pure lipid to $R_i = 1$. The optical D- and L-isomers gave identical results.**

## Introduction

The physical state of a lipidic bilayer is primarily governed by the length and degree of unsaturation of the hydrophobic chains, and by the charge and dimensions of the polar headgroups. External factors affecting the headgroup state can also modify the fluidity of the hydrophobic core. For example, the gel to liquid-crystalline phase transition of methylphosphatidic acid dispersions is shifted from 28°C at pH > 8, where the headgroup is charged, to 48°C in the neutral state at pH 3 [1–3]. Specific ions, like $Ca^{2+}$, also lead to important modifications of the temperature behavior of various phospholipids [2–7].

In the present study, we focused on the effects of polylysine on the thermotropic behavior of dipalmitoylphosphatidylglycerol (DPPG) bilayers. Polylysine is a cationic polypeptide frequently used as a model of extrinsic protein. At neutral pH, it binds electrostatically to negatively charged phospholipids, like DPPG. According to Raman spectroscopy data, this interaction induces a conformational change of poly(L-lysine) (mol. wt. 150 000) which goes from a random coil to an $\alpha$-helix structure, while the lipidic bilayer is stabilized [8]. This ordering effect results in a decrease of the number of *gauche* bonds within the acyl chains in the gel phase, and an increase of the interchain interactions and of the temperature of the gel to liquid-crystalline phase transition. Similar effects were also shown to occur with other anionic phospholipids, for example dipalmitoylphosphatidic acid [7–11]. The Raman results also indicate that

polylysine can lead to a change of the lipid chain-packing lattice at lipid/lysine molar ratio ($R_i$) greater or equal to 1, or to a lateral phase separation below equimolarity. We are here concerned with this latter phenomenon, and with simple shifts of the main transition temperature.

Although the thermotropic behavior of lipid bilayers can be monitored by several techniques, 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence polarization is particularly convenient for such a study, owing to its rapidity and simplicity, which allow to realize more extensive investigations. Since the diphenylhexatriene fluorescent probe is very hydrophobic, it spontaneously inserts into the lipophilic core of the bilayer. Its fluorescence polarization degree depends on its rotational mobility, which increases when lipid molecules go through the gel to liquid-crystalline phase transition [12].

Polylysine is widely used in membrane research and its interaction with acidic lipids is thought to be well understood. However, in previous studies the authors seldom worried about the possible influence of its degree of polymerization and we felt it could modify the way it affects the thermotropic properties of phospholipids. Therefore, using diphenylhexatriene polarization, we have investigated the effect of polypeptides with various lengths on the thermotropic behavior of unilamellar vesicles and multilamellar dispersions of DPPG, as a function of the lipid/lysine residue molar ratio.

## Materials and Methods

*Materials.* Dipalmitoylphosphatidylglycerol (ammonium salt) and polylysines (bromide salts) were supplied by Sigma Chemical Co. (St. Louis, MO) and used without further purification. All samples were made using a 100 mM phosphate buffer (pH 7.0) with EDTA 10 mM. For a series of experiments, a stock dispersion was prepared by vortex shaking the weighed lipid-buffer mixture at 55°C. To get unilamellar vesicles, such dispersions were then sonicated at this temperature for two 30 second cycles. The fluorescent probe diphenylhexatriene, from Aldrich Chemical Co., solubilized in tetrahydrofuran and stored below −5°C, was then added to yield a diphenylhexatriene (DPH) concentration of 0.5 to 1 percent relative to the lipid molarity. Aliquots of this DPPG-DPH dispersion were diluted into 3 ml of phosphate buffer containing the required amount of polylysine, in order to get a final lipid concentration of approx.

