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Shifts in chain-melting transition temperature of liposomal membranes by polymer-grafted lipids

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Abstract

The chain-melting transition temperature of dipalmitoyl phosphatidylcholine (DPPC) bilayer membranes containing poly(ethylene glycol)-grafted dipalmitoyl phosphatidylethanolamine (PEG-DPPE) was determined by optical turbidity measurements. The dependence on content, X_p, of PEG-DPPE lipid was studied for different polar headgroup sizes, n_p, of the polymer lipid, throughout the lamellar phase of the mixtures with DPPC. Mean-field theory for the polymer brush regime predicts that the downward shift in transition temperature should vary with polymer size and content as $n_p X_p^{5/3}$ ($\sim n_p X_p^{11/6}$ for scaling theory). Any shift induced by the charge on PEG-lipids is independent of polymer size. These predictions are reasonably borne out for the longer polymer lipids (PEG molecular masses 750, 2000 and 5000 Da). Transition temperature shifts in the lamellar phase, before the onset of micellisation, are in the region of -1 to -2 °C (± 0.1–0.2 °C) in reasonable accord with theoretical estimates of the lateral pressure exerted by the polymer brush. Shifts of this size are significant to the design of liposomes for controlled release of contents by mild hyperthermia.

Keywords: Polymer lipid; PEG-lipid; Liposome; Chain-melting transition; Controlled release; Mild hyperthermia; Optical turbidimetry

1. Introduction

Following the establishment of a maximum in permeability of lipid membranes for polar ionic solutes at the gel–fluid phase coexistence region [1–3], Needham et al. [4,5] have developed a liposomal formulation for drug delivery in which release can be triggered by mild hyperthermia that can be applied topically. The liposome composition is based on dipalmitoyl phosphatidylcholine (DPPC, transition temperature, 41.5 °C) to which is added on the order of 10 mol% of the single-chain lipid 1-palmitoyl lysophosphatidylcholine. The function of the lysolipid additive is to bring the chain-melting transition (which is reduced to 39–40 °C) closer to physiological temperatures, and to optimise the release characteristics [4]. To prolong the lifetime in the circulation, it is also necessary to add a polymer-grafted lipid, PEG:2000-phosphatidylethanolamine [5].

We have shown previously that, in the brush regime, polymer-grafted lipid additives exert a positive lateral pressure in lipid membranes [6,7]. This tendency towards lateral expansion will favour the fluid membrane phase and hence will depress the chain-melting transition temperature. Even small shifts in transition temperature are of practical relevance because the ultimate aim is to achieve directed chemotherapy of solid tumors by utilising only mild topical hyperthermia that is readily achieved in the clinic [5]. One of the design parameters for development of temperature-sensitive liposomes is therefore the influence of polymer-lipid content on chain melting.

In the present paper, we analyse the chain-melting transition of polymer-grafted liposomes theoretically, and we demonstrate experimentally that the shifts in transition temperature that are induced by polyethylene-glycol (PEG)-grafted lipids are of the size predicted. So as to concentrate on the effects of the polymer brush, we use PEG-grafted dipalmitoyl phosphatidylethanolamine (DPPE) that has a fatty acyl chain composition identical to that of DPPC, which constitutes the parent liposome. Systematic variation of the polymer size and polymer-lipid content of the liposomes reveals the parameters that are important for the design of temperature-sensitive liposomes, which are destined for applications in drug delivery.

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2. Materials and methods

2.1. Materials

Poly(ethylene glycol)-lipids (PEG-lipids), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-poly(ethylene glycol) with average polymer molecular weights of 350, 750, 2000 or 5000 Da (PEG:350-DPPE, PEG:750-DPPE, PEG:2000-DPPE, and PEG:5000-DPPE, respectively) were from Avanti Polar Lipids (Birmingham, AL). 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was from Sigma (St. Louis, MO). Reagent grade salts for the 10 mM phosphate buffer solution (PBS) at pH = 7.2 were from Merck (Darmstadt, Germany).

2.2. Spectrophotometric measurements

Samples for turbidity measurements were prepared by dissolving the required amounts of DPPC and PEG-DPPE in chloroform–methanol. The solvent was evaporated in a nitrogen gas stream and then kept under vacuum overnight. The dried lipid samples were hydrated with PBS at pH 7.2, by heating and vortexing for ca. 30 min at 45 °C. The lipid suspensions were transferred to 3-ml quartz cells of 1-cm optical path, and incubated at 4 °C before measurement. The final lipid concentration was ca. 2 mg/ml.

