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Interfacial interactions between poly[L-lysine]-based branched polypeptides and phospholipid model membranes

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Abstract

The interaction of five poly[L-lysine]-derived branched chain polypeptides of poly[Lys(X_i)] (X_iK) or poly[Lys(X_i-DL-Ala$_m$)] (XAK) with lipid bilayers (DPPC and DPPC/PG, 8:2) was studied by fluorescence polarization techniques. Two fluorescent probes, DPH and TMA-DPH, were utilized to monitor changes of motion in the internal and/or in the polar head regions, respectively. Results indicate that the interaction of polypeptides with neutral (DPPC) bilayers is mainly dependent on the polarity and electrical charge of side chains. The amphoteric E_iK shows the highest level of interaction. Polycationic polypeptides (H_iK, P_iK, TAK) have a relatively small effect on the transition temperature of the lipids, while the polyanionic Succ-EAK has no effect at the alkyl chain region of the bilayer. Data with TMA-DPH indicate the lack of pronounced interaction between the polypeptides and the outer surface of the liposome. Similar tendency was documented for DPPC/PG vesicles. Polypeptides, H_iK, and P_iK induce significant changes in the transition temperature, thus indicating their insertion into the hydrophobic core of the bilayer without marked effect on the polar head region. Results suggest that these polypeptides (except E_iK) have no destabilizing effect on liposomes studied. These properties are considered as beneficial for their use as safe carriers for bioactive molecules.

Abbreviations: DPH: (1,6-diphenyl-1,3,5-hexatriene); TMA-DPH: [1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5 hexatriene *p*-toluensulfonate]; DPPC: dipalmitoyl phosphatidylcholine; PG: phosphatidylglycerol

1. Introduction

Cells take up macromolecules, including proteins and bioconjugates, derived from proteins or polypeptides by endocytosis via specific receptors with broad specificity (e.g., mannose or scavenger) [1]. During the internalization process macromolecules have to interact with the cell membrane and they may influence its fluidity. This could result in altered lateral receptor mobility and thus several receptor-related functions such as cell adhesion [2]. It was observed that decrease of Ca^{2+} response is associated with decreased membrane fluidity in prion-infected cells [3]. Some hydrophilic molecules, even negatively charged peptides, can penetrate the membrane [4]. Polycationic polymers such as polylysine [5] or histidylated polylysine [6] are able to perforate the cell membrane and thus serve as carriers of nucleic acids. In the course of gene or drug delivery into living cells it is important that the carrier molecule not disturb the balance of the cell membrane [7]. The selection and/or design of macromolecules of bioconjugates of drugs, genes, or epitopes could be improved by establishing structure–function relationship between the structure of the macromolecule and its capability to interact with phospholipid bilayers mimicking biological membranes.

Polymeric polypeptides based on poly[L-lysine] have been widely developed to explore their potential uses as delivery systems. Various drugs such as methotrexate, daunomycin, amyloride, and GnRH analog coupled to these carrier molecules exhibited markedly improved efficacy [8,9]. For example, coupling of daunomycin to poly[Lys(Glu$_i$-DL-Ala$_m$)] (EAK) resulted in compensation for the immunosuppressive effect of the drug and produced 66–100% long-term survivors (>60 days) against L10 leukaemia in

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mice [10]. Carrier-dependent anti-*Leishmania donovani* effects of methotrexate branched polypeptide conjugates were observed in vitro and in vivo [11].

Recently a systematic study was initiated to investigate the interaction between branched chain polypeptides with the general formula of poly[Lys(X_i-DL-Ala$_m$)] (XAK), where $i \cong 1$ and $m \cong 3$, and phospholipid mono- or bilayers with different compositions [12–14]. These polypeptides contain oligopeptide side chains composed of oligo[DL-alanine] and an additional amino acid (X) coupled to the α-amino group of the terminal alanine. The results with these polypeptides indicate that the side-chain composition, namely the charge and length of the branches, markedly influence its effect on model membranes.