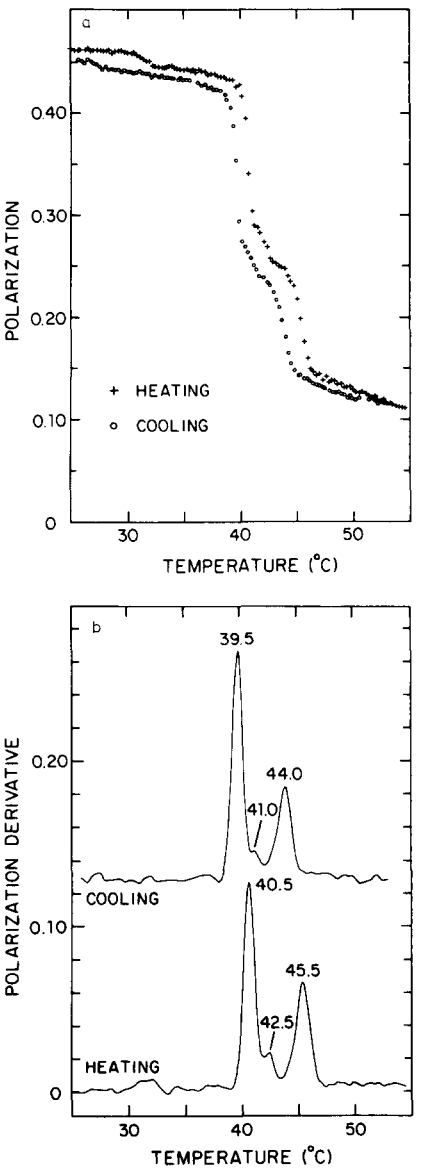


Fig. 1. (a) Effect of temperature on the fluorescence polarization degree of diphenylhexatriene embedded in unsonicated dispersions of DPPG in the presence of poly(L-lysine) with mol. wt. 60000 at a lipid to lysine molar ratio of 5. 100 mM phosphate buffer (pH = 7.0)/10 mM EDTA. (b) Derivatives of the curves shown in (a).

30 μM. These samples were incubated for 10 to 15 min at 55°C, shaking from time to time on vortex mixer, and allowed to cool down before the experiment.

*Fluorescence measurements.* The degree of polarization $P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$ was determined on a home built, entirely computerized apparatus described elsewhere [13]. The fluorescence intensities parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the polarization of the excitation light at 360 nm were observed simultaneously. Each data point is the average of ten measurements. The sample temperature was controlled with thermoelectric heat pumps and the heating rate of 10 Cdeg/h was automatically monitored. Steady-state polarization measurements at constant temperature were performed on an SLM 4000 spectrofluorometer.

Fig. 1a illustrates a typical temperature profile for an unsonicated dispersion. The large number of data points allows a very good definition of lipid order-disorder transitions with multiple steps. Transition temperatures are determined by the maxima of the polarization derivative curves (Fig. 1b). The cooling profile shows a hysteresis of approximately 1 Cdeg. Additional heating scans gave the same curve as for the first heating which demonstrates that the lipid-peptide complexes are very stable and are not affected by several heating and cooling cycles.

## Results

*Phase separations induced by polylysines*

Fig. 2 shows the temperature profiles obtained by the technique of diphenylhexatriene fluorescence polarization when poly(D-lysine) with a molecular weight of 100000 was added to multilamellar liposomes. On the pure lipid curve, the pretransition and main transition appear at 31.5 and 40.5°C, respectively. These values agree fairly well with those obtained by Raman spectroscopy [8], 33 and 40°C, and differential scanning calorimetry [14], 31 and 41°C, respectively. As the polypeptide concentration is increased, the pretransition slowly disappears and at equimolar ratio, it has completely vanished. For DPPG to lysine molar ratios ($R_i$) greater than 1, the gel-to-fluid transition comprises three steps at temperatures of