Optical density measurements were made at 400 nm with a Jasco 7850 spectrophotometer, equipped with a Peltier thermostatted cell holder (model EHC-441) and temperature programmer (model TPU-436; accuracy ± 0.1 °C). A heating rate of 1 °C/min was used. Data acquisition and manipulation were carried out with the built-in microcomputer accessory of the spectrophotometer.

3. Theoretical background

3.1. Transition temperature shifts

A perturbation calculation shows that the shift in chain-melting transition temperature of phospholipid membranes induced by an interaction that contributes an additional free energy $\Delta F_{\rm int}$ is given by (see e.g., Cevc and Marsh [8]):

$$\Delta T_{\rm t} = \delta\Delta F_{\rm int}/\Delta S_{\rm t} \tag{1}$$

where $\delta\Delta F_{\rm int}$ is the change in free energy of the additional interaction at the phase transition and $\Delta S_{\rm t}$ is the transition entropy for chain melting. Both are given as per mole of lipid.

The interaction energy can be expressed in terms of a lateral pressure increment: $\Delta\Pi_{\rm lat} = -(1/N_A)\partial\Delta F_{\rm int}/\partial A_{\rm l}$, where $A_{\rm l}$ is the membrane surface area per lipid molecule, giving the following expression for the shift in transition temperature:

$$\Delta T_{\rm t} = -N_A(\Delta\Pi_{\rm lat}/\Delta S_{\rm t})\Delta A_{\rm t} \tag{2}$$

where $\Delta A_{\rm t}$ is the increase in area per lipid molecule on chain melting, and N_A is Avogadro's number. In principle, there are several contributions to the lateral pressure increment:

$$\Delta\Pi_{\rm lat} = \Pi_{\rm p}^{\rm brush} + \Delta\Pi_{\rm el} + \Delta\Pi_{\rm chain} \tag{3}$$

where $\Pi_{\rm p}^{\rm brush}$ is the lateral pressure contributed by the grafted polymer brush, $\Delta\Pi_{\rm el}$ represents any contribution from change in surface electrostatics of the membrane, and $\Delta\Pi_{\rm chain}$ is the difference in lateral pressure exerted by the chains of the host and polymer-grafted lipids. We consider primarily the lateral pressure in the polymer brush. Any electrostatic contribution is screened out at high ionic strength, and $\Delta\Pi_{\rm chain} = 0$ for identical chain compositions of host and grafted lipids. Neither of these two latter possible contributions depend on the size of the grafted polymer.

The free energy associated with the polymer brush is given in the scaling and mean-field theories of polymer physics by Refs. [9,10]:

$$F_{\rm p}^{\rm brush} = N_A X_{\rm p} k_B T n_{\rm p} a_{\rm m}^{2m_F} (X_{\rm p}/A_{\rm l})^{m_F} \tag{4}$$

where $n_{\rm p}$ is the number and $a_{\rm m}$ the size of monomer units in the polymer, and $X_{\rm p}$ is the mole fraction of polymer lipids. The exponent m_F is 5/6 in the Alexander-De Gennes scaling theory and 2/3 in mean-field theory; k_B is Boltzmann's constant and T is the absolute temperature. A uniform surface density, $X_{\rm p}/A_{\rm l}$, of polymer lipid is assumed. In Eq. (4), the free energy, $F_{\rm p}^{\rm brush}$, is expressed as per mole of lipid. Therefore, the lateral pressure ($\Pi_{\rm p} = -N_A^{-1}\partial F_{\rm p}/\partial A_{\rm l}$) created by the polymer brush is given by:

$$\Pi_{\rm p}^{\rm brush} = m_F k_B T n_{\rm p} a_{\rm m}^{2m_F} (X_{\rm p}/A_{\rm l})^{m_F+1} \tag{5}$$

From Eqs. (2) and (5), the shift in chain-melting transition temperature induced by the polymer lipid is finally given by:

$$\Delta T_{\rm t} = -\frac{N_A k_B T_{\rm t}}{\Delta S_{\rm t}} \left(\frac{\Delta A_{\rm t}}{A_{\rm l}}\right) \left(\frac{a_{\rm m}^2}{A_{\rm l}}\right)^{m_F} m_F n_{\rm p} X_{\rm p}^{m_F+1} \tag{6}$$

Typical mean values for the lipid molecular area and its change on chain melting are: $A_{\rm l} \approx 0.55$ nm^2 and $\Delta A_{\rm t}/A_{\rm l} \approx 0.18$, respectively, e.g., for phosphatidylcholines [11]. For a PEG polymer, the effective monomer size is close to: $a_{\rm m} \approx 0.39$ nm [12].