The aim of the present study was to establish a correlation between the structural properties of the polymers and their influence on the fluidity of phospholipid bilayers using a new group of polylysine-based compounds with the general formula (poly[Lys(X_i)]) X_iK. These polypeptides possess a single amino acid (X = Pro, His, or Glu) coupled to the ε-amino groups of the polylysine backbone. This study was extended by the inclusion of two new XAK type of polypeptides with polycationic (poly[Lys-(Thr$_i$-DL-Ala$_m$)] (TAK)) or anionic (poly[Lys-(Succ-Glu$_i$-DL-Ala$_m$)] (Succ-EAK)) character. The chemical structure of these polymers is given in Fig. 1.

In this communication we describe our findings concerning the interaction of branched polypeptides with phospholipid bilayers with two different lipid compositions: dipalmitoyl phosphatidyl choline (DPPC) and DPPC containing 20% negatively charged phosphatidyl glycerol (PG). Using two fluorescent probes, DPH and TMA-DPH [15], as reporter molecules incorporated into the liposomes, we found that polymeric polypeptides studied, except the amphoteric E_iK with Glu, have no destabilizing effect on liposomes.

2. Experimental

Abbreviations for amino acids and their derivatives follow the revised recommendation of the IUPAC-IUB Committee on Biochemical Nomenclature, entitled "Nomenclature and Symbolism for Amino Acids and Peptides" (recommendations of 1983). Nomenclature of branched polypeptides is in accordance with the recommended nomenclature of graft polymers (IUPAC-IUB Commission on Biochemical Nomenclature, (1984) [16]. For the sake of brevity, codes of branched polypeptides were constructed by us using the one-letter symbols of amino acids (Table 1). The abbreviations used in this paper are the following: X_iK, poly[Lys(X_i)], X = Pro (P_iK), His (H_iK), or Glu (E_iK); poly[Lys(X_i-DL-Ala$_m$)], X = Thr (TAK) or Succ–Glu (Succ–EAK). All amino acids are of L-configuration unless otherwise stated. Z: benzyloxycarbonyl; Boc: *tert*-butyloxycarbonyl; Pcp: pentachlorophenyl; NCA: *N*-carboxy anhydride; Succ: succinyl; DMSO: dimethylsulfoxide.

2.1. Chemicals

DPH, TMA-DPH, DPPC, and PG were from Sigma. Amino acids, acetic acid, and diethyl ether were from Reanal (Budapest, Hungary). Benzyloxicarbonyl chloride, 1-hydroxy benzotriazole, and diethylamine were purchased from Fluka (Buchs, Switzerland), while pentachlorophenol was from Merck (Darmstadt, Germany).

2.2. Synthesis

Experimental details of the synthetic procedures were described previously [17–19]. Briefly, poly[L-Lys] was prepared by the polymerization of *N*-carboxy-*N*-benzyloxycarbonyllysine anhydride under conditions that allowed a