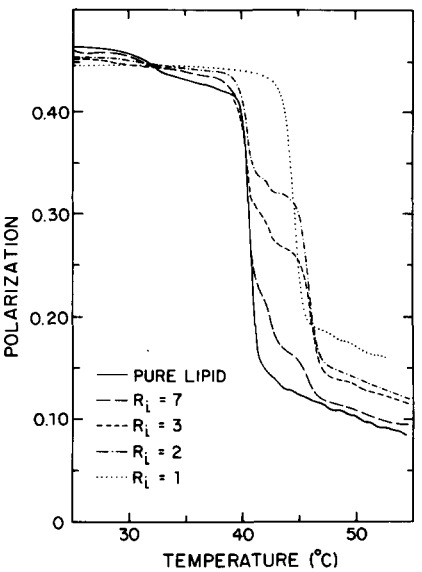


Fig. 2. Effect of temperature on the fluorescence polarization degree of diphenylhexatriene embedded in unsonicated dispersions of DPPG in the presence of poly(D-lysine) with mol. wt. 100000 at different lipid to lysine molar ratios ($R_i$). 100 mM phosphate buffer (pH = 7.0)/10 mM EDTA.

40.5, 42.5 and 46.0°C, revealing the formation of three distinct phases within the bilayer. For $R_i \leqslant 1$, the temperature profiles only show a single-step transition, centered at 44.5°C. An alternative way to present the thermotropic behavior of the lipid is proposed in Fig. 3, where the transition tempera-

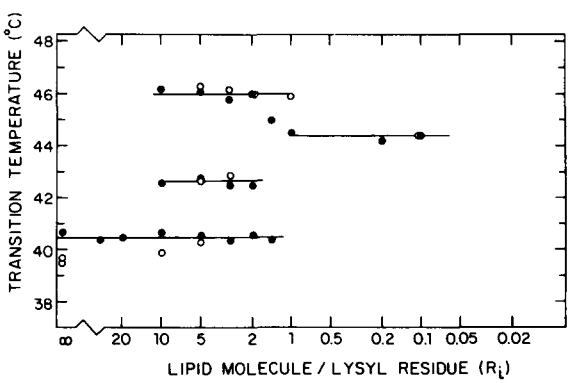


Fig. 3. Transition temperatures of DPPG bilayers in the presence of poly(L-lysine) with mol. wt. 180000 versus lipid to lysine molar ratio ($R_i$), as observed by fluorescence polarization of diphenylhexatriene probe. 100 mM phosphate buffer (pH = 7.0)/10 mM EDTA. ●, unsonicated dispersions; ○, sonicated dispersions.

tures are plotted against the inverse of the lipid to lysine molar ratio, for poly(L-lysine) with mol. wt. 180 000. This figure emphasizes the change of effects occurring at equimolarity. Poly(L-lysine) with mol. wt. 60 000 has led to the same results (as seen on Fig. 1), three transitions being observed on the heating scan at 40.5, 42.5 and 45.5°C, for $R_i = 5$.

Hence, poly(D-lysine) and poly(L-lysine) with mol. wt. 60 000 or more (corresponding to a degree of polymerization greater than 200) all generated identical modifications of the thermotropic behavior of DPPG. In the following, polypeptides in this range will be referred to as 'long polylysines'.

A few number of experiments were also performed with a 1:1 copolymer of L-lysine and L-alanine with mol. wt. 37 000, which represents a polypeptidic backbone having about the same length as poly(L-lysine) with mol. wt. 60 000. The effects observed somewhat resemble those of long polylysines: a single, positively shifted transition (44.5°C) below $R_i = 1$, and phase separation at $R_i > 1$. However, whether there was two (40.5 and 44.0°C) or three (40.5, 42.0 and 44.0°C) phase transitions at low peptide concentration is not clear and temperature shifts were not as important as with long polylysines.

Fig. 4a shows that poly(L-lysine) with mol. wt. 4000 failed to induce any observable phase separation nor any significant shift of the gel-to-fluid phase transition temperature; at very high lysine concentration ($R_i \leqslant 0.1$), the shift was always smaller than +1 Cdeg. However, larger positive shifts were induced when unilamellar vesicles were used instead of the usual multilamellar liposomes (Fig. 4b). For the pure lipid, sonication results in a loss of cooperativity of the gel-to-fluid transition, now appearing at 39.5°C, as well as in the abolition of the pretransition. These effects are attributed to the packing constraints on the acyl chains imposed by the small radius of curvature of the vesicles [15,16] and also to the decrease of the maximal size of cooperative units [17]. Upon addition of the short polylysine, the cooperativity of the main transition is gradually increased and its temperature is positively shifted by almost two degrees, reaching the value found with unsonicated dispersions.