Taking calorimetric values of $\Delta S_{\rm t} = 115$ J mol^{-1} K^{-1} and $T_{\rm t} = 314$ K, appropriate to DPPC (see e.g., Marsh [11]), Eq. (6) then yields estimates of the shift in transition temperature of DPPC induced by PEG-DPPE lipids of various polymer sizes that are given in Table 1. These shifts are calculated for the maximum content of PEG-lipid in DPPC lamellar membranes before micelles begin to form. Values for the PEG-DPPE content, $X_{\rm p}^{\rm on}$, at the onset of micellisation are taken from spin-label EPR measurements by Belsito et al. [13] and Montesano et al. [6]. Longer

polymer lipids create a greater lateral pressure and therefore are more effective in reducing the transition temperature. In Table 1, however, somewhat larger shifts in transition temperature are predicted for the shorter polymer lipids, because a higher content of PEG-lipid can be incorporated prior to micelle formation. The dependence of the shift in transition temperature on polymer lipid content that is predicted by Eq. (6), is given in Fig. 1 for DPPC mixed with PEG-lipids of different sizes. On the whole, the predicted shifts are relatively modest, on the order of 1–2°, but as previously explained in the Introduction, these are of practical relevance to the design of liposomes for controlled release by mild hyperthermia [4,5].

The above can be contrasted with the shift in transition temperature induced by a solute that does not undergo chain melting and is immiscible with the gel-phase lipid. The entropy of mixing then gives: $\delta\Delta F_{\mathrm{int}} = N_A k_B T_t \ln(1 - X_p)$ for the free energy decrement in Eq. (1). In this case, the classical freezing-point depression is: $\Delta T_t = -0.69, -1.65, -3.16$ and -5.64 K for $X_p = 0.03, 0.07, 0.13$ and 0.22, respectively. It can be assumed that, at relatively low mole fractions, polymer lipids mix well with phosphatidylcholine lipids of equal chain length (cf. analogous negatively charged phosphatidylglycerols in Marsh [11]). Therefore, no classical freezing-point depression by PEG-lipids would be expected in the absence of interactions within the polymer brush.

The electrostatic lateral pressure, Π_{el}, of a bilayer membrane can be estimated from Gouy-Chapman diffuse double layer theory [8,14]. Using this expression for Π_{el}, together with Eq. (2), gives rise to the following expression for the shift in transition temperature by lipid surface charge:

$$\Delta T_t^{\mathrm{el}} = -\left(\frac{RT_t}{e\Delta S_t}\right)\sqrt{\frac{\varepsilon R T_t I}{125\pi}}\left[\sqrt{1 + \frac{500\pi}{\varepsilon R T_t I}\left(\frac{eX_p}{A_l}\right)^2} - 1\right]\Delta A_t \tag{7}$$

where e is the electron charge, ε is the aqueous dielectric constant, I is the ionic strength and $R = N_A k_B$. Because simple electrostatic double-layer theory is known to overestimate the membrane surface potential (see e.g., Cevc and Marsh [8]), Eq. (7) provides an upper estimate for the decrease in transition temperature induced by the negative charge on the PEG-lipid. With an ionic strength $I = 0.1$, the predicted electrostatic shift is $\Delta T_t^{\mathrm{el}} = -0.03, -0.14, -0.44$ and -1.03 K for $X_p = 0.03, 0.07, 0.13$ and 0.22, respectively. The electrostatic shift is therefore expected to be less than the polymer-induced shift for all cases, except for high concentrations of the short polymer lipid.

Table 1
Shifts in chain-melting transition temperature of DPPC bilayer membranes at maximum content, X_p^{on}, of PEG-DPPE lipids with different polymer sizes, n_p, predicted from Eq. (6) by mean-field and scaling theories[a]

Lipid	n_p	X_p^{on}	ΔT_t^{MF} (°C)[b]	ΔT_t^{SC} (°C)[c]
PEG:350-DPPE	8	0.22 ± 0.07	-0.74 ± 0.4	-0.58 ± 0.34
PEG:750-DPPE	17	0.13[d]	−0.66	−0.47
PEG:2000-DPPE	45	0.07 ± 0.03	-0.62 ± 0.44	-0.40 ± 0.32
PEG:5000-DPPE	114	0.03 ± 0.01	-0.38 ± 0.2	-0.21 ± 0.13

[a] Mole fractions of polymer lipid, X_p^{on}, at the onset of micelle formation, together with uncertainty ranges, are from Belsito et al. [13] and Montesano et al. [6].
[b] Mean-field theory.
[c] Scaling theory.
[d] Interpolated value from Montesano et al. [6].