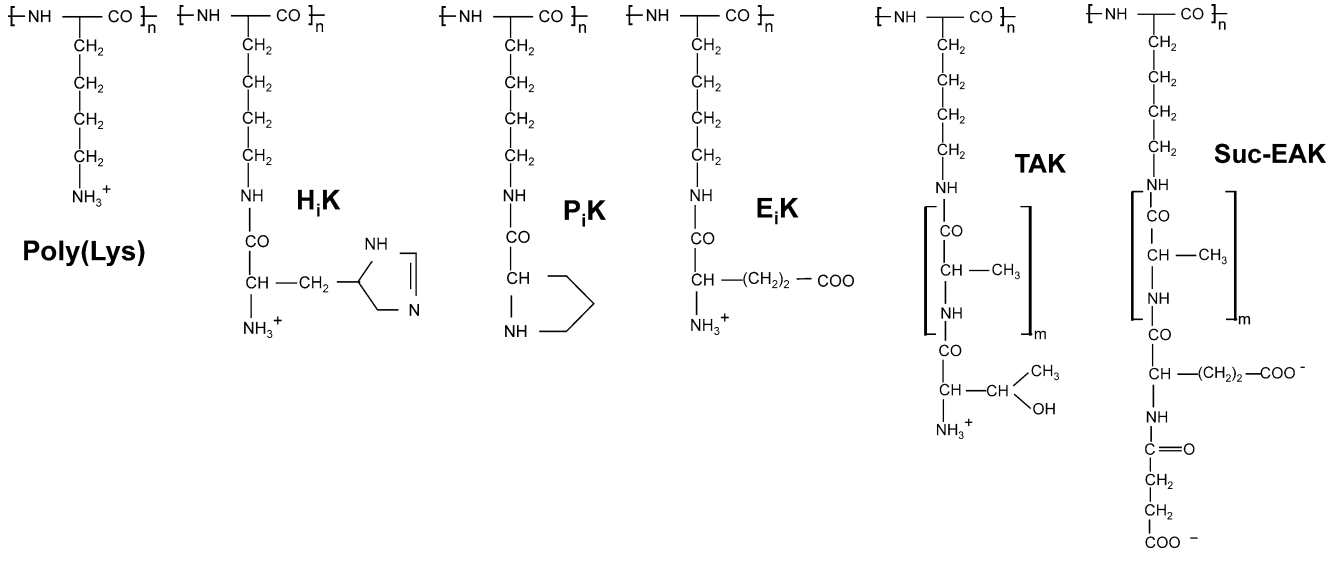

Fig. 1. Schematic presentation of XK type polypeptides, where X = Pro (PK), X = Glu (EK), X = His (HK), and of XAK type polypeptides, where X = Succ–Glu (Succ–EAK), X = Thr (TAK), derived from poly[L-Lys].

Table 1
Characteristics of branched chain polymeric polypeptides with poly[Lys(X_i)] or poly[Lys(X_i-DL-Ala$_m$)] formula

Polymer	Abbreviation[a]	Amino acid composition[b]			$\overline{DP}_n$[c]	$\overline{M_w}$[d] ± 5%
		Lys	Ala (m)	X (i)		
Poly[Lys(Pro$_i$)]	P$_i$K	1.00	–	0.95	84	24800
Poly[Lys(His$_i$)]	H$_i$K	1.00	–	0.56	93	15400
Poly[Lys(Glu$_i$)]	E$_i$K	1.00	–	1.00	94	25700
Poly[Lys(Thr$_i$-DL-Ala$_m$)]	TAK	1.00	3.10	1.00	60	29300
Poly[Lys(Succ-Glu$_i$-DL-Ala$_m$)]	Succ-EAK	1.00	4.00	1.00	80	51400

[a] Code and branched chain polymeric polypeptides based on one-letter symbol of amino acids.
[b] Amino acid composition was determined by amino acid analysis as after hydrolysis in 6 M HCl at 105 °C for 24 h.
[c] Number of average degree of polymerization determined by sedimentation equilibrium measurements.
[d] Average molecular mass of polymers, calculated from the average degree of polymerization (DP_n) of poly[Lys] and of the side chain composition.

number average degree of polymerization (DP_n) approximately 100. Protecting groups were cleaved by HBr in acetic acid (35%, m/V). The size of the polypeptides were determined by sedimentation equilibrium analysis, the average relative molar masses ($\overline{M_w}$ and $\overline{M_z}$) were determined, and the polydispersity factor ($\overline{M_z}/\overline{M_w}$) and an average degree of polymerization ($\overline{DP}_n$) were calculated. Data are given in Table 1.

2.3. Poly[Lys(X_i)](X_iK) polypeptides

The amino acids were coupled to the ε-NH_2-group of lysine as protected pentachlorophenyl ester derivatives (Z-X-OPcp, where X = Glu or Pro; Boc-X-OPcp, where X = His) using the in situ active ester method. Z or Boc protecting groups were cleaved with HBr or acetic acid containing 35% HBr (m/V) [18].