Experiments using unilamellar vesicles were also carried out with long polylysines, but phase separations were much less conspicuous because of the flattening of temperature profiles. However, we have observed the same effects as with unsonicated dispersions, namely the segregation in three phases above $R_i = 1$ and a positive temperature

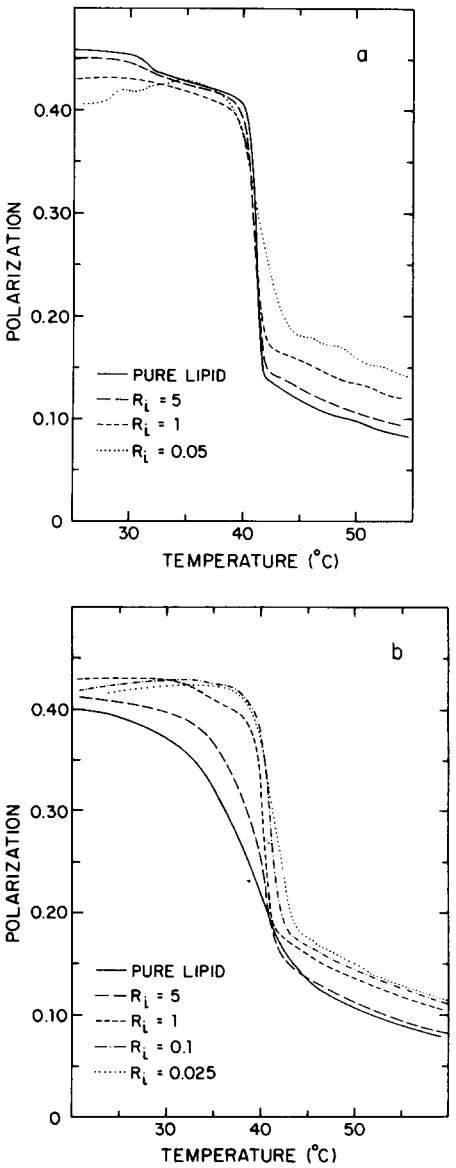


Fig. 4. Effect of temperature on the fluorescence polarization degree of diphenylhexatriene embedded in DPPG bilayers in the presence of poly(L-lysine) with mol. wt. 4000 at different lipid to lysine molar ratios ($R_i$). 100 mM phosphate buffer (pH = 7.0)/10 mM EDTA. (a) Unsonicated dispersions. (b) Sonicated dispersions.

shift for $R_i < 1$ (see Fig. 3, open circles). As with short polylysine, the transition cooperativity was restored by the polypeptide.

To further investigate the effect of the degree of polymerization on the influence of polylysine on DPPG bilayers, we have finally performed similar experiments using poly(L-lysine) with mol. wt. 17000, corresponding to approx. 80 residues (Figs. 5 and 6). The thermotropic behavior observed here is much more complex than in both preceding cases. A transition at the same temperature (40.5°C) as that of the pure lipid is seen at up to ten lysines per lipid molecule, $R_i = 0.1$, and phase separation exists on this whole range, while it was never observed with other polypeptides at $R_i < 1$. From pure lipid to $R_i = 1$, Fig. 6 shows a second phase transition, at 42.0°C, which soon disappears. A third one is seen at 43.5°C, from $R_i = 10$ to $R_i = 1$ where a high melting one with $T_c =$ 47.0°C abruptly appears. This latter coexists with the pure-lipid-like one (shallow, $T_c = 40.5$°C) and the high poly(L-lysine)-concentration ($T_c = 45.0$°C) phase transition, down to $R_i = 0.2$ or 0.1. The temperature of the single transition at $R_i < 0.1$, 45.0°C, can be compared to the one (44.5°C) obtained with long polylysines at high lysine concentration.