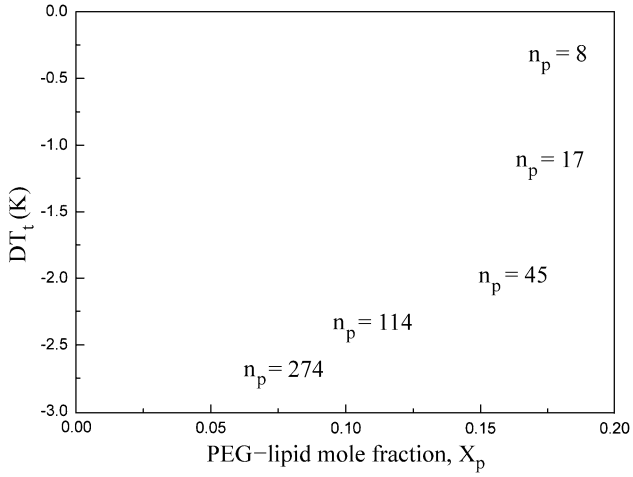

Fig. 1. Shift (ΔT_t) in chain-melting transition temperature of DPPC bilayer membranes as a function of mole fraction, X_p, of PEG-lipids with different polymer sizes, n_p, predicted from Eq. (6) by mean field (solid lines) and scaling (dashed lines) theories.

4. Results and discussion

Measurements were all confined to the lamellar phase of DPPC/PEG-DPPE mixtures, i.e., to contents of polymer lipid below those at which micelle formation begins. The mole fractions of PEG-lipid at the onset of micellisation have been determined as: $X_{\mathrm{PEG}}^{\mathrm{on}} = 0.22 \pm 0.07$, 0.07 ± 0.03 and 0.03 ± 0.01 for mixtures of DPPC with PEG:350-DPPE, PEG:2000-DPPE and PEG:5000-DPPE, respectively [6,13]. These values and their range of uncertainty were determined by using spin-labelled phospholipids to detect micelle formation, which was quantitated by fitting to the lever rule for coexisting phases in two-component systems. For PEG:750-DPPE, an interpolated value is: $X_{\mathrm{PEG}}^{\mathrm{on}} = 0.13$ (see Montesano et al. [6]).

4.1. Transition temperature shifts

Chain melting is accompanied by an increase in lipid partial specific volume [15] and hence, from the Clausius–Mossotti relation, by a decrease in refractive index. The latter is reflected as a decrease in light scattering and turbidity. The temperature dependence of the optical density of DPPC dispersions and of DPPC containing PEG-DPPE

lipids of different polymer lengths is given in Fig. 2. The sharp discontinuity at the chain-melting temperature (T_t = 41.5 °C) of DPPC membranes and the downward shift in transition temperature on adding PEG-grafted lipids of equal hydrocarbon chain length is clearly seen. With a slight hysteresis, the OD measurements are reversible and increases in turbidity are observed upon cooling of the sample through the phase transition temperature (data not shown).

Note that, in the case of any micellar contamination, it is the lamellar component that gives rise to the sharp chain-melting transition [6]. Micelles of PEG-lipids do not exhibit cooperative chain melting in this temperature range.

The dependence of the chain-melting temperature on content of polymer lipid is given in Fig. 3 for admixtures with PEG:750-DPPE, PEG:2000-DPPE or PEG:5000 DPPE. Based on Eq. (6), non-linear least-squares fits of the following expression:

$$T_t = T_t^o(1 - b_p X_p^{m_F+1}) \quad (8)$$

to the dependence of the transition temperature on polymer lipid content, X_p, are also given in Fig. 3. The general trend of decreasing transition temperature with increasing content of polymer lipid is reasonably described by Eq. (8), for all PEG-lipids except the shortest, i.e., PEG:350-DPPE (data not shown). For admixtures of PEG:350-DPPE restricted to the range X_p = 0.05–0.23 corresponding solely to the brush regime [16], the transition temperature remains approximately constant, and even increases a little. It is possible that the scaling and mean-field polymer theories are not strictly applicable to such short polymers as PEG:350, which contains only eight monomers.