2.4. Poly[Lys(X_i-DL-Ala$_m$)](XAK) polypeptides

XAK-type polypeptides were synthesized by grafting short oligomeric DL-Ala chains to the ε-NH_2-group of lysine residues using N-carboxy-DL-Ala-anhydride [17]. The suitably protected and activated Glu/Thr residue was coupled to the end of branches of AK by amide bond [19]. After the removal of protecting groups, samples were dialyzed against distilled water, freeze-dried, and characterized. Poly[Lys-(Succ-Glu$_i$-DL-Ala$_m$)] (Succ-EAK) was prepared from EAK as follows: 10 mg (18.5 mmol) EAK was dissolved in 2 ml of 0.1 M carbonate buffer (pH 9.2). To this solution 220 µl (220 mmol) succinic anhydride dissolved in DMSO ($c = 100$ mg/ml) was added in aliquots with continuous stirring in 30 min. The pH was adjusted between 9.0 and 9.2 with 0.1 M NaOH. After 4 h the solution was dialyzed against distilled water for 2 days and then freeze-dried.

2.5. Amino acid analysis

The amino acid constitution of the polymeric polypeptides was determined by amino acid analysis using a Beckman 6300 analyzer (Fullerton, USA). Prior to the analysis, the samples were hydrolyzed in 6 M HCl in sealed and evacuated tubes at 105 °C for 24 h.

2.6. Preparation of liposomes

Liposomes were prepared using DPPC or a mixture of DPPC and PG (80/20, mol/mol) [20] as follows. Either 20 mg DPPC or 16 mg DPPC and 4 mg PG were dissolved in chloroform were mixed in a round-bottomed flask and solvent was eliminated by rotary evaporation from the clear solution at 55 °C in vacuum for 15 min. The lipid film was dried in high vacuum for 1 h and hydrated with 4 ml of 0.1 M sodium acetate adjusted to pH 7.4. This suspension containing multilamellar vesicles (MLV) was then sonicated using an ultrasounds tip, with the tube immersed in ice for 8×2 min. Between the sonication events N_2-flux was applied for 1 min. Finally, the suspension of small unilamellar vesicles was placed into a 55 °C water-bath for 1 h for annealing, followed by centrifugation to eliminate metal debris and remaining MLV. The size of the liposomes, measured in a Malvern Autosizer [21], was lower than 80 nm in diameter.

2.7. Fluorescence studies

Fluorescence studies were carried out by measuring changes in fluorescence intensity and polarization of TMA-DPH and DPH probes located in the bilayers, using a PE-LS50 spectrofluorometer provided with a four cuvettes thermostated holder. Liposomes (SUVs) prepared as described above were incubated with different concentrations of fluorescent probes in the dark at 55 °C for 60 min. Fluorescence intensity was measured to determine the PL/probe relationship saturation. Once selected the optimal value, liposomes were mixed either with polymer solution (0.2 mg/ml) or with 0.1 M sodium acetate (pH 7.4), used as reference, and fluorescence intensity as well as polarization were measured. This process was carried out by duplicate, and temperature was increased from 28 to 55 °C step by step leaving enough time between lectures to allow the system to equilibrate. Excitation and emission wavelengths were 355–430 nm for TMA-DPH or 365–425 nm for DPH. The degree of fluorescence polarization was calculated according to Shinitzky and Barenholz [22] applying the equation

$$P = \left(I_{\parallel} - I^*_{\perp} G\right) \Big/ \left(I_{\parallel} + I^*_{\perp} G\right), \tag{1}$$

where $I_{\parallel}$ and $I_{\perp}$ are the intensities measured with its polarization plane parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to that of the exciting beam. G is a factor used to correct polarization of the instrument and is given by the ratio of vertically to horizontally polarized emission components when the excitation light is polarized in the horizontal direction.

3. Results and discussion

3.1. Chemical structure of the polymeric polypeptides

Two types of branched polypeptides derived from linear polylysine were prepared. In the case of polymers described with the formula of poly[Lys(X_i)](X_iK) a single amino acid (Glu, His, or Pro) was coupled to the ε-NH_2 groups of the lysine residues using in situ active ester method (Fig. 1). The oligo (DL-Ala) side chains of poly[Lys(X_i-DL-Ala$_m$)] (XAK) polymers was grafted to the ε-NH_2 groups of the lysine residues by polymerization of DL-Ala-*N*-carboxy anhydride where the ε-NH_2 groups of the polylysine served as multifunctional initiator. To the α-NH_2 groups of the terminal alanine residues of the branches a further amino acid (X = Glu or Thr) was coupled and derivatized by succinylation (Succ-Glu) (Fig. 1). The polypeptides were characterized by their average relative molar mass and the composition of the side chain. The characteristics of these macromolecules are summarized in Table 1.