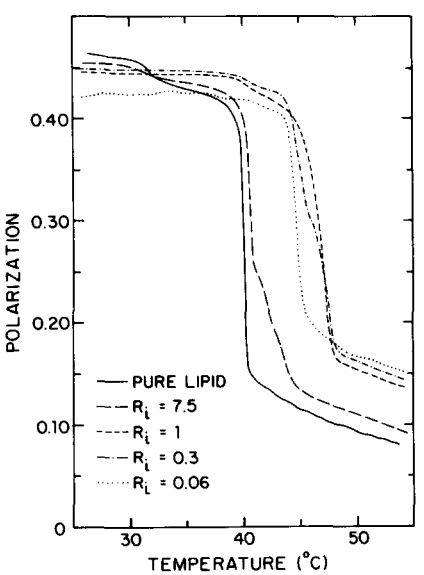


Fig. 5. Effect of temperature on the fluorescence polarization degree of diphenylhexatriene embedded in unsonicated dispersions of DPPG in the presence of poly(L-lysine) with mol. wt. 17000 at different lipid to lysine molar ratios ($R_i$). 100 mM phosphate buffer (pH = 7.0)/10 mM EDTA.

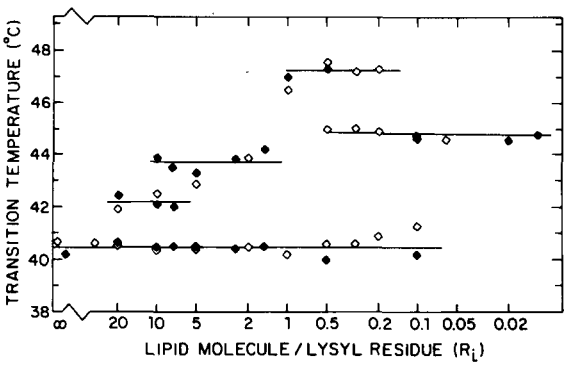


Fig. 6. Transition temperatures of DPPG unsonicated dispersions in the presence of poly(L-lysine) with mol. wt. 17000 versus lipid to lysine molar ratio ($R_i$), as observed by fluorescence polarization of diphenylhexatriene probe. 100 mM phosphate buffer (pH = 7.0)/10 mM EDTA. Different symbols indicate different sets of experiments.

*Effects of polylysine on diphenylhexatriene fluorescence polarization in the fluid phase*

As mentioned above, the binding of polylysine to DPPG results in the disappearance of the lipid pretransition. Below the pretransition temperature, there is a gradual decrease of diphenylhexatriene fluorescence polarization as the polypeptide is added (see Fig. 2). This increase of the probe mobility indicates that the interaction affects the packing of the acyl chains in the gel phase.

On the opposite, polylysine leads to an augmentation of the degree of polarization in fluid phase. Should one conclude that, as cholesterol, polylysine fluidifies the bilayer in its gel phase but rigidifies it in the liquid-crystalline phase? In fact, all investigated proteins and peptides that can bind to phospholipid bilayers generate such effects in a qualitative way, irrespective of the type of interaction they may have with the lipid [18,19]. Therefore, these changes in $P$ cannot shed any light on the nature of the interaction. Nevertheless, we noticed that both the decrease of $P$ in gel state and its increase in fluid state always seemed to be maximized at equimolar lipid to lysine ratio. Fig. 7 shows that for long polylysine, the increase of the probe degree of polarization at 55°C is linear