The fitting parameters according to Eq. (8) are given in Table 2 for the different polymer lipids. Because the measured shifts in transition temperature are intrinsically

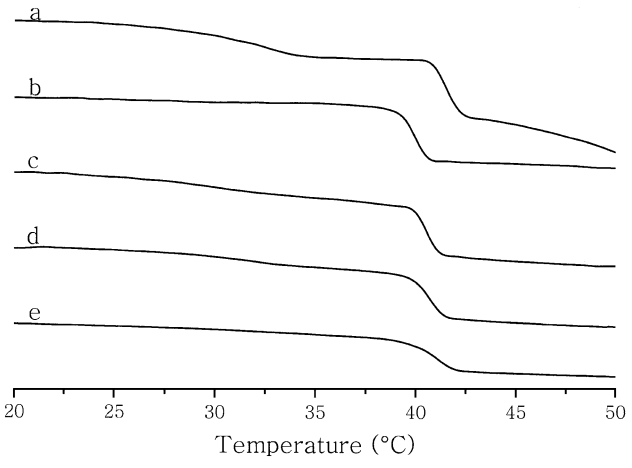

Fig. 2. Temperature dependence of the optical density at 400 nm for dispersions of: DPPC (a), and of DPPC containing 20 mol% of PEG:350-DPPE (b), 12 mol% of PEG:750-DPPE (c), 7 mol% PEG:2000-DPPE (d), and 4 mol% PEG:5000-DPPE (e).

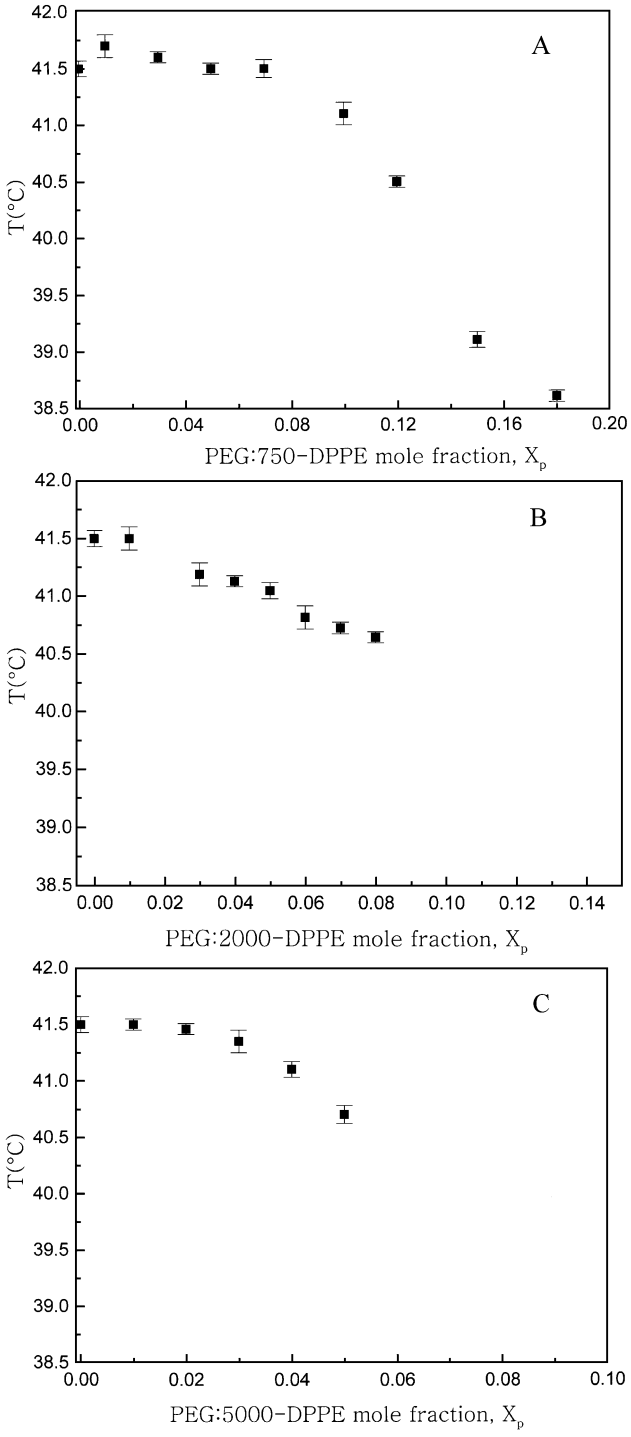

Fig. 3. Dependence of the chain-melting temperature, T_t, of DPPC on content of PEG-DPPE polymer lipid: PEG:750-DPPE (A), PEG:2000-DPPE (B) and PEG:5000-DPPE (C).