Regarding the amino acid composition and the degree of substitution by amino acid X each polymer had a different overall electrical charge that has been estimated as follows: E_iK possessing Glu residues at almost each side chain can be considered as a neutral, but zwitterionic molecule. Polymers TAK and P_iK can be considered as a polycation bearing a positive charge at the terminal of each monomer side chain, while H_iK, due to the intrinsic basicity of the imidazole ring, behaves as a polycation, but with two positive charges at the side terminal of the monomer unit. Due to the presence of succinylated Glu at the end of the branches Succ-EAK polymer has two negative charges per monomer side chain.

Based on the length of the branches two groups of polypeptides can be classified. Polymers like H_iK, P_iK, and E_iK have short and similar side chains (equivalent to 8 carbon atoms), but the length of the branches of TAK and Succ-EAK is equivalent to 17 and 24 carbon atoms, respectively. All these parameters will condition their further interaction with lipid bilayers.

3.2. Interaction of polymers with phospholipid bilayers

The interaction between branched polypeptides and phospholipid membranes was investigated using lipid bilayers with DPPC and DPPC/PG (80/20, mol/mol). A fluorescent probe with positive electrical charge TMA-DPH as well as a hydrophobic one, 1,6-diphenyl-1,3,5-hexatriene (DPH), were used to analyze the effect of polymers on the outer surface and on hydrophobic core of bilayers.

For these studies small unilamellar vesicles (SUVs) were used, so that light scattering was not important. Changes in fluorescence intensity and/or polarization of probes in bilayers were determined as a function of temperature. Transition from gel to liquid crystalline state ($T_c \sim 40$–$41.5\,^{\circ}$C for DPPC and $T_c \sim 32\,^{\circ}$C for DPPC/PG, 80/20) was characterized by the analysis of T_c values in the presence or absence of polymers. The optimal probe/phospholipid molar ratios were determined by incubation of plain liposomes with increasing volumes of concentrated solutions of each label followed by fluorescence intensity measurements. Up to a certain label/lipid relationship there was no increase in the fluorescence of the samples thus indicating that the system was already saturated and stable. Results obtained were as follows: TMA-DPH:phospholipid = 1:144 (mol/mol), and DPH:phospholipid = 1:240 (mol/mol) [12]. Under conditions applied (neutral pH, low polymer concentration, probe/phospholipid ratio) liposomes containing 20% PG were negatively charged while those composed of DPPC could be considered neutral.

Changes in fluorescence intensity as well as polarization of DPH or TMA-DPH after incubation of DPPC and DPPC/PG liposomes with polymer solution or acetate solution are described in the next paragraph and summarized in Figs. 2–5.

3.3. Polarization fluorescence studies with DPH

Liposomes labeled with a hydrophobic fluorophore, DPH, located in the inner core of the bilayer, were mixed with polymer solutions and fluorescence intensity and polarization were measured at different temperatures. Due to their high hydrophobicity, DPH molecules, after incubation with liposomes at temperatures above T_c, are able to penetrate the external part of the bilayer and localize mainly in its internal core. This location, in between the acyl chains of phospholipids, makes them extremely sensitive to the microviscosity of their environment. Any change in the motion of acyl chains affects their own motion and results in changes in the polarization or anisotropy of the samples.