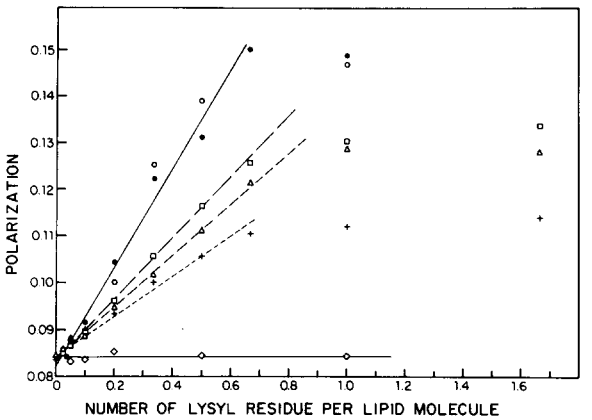


Fig. 7. Fluorescence polarization degree ($P_{55}$) of diphenylhexatriene embedded in DPPG bilayers in the fluid state at 55°C versus lipid to lysine molar ratio ($R_i$). 100 mM phosphate buffer (pH = 7.0)/10 mM EDTA. Poly(L-lysine) with mol. wt. 180000: $P_{55}$ as measured on heating scans of unsonicated (●) and sonicated (○) dispersions. For other symbols, successive aliquots of polylysine were added and $P_{55}$ measured after a 10 min equilibration period. □, $(Lys, Ala)_{1:1}$ with mol. wt. 37000; △, poly(L-lysine) with mol. wt. 14000; +, poly(L-lysine) with mol. wt. 4000; ◇, NaCl.

against the number of lysine residues per lipid molecule, up to approx. 0.7 where the 'limiting value' is nearly reached. The $P$ values were taken on the temperature profiles obtained for a series of experiments with poly(L-lysine) (mol. wt. 180000) and sonicated (open circles) or unsonicated (filled circles) DPPG dispersions. Very similar results are obtained when isothermal binding experiments are done by adding aliquots of the required polypeptide to a single lipid sample and measuring the diphenylhexatriene degree of polarization after a ten minutes incubation (Fig. 7.). The increase of $P$ is still linear up to $R_i^{-1} \approx 0.7$. A control experiment with NaCl gave no significant increase on the same range. Beside the linear effect below equimolar ratio, this figure points out a relation between the importance of the effect and the polypeptide length, the longest ones leading to maximal increases.

## Discussion

The results presented here clearly show that polylysine induces major alterations of the thermotropic behavior of DPPG bilayers. The cooperativity of multistep transitions obtained with long and medium polylysines demonstrates the formation of distinct domains with reasonable size. Lateral phase separation caused by polylysine has already been reported for membranes composed either of a single, anionic lipid [8,20,21] or of a mixture of a charged and a neutral, zwitterionic lipid [7,9–11]. However, the membrane thermotropic behavior never revealed so complicated and no author ever mentioned the presence of a triphasic gel-to-fluid transition.

Among the various lengths of polylysine investigated, three types of behavior were found, and consequently the polypeptides can be divided as (a) long polylysines for mol. wt. $\geqslant 60000$, (b) short polylysines with mol. wt. 3500 or 4000 and (c) medium polylysines of intermediate length between short and long ones.

For long polylysines, the temperature profiles reveal a triphasic gel to liquid-crystalline phase transition at $R_i > 1$. The first step, at 40.5°C, obviously pertains to a phase composed of unbound lipids; as expected, its amplitude decreases from the pure lipid to the equimolar ratio. The transition at 45.5°C (or 46.0°C) corresponds to the maximal bilayer stabilization and it grows in intensity up to $R_i = 1$. Accordingly this phase likely contains completely saturated DPPG. The neutralization of the charged headgroups which eliminates the electrostatic repulsion between them allows a better packing of the chains and then stabilizes the ordered phase. The amplitude of the intermediate transition (at 42.5°C) first increases with polylysine concentration, but then diminishes and has disappeared at equimolarity. The organization of the corresponding lipid-peptide complex is obviously different than that of the higher melting temperature phase. One possibility could be a different conformation of bound polylysine, perhaps giving another binding stoichiometry such as one lysine for each second lipid. Because this phase is less stable than the higher melting temperature one, it would be replaced by this latter as equimolarity is reached. One can also imagine a bilayer with bound polylysine only on one side, where the perturbation could be transmitted across the bilayer through the influence of the new spontaneous curvature due to the lysine binding [10,18]. At $R_i \leqslant 1$, the single phase transition observed