small, the inherent precision is not very high, but sufficient to distinguish trends. Additionally, the effects observed are large enough to be of relevance clinically. According to Eq. (6), the parameter $b_p (= m_F(N_A k_B/\Delta S_t)(\Delta A_t/A_l)(a_m^2/A_l)^{m_F} n_p)$ should be directly proportional to the polymer size, n_p. From Table 2, this is seen to be approximately the case

Table 2
Parameters, b_p, fitting the dependence on PEG-lipid content of the shift in DPPC chain-melting transition temperature, T_t^o, according to Eq. (8) for mean-field and scaling theories[a]

Lipid	T_t^o (K)[b]	b_p^{MF} [b]	T_t^o (K)[c]	b_p^{SC} [c]
PEG:750:DPPE	315.1 ± 0.2	0.18 ± 0.02	314.9 ± 0.1	0.24 ± 0.02
PEG:2000-DPPE	314.57 ± 0.04	0.18 ± 0.01	314.54 ± 0.04	0.25 ± 0.02
PEG:5000-DPPE	314.72 ± 0.06	0.37 ± 0.05	314.70 ± 0.05	0.61 ± 0.07

[a] Error ranges are determined from χ^2 and the diagonal elements of the variance–covariance matrix for the fits.
[b] Mean-field theory.
[c] Scaling theory.

when comparing the three polymer lipids PEG:750-DPPE, PEG:2000-DPPE and PEG:5000-DPPE. Note that neither the charge on the PEG-lipids nor the lipid (DPPE) to which the PEG is attached can explain the dependence of the shift in chain-melting transition on polymer size, n_p. A 1° depression in transition temperature is achieved by approximately 12 mol% DPPE-PEG:750, 8 mol% DPPE-PEG:2000 and 5 mol% DPPE-PEG:5000 (see Fig. 3). These differences must be attributed directly to the PEG-polymer.

4.2. Comparison with other measurements

Previous studies involving non-matched hydrocarbon chain lengths have reported increases in transition temperature on adding PEG-distearoyl phosphatidylethanolamine (DSPE) lipids to DPPC [17,18]. Control experiments with the non-polymer lipid DSPE indicate that the reported upward shifts are attributable to the increased hydrocarbon chain length of the polymer lipid [18]. This corresponds to a negative value of $\Delta\Pi_{chain}$ in Eq. (3). The net transition temperature shift is then given by $\Delta T_t^{(tot)} = \Delta T_t^{(p)} + \Delta T_t^{(chain)}$, where the polymer contribution, $\Delta T_t^{(p)}$, to the shift in chain-melting transition temperature is negative, but the contribution from chain mismatch, $\Delta T_t^{(chain)}$, is positive as indicated by measurements with the ungrafted DSPE. For shorter polymers with molecular masses up to PEG:3000-DSPE, the effects of chain mismatch dominate and the DPPC chain-melting temperature increases with polymer-lipid content. Admixture of PEG:5000-DSPE hardly shifts the DPPC phase transition at all; the two contributions then compensate almost exactly. Finally, for the very long polymer in PEG:12000-DSPE, the polymer contribution dominates and the transition temperature decreases with increasing polymer-lipid content [18], in agreement with the present results.

5. Conclusions and implications

The size of the shifts in chain-melting transition that are induced by admixture of PEG-lipids is such that this must be taken into account in the formulation of temperature-sensitive liposomes. Indeed, it could provide an additional means for tuning the chain-melting transition temperature to the near-physiological range. In principle, the polymer-lipid contribution can be varied by means of both polymer size and polymer-lipid content. The theoretical calculations presented here should aid in this aspect of hyperthermic liposome design. As already noted, mismatch in fatty acyl chain length between polymer-lipid and host lipid will also influence the size of transition temperature shifts. For PEG-lipids with chains longer than those of the host, the effect will oppose that of the polymer and a fine balance might be achieved. Consideration of these basic principles could help to develop and further optimise liposomes that are directed for topical release in systemic pharmacological applications.

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