The Effects of Ethylene Oxide Containing Lipopolymers and Tri-Block Copolymers on Lipid Bilayers of Dipalmitoylphosphatidylcholine

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ABSTRACT A comparative study is conducted on the influence of two types of polymeric compounds on the phase behavior of 1,2-dihexadecanoyl-s,n-glycero-3-phosphotidylcholine ($DC_{16}PC$) lipid bilayers. The first polymeric compound is a lipopolymer, with two different lengths of a hydrophilic polyethylene oxide moity, anchored to the bilayer by a 1,2-dioctadecanoyl-s,n-glycero-3-phosphoethanolamine ($DC_{18}PE$) lipid. The second type, which is a novel type of membrane-spanning object, is an amphiphilic tri-block copolymer composed of two hydrophilic stretches of polyethylene oxide separated by a hydrophobic stretch of polystyrene. Hence the tri-block copolymer may act as a membrane-spanning macromolecule mimicking an amphiphilic protein or polypeptide. Differential scanning calorimetry is used to determine a partial phase diagram for the lipopolymer systems and to assess the amount of lipopolymer that can be loaded into $DC_{16}PC$ lipid bilayers before micellization takes place. Unilamellar and micellar phase structures are investigated by fluorescence quenching using bilayer permeating dithionite. The chain length-dependent critical lipopolymer concentration, denoting the lamellar-to-micellar phase transition, compares favorably with a theoretical prediction based on free-energy considerations involving bilayer cohesion and lateral pressure exerted by the polymer chains.

INTRODUCTION

Lipid mixtures of phospholipids and lipopolymers (polyethylene oxide headgroup coupled to 1,2-dioctadecanoyl-s,n-glycero-3-phosphoethanolamine ($DC_{18}PE$)) have attracted much attention in recent years, both experimentally (Baekmark et al., 1995; Bedu-Addo and Huang, 1993; Blume and Cevc, 1993; Blume et al., 1993; Hristova et al., 1995; Kenworthy et al., 1995a,b; Kuhl et al., 1994; Lasic et al., 1991; Lasic, 1994; Lasic and Papahadjopoulos, 1995; Maruyama et al., 1995; Parr et al., 1994; Torchilin et al., 1994b; Warriner et al., 1996; Woodle and Lasic, 1992) and theoretically (Hristova and Needham, 1994, 1995; Torchilin et al., 1994a). These systems are interesting because they may serve as models for the cell glycocalyx. Another reason for the strong interest in these systems lies in the enhanced in vivo circulation time in the bloodstream of liposomes made from lipid mixtures of lipopolymers and phospholipids (and possibly cholesterol). The prolonged lifetime is generally ascribed to a steric stabilization of the liposomes exerted by the polymeric heads (Lasic et al., 1991). Such liposomes are therefore of medical interest as potential liposomal drug delivery systems (Blume and Cevc, 1993; Blume et al., 1993; Lasic et al., 1991; Lasic, 1994; Lasic and Needham, 1995; Lasic and Papahadjopoulos, 1995, 1996; Maruyama et al., 1995; Parr et al., 1994; Torchilin et al., 1994b; Woodle and Lasic, 1992), and clinical studies have been undertaken (Lasic and Papahadjopoulos, 1995, 1996), so far with promising results.

However, the previously assumed general stabilization of the vesicles by the polymer at all lipopolymer concentrations up to ~60% of lipopolymer, at which a transition to spherical micelles is observed (Lasic et al., 1991), has recently been questioned (Hristova and Needham, 1994, 1995; Hristova et al., 1995; Kenworthy et al., 1995a,b; Warriner et al., 1996). Hristova and Needham (1994, 1995) proposed that high water concentrations, above some lipopolymer threshold concentration, n_{tr}, depending on the system in question, the vesicles will break down and form nonspherical micellar lipid phases. This was later verified by x-ray studies (Hristova et al., 1995; Kenworthy et al., 1995a,b). For mixtures of $DC_{14}PE$ with $DC_{14}PE\text{-}EO_{45}$ or $DC_{14}PE$ with $DC_{14}PE\text{-}EO_{113}$ (where EO_N is polyethylene oxide of monomer number N) at changing water content (from dilute to concentrated), Warriner et al. (1996) observed the formation of lamellar gels using x-ray diffraction.

In the present paper we present a comparative study of the properties of unilamellar vesicles in the presence of two different kinds of polymeric molecules: polyethylene oxide (PEO) lipopolymers with two different polymer chain lengths and a specific amphiphilic tri-block copolymer. Both studies were carried out at high water concentrations