indicates that the whole membrane is in a uniform state, apparently unaffected by excess polylysine. This phase can thus be correlated to the higher melting temperature phase above $R_i = 1$. The difference between their melting temperatures could arise from a reorganization of the lipidic array occurring only after complete saturation, as seen by Raman spectroscopy [8]. For $R_i < 1$, the fusion of lysine-bound domains is prevented by elastic forces [22] and this can justify the requirement of total neutralization prior to rearrangement.

Sonication does not seem to affect the interaction between DPPG and polylysine, whatever its molecular weight is. However, the increasing cooperativity of the transition indicates that the unilamellar structure is gradually destroyed as the polypeptide is added. At equimolarity, sonicated and unsonicated dispersions give identical temperature profiles, suggesting that the bilayer organization in both cases has evolved in such a way that the physical state of the lipid molecules is the same. The implied modification of the unilamellar structure may be facilitated by the presence of defects between polylysine-bound domains and the surrounding unperturbed phase.

Short polylysine (mol. wt. 4000), that is approx. 20 residues, fails to induce any phase separation and has a much weaker effect on the gel-to-fluid phase transition (Fig. 4). One may then question about its affinity for DPPG bilayers. The visible aggregation of lipidic particles produced upon addition of any investigated polylysine leaves no doubt about the affinity of polylysine for DPPG. Moreover, the aggregation phenomenon is more extensive with the short polylysine. The variations of diphenylhexatriene degree of polarization in fluid state (Fig. 7) tend to prove that all investigated polylysines do in fact bind to DPPG molecules with a 1 : 1 stoichiometry corresponding to the complete neutralization of both the lipid and the peptide charges. The complete restructuration of unilamellar vesicles at $R_i = 1$ confirms this proposed 1 : 1 stoichiometry and the strong affinity of DPPG for polylysine of any molecular weight.

Hence, short polylysine really binds to DPPG, with the same stoichiometry as long polylysines. Therefore, we should examine more closely the driving forces of the interaction between DPPG and polylysine since the stabilizing effect of long polylysines does not appear to be a simple matter of charge neutralization, which is equally feasible by short polylysine.

DPPG-polylysine interaction involves a hydrophobic component. The cationic polypeptide can bind also to neutral lipids. The phase transition of dimyristoylphosphatidylcholine has been found to be increased by 4°C upon addition of medium poly(L-lysine) [23], at neutral pH. High ionic strength prevents the binding of poly(L-lysine) to phosphatidylcholine but salt addition does not disrupt the already formed complexes [24]. Actually, many authors [24–26] have reported that polylysine binding involves hydrophobic effects in addition to the initial electrostatic interaction.

The electrostatic interaction is itself quite complex. The simple pH titration of DPPG induces a 15 Cdeg increase of the transition temperature while the lipid goes from neutral pH to the fully protonated form [14]. Calcium ions produce much larger effects because they can form salt bridges between lipids. Ammonium groups have also been shown to induce a larger increase of $T_c$ than other monovalent cations like $Na^+$, $K^+$ [27]. The ionic strength may by itself affect the transition temperature of acidic lipids through modifications of the electrical double layer [22]. So, electrostatic interactions may involve pH, ionic strength and more specific structural effects.

DPPG-poly(L-lysine) interaction was shown to imply structural effects. According to Raman spectroscopy [8] and circular dichroism [25] data, long polylysine goes from disordered to $\alpha$-helical conformation upon binding to acidic bilayers. This structural modification may be of primary importance and one can imagine that the distance between neighbouring lateral chains of polylysine could govern the spacing between lipid molecules. Consequently, a polypeptide unable to assume an $\alpha$-helical conformation will not affect in the same way the thermotropic properties of the lipid. This could explain why the monomer [23] and the short polylysine have none or only small effects. Actually, preliminary results obtained by Raman spectroscopy [32] indicate that short polylysine does not form an $\alpha$-helix when it binds to DPPG bilayers.