Polarization/temperature curves recorded for the five polymers under study and DPPC liposomes indicated that all of them except Succ-EAK interact with the internal core of bilayers. Figures 2a–2c show this process recorded for E_iK, H_iK, and P_iK. One can appreciate that differences are larger at temperatures under or around T_c. Moreover, for E_iK, the polarization curve runs under that of the reference, indicating a depolarization of the probe that reflects a fluidification of the system. In contrast, for H_iK and P_iK, the polarization values corresponding to the samples are slightly higher, suggesting a rigidifying effect. Transition temperatures calculated from the first derivatives are given in Table 2. A very strong effect was observed for the transition temperature in DPPC bilayer with DPH for E_iK ($\Delta T = -8.4$). This

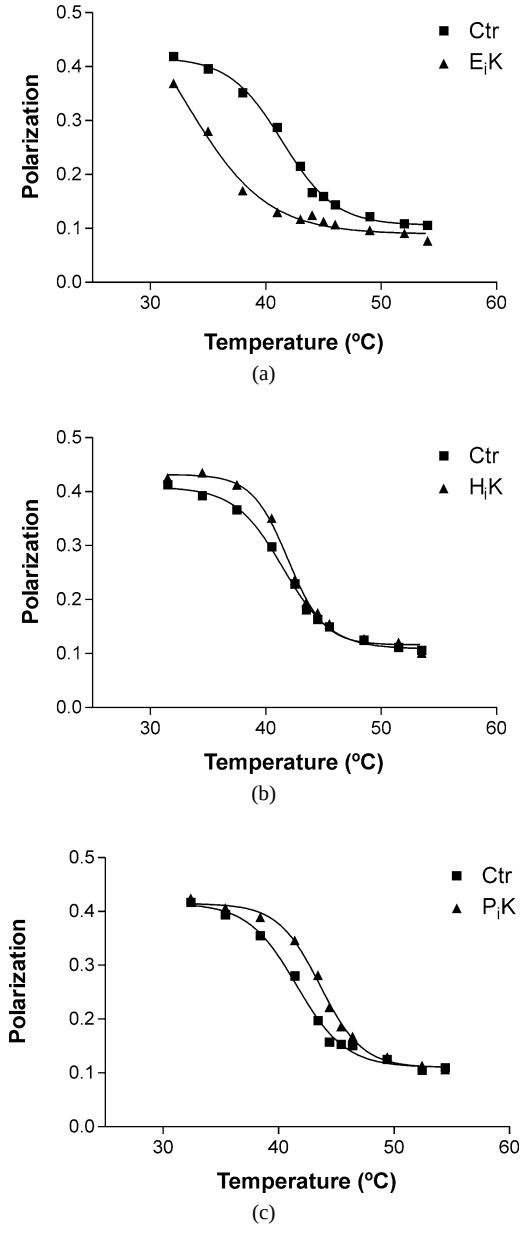

Fig. 2. Polarization/temperature curves recorded for DPH/DPPC vesicles in the presence or absence of branched polypeptides: (a) poly[Lys($Glu_{1.0}$)] (E_iK) —▲—; (b) poly[Lys($His_{0.56}$)] (H_iK) —▲—; (c) poly[Lys($Pro_{0.95}$)] (P_iK) —▲—; control —■—.

finding could be explained by pronounced ionic interaction between the amphoteric polypeptide and DPPC possessing also one positive and one negative charge per molecule. The attraction forces generated changes in the outer surface of the bilayer. Interestingly P_iK, one of the three polycationic polypeptides studied, interacted with DPPC and exhibited a somewhat higher effect on the transition temperature ($\Delta T = 2.0$) than the other two compounds (for both TAK and H_iK $\Delta T = 0.6$). This difference in the effect of the three polycationic polypeptide could be explained perhaps by steric and electronic factors. Polypeptide TAK contains positively charged α-amino groups at the end of the branches; however, the distance between the polypeptide backbone and the amino group is relatively large as compared to that of the polypeptide backbone and the imino group in P_iK. This could result in a sterically more favorable close interaction for P_iK with the amphoteric DPPC. In case of H_iK we have not only one, but two positive charges (the α-amino group and the imidazole moiety in the side chain) in the branches. The neighboring arrangement of these groups generate higher charge density and upon interaction with DPPC not only is the attraction between H_iK and the phospholipid present, but also the repulsion between the positive charges of H_iK and DPPC exists. The lack of such "built-in" double density repulsion could result in more pronounced interaction of P_iK with DPPC. Differences with a similar tendency in the binding of polycationic polypeptides with the negatively charged DPPC-PG liposome were also observed (Table 3). In this case the most marked effect of P_iK as compared to H_iK or TAK could also be explained by the above structure-based interpretation.