In view of the respective behaviors of short and

long polylysines, one could expect that intermediate polymers first react as short ones and, at a given molecular weight allowing the formation of $\alpha$-helix, begin to give the effects characteristic of long polylysines. Figs. 5 and 6 reveal that poly(L-lysine) with mol. wt. 17000 (approx. 80 residues) actually induces a very complicated thermotropic behavior. For $R_i > 1$, there is a phase separation analogous to the one observed with long poly(L-lysine). If we use the same arguments, the transition at 43.5°C would thus correspond to patches of lipids bound to lysine residues according to a 1 : 1 stoichiometry; the phase melting at 42.0°C would be a semi-stabilized, partially bound lipids region while a remaining pure lipid phase would be responsible for the lower transition, at 40.5°C. The smaller temperature shift for medium poly(L-lysine), 3.0 and 1.5°C instead of 5.0 (or 5.5) and 2.0 for long poly(L-lysine), are easily explained by the assumption that medium poly(L-lysine) can only form imperfect helices. At equimolar ratio, these two shifted transitions disappear and a new one is observed at 47.0°C. This highly shifted phase is probably the most striking feature about polylysine of medium length. Relying on what has been said before about DPPG-polylysine binding stoichiometry, one can assume that at this point, all the lipid headgroups are neutralized, so that domains of bound lipids may come into contact and fuse together. Any further addition of polylysine in these large areas results in a molar excess and the binding of each lysyl side chain to a specific DPPG headgroup becomes impossible. As the binding of helical polylysine is more stable than that of the unordered peptide, one may suppose that helical portions of the peptide preferentially bind while the unordered parts dangle above the bilayer surface. These dangling ends can attach to a second bilayer, causing a more pronounced dehydration that could well be responsible for the large temperature shift and the more extensive aggregation. For $1 < R_i < 0.1$, there is still a tiny transition at 40.5°C, probably due to the poor organization of the polypeptide on the lipidic surface. The presence of unbound lipids in this range favors the preceding proposal of bilayers fastening by the unordered ends. The single phase remaining at very high polylysine concentration appears at $R_i = 0.5$. Because of the close similarity between transition temperatures, this phase (45.0°C) is compared with the single phase (44.5°C) observed with long polylysines at low $R_i$.

We were also interested by the effects of the optical isomerism of the polypeptide on the nature of its interaction with lipids. In a monolayer study [24], Shafer has found differences in the interaction between D- and L-isomers of long polylysine with neutral phosphatidylserine films, and he has suggested that the optically active carbon of lysine is juxtaposed to the optically active carbon of the lipid. In the present study, no difference has been detected between the effects induced by D- and L-isomers of polylysine with high degree of polymerization.

The results discussed above clearly demonstrate the dramatic influence of the polypeptide degree of polymerization on the interaction between polylysine and DPPG bilayers. The striking differences are related to the ability of each peptide to form an $\alpha$-helix. Whatever their structure is, all the investigated polypeptides bind to DPPG molecules according to a 1 : 1 stoichiometry. Above this lipid to peptide molar ratio, polylysines with mol. wt. 17000 or more lead to a phase separation where two new phases melting at temperatures higher than that of the pure lipid appear. At high or very high lysine to lipid ratio, no domain formation is observed for long (mol. wt. $\geqslant$ 60000) or medium (mol. wt. 17000) polylysine and the single phase detected has a positively shifted gel-to-fluid transition. Short polylysine (mol. wt. 4000, that is approx. 20 residues) does not induce any lateral phase separation and has a very limited effect on the transition temperature. All classes of polylysine exhibit identical effects on small unilamellar vesicles or on multilamellar liposomes, except that in the first case one can observe the gradual destruction of the vesicles structure.

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