Plain liposomes composed of DPPC/PG (8/2, molar) labeled with DPH showed, as was to be expected, polarization/temperature changes, softer than those composed of pure DPPC, indicating a decrease in the cooperativity of the transition. After incubation with H_iK and P_iK, polarization/temperature curves adjusted better to a sigmoid, as can be appreciated in Figs. 3a, 3b and Table 3. These results suggest that the presence of polymer molecules, H_iK and P_iK, in the media has a rigidifying effect, increasing the cooperativity of the process. This effect is also stronger at low temperatures and disappears almost completely at temperatures around T_c (Table 3). TAK the third polycationic

Table 2
Effect of polymeric constructs on the transition temperature of DPPC bilayers

Polymer	Code	ΔT ($T - T_c$)[a]	
		DPH	TMA-DPH
Poly[Lys($His_{0.56}$)]	H_iK	0.6	−1.7
Poly[Lys($Pro_{0.95}$)]	P_iK	2.0	−0.3
Poly[Lys($Glu_{1.0}$)]	E_iK	−8.4	0
Poly[Lys($Thr_{1.0}$-DL-$Ala_{3.1}$)]	TAK	0.6	0.2
Poly[Lys(Succ-$Glu_{1.0}$-DL-$Ala_{4.0}$)]	Succ-EAK	0	0

[a] Transition temperatures were calculated from the first derivative of polarization versus temperature sigmoidal curves.

Table 3
Effect of polymeric constructs on the transition temperature of DPPC-PG (8:2) bilayers

Polymer	Code	ΔT ($T - T_c$)[a]	
		DPH	TMA-DPH
Poly[Lys($His_{0.56}$)]	H_iK	4.9	2.8
Poly[Lys($Pro_{0.95}$)]	P_iK	7.0	−0.7
Poly[Lys($Glu_{1.0}$)]	E_iK	0.1	0
Poly[Lys($Thr_{1.0}$-DL-$Ala_{3.1}$)]	TAK	1.0	0
Poly[Lys(Succ-$Glu_{1.0}$-DL-$Ala_{4.0}$)]	Succ-EAK	0	0

[a] Transition temperatures were calculated from the first derivative of polarization/temperature sigmoidal curves.

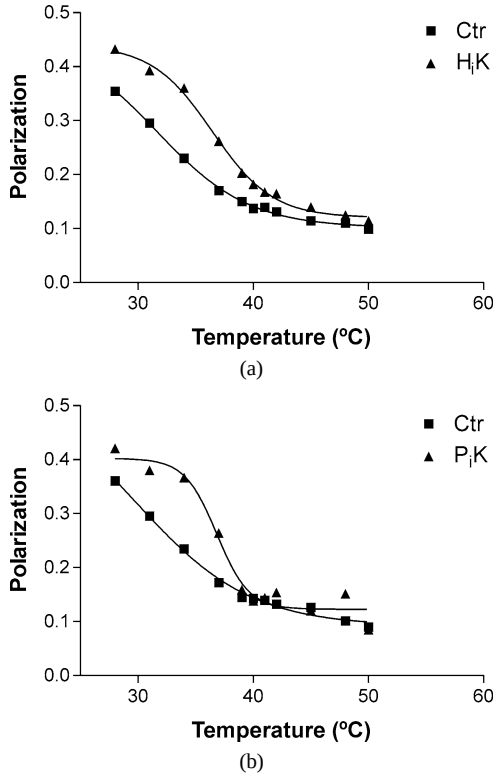

Fig. 3. Polarization/temperature curves recorded for DPH/DPPC-PG (8:2) vesicles in the presence or absence of branched polypeptides. (a) poly[Lys(His$_{0.56}$)] —▲—; (b) poly[Lys(Pro$_{0.95}$)] —▲—; control —■—.

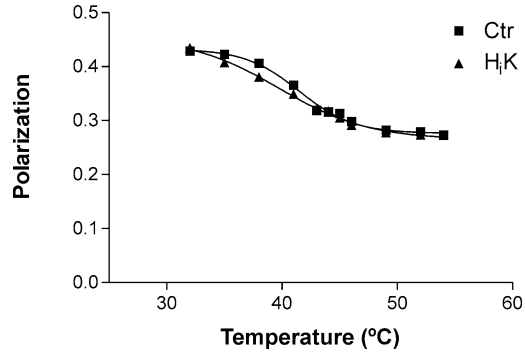

Fig. 4. Polarization/temperature curves recorded for TMA-DPH/DPPC vesicles in the presence or absence of branched polypeptide poly[Lys(His$_{0.56}$)] (H_iK) —▲—; control —■—.

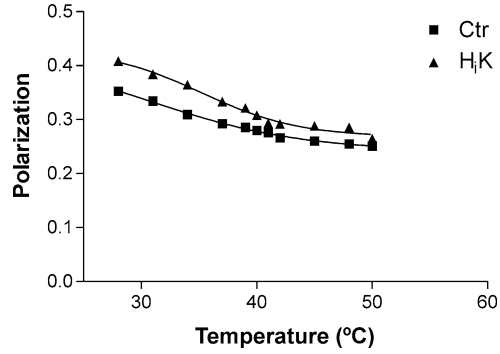

Fig. 5. Polarization/temperature curves recorded for TMA-DPH/DPPC-PG (8:2) vesicles in the presence or absence of branched polypeptide poly[Lys(His$_{0.56}$)] (H_iK) —▲—; control —■—.

structure has only a small rigidifying effect with a temperature increase of 1 °C (not shown). E_iH and Succ-EAK show no interaction with vesicles in this experiment.

3.4. Polarization fluorescence studies with TMA-DPH

Experiments carried out with liposomes saturated with TMA-DPH showed soft interactions between the lipid polar zone of the bilayer and the polymeric constructs (Tables 2 and 3). As a common trend polarization/temperature curves show a lower slope (without sudden changes around the T_c), than was previously described for samples involving DPH. This behavior has already been described [23] for other molecules that localize at the polar heads of phospholipids, and is mainly due to the external localization of probe molecules far away from the acyl chains that experience the fusion process. In most cases it was not possible to adjust the curves to a sigmoid and only qualitative differences between samples and references could be reported. Liposomes composed of DPPC showed no interaction with polymers except with the histidine containing derivative (Table 2). The presence of this polymer in the incubation media promotes a soft fluidification of the bilayers for lipids in gel state; T_c thus is 1.7 °C lower than that of the reference (DPPC), Fig. 4; up to this transition polarization/temperature curves are superimposable for both samples (reference and H_iK).

Concerning vesicles composed of DPPC/PG (0.8/0.2) saturated with TMA-DPH, the presence of polymers in the media, except for the H_iK derivative, has almost no effect on the microviscosity of the system (Fig. 5, Table 3). One can suggest that the presence of a certain percentage of negative charge on the surface of the liposome can attract the positively charged polymer side chains and this interaction results in a restriction in motion of the atoms involved in this area. Other polymers have no effect on this parameter.

Comparing the physicochemical results obtained with the two liposomal compositions and the five poly[L-lysine] derivatives, one can conclude that these polymeric constructions are by themselves safe, as far as the lack of destabilization of bilayers is concerned. All of them modify very softly the microviscosity of bilayers, and in some cases (H_iK/DPPC-PG) a beneficial effect has been reported as their presence results in an improvement of compactness of bilayers. The only exception is the glutamic acid derivative, which has a fluidifying effect that could eventually compromise the stability of vesicles.

Nevertheless, one can not exclude the possibility that this drawback could be beneficial if the direct interaction of polymers (E_iK) with cellular membranes was concerned, as this local fluidification could help the penetration of polymer-associated molecules into the cells.

Acknowledgments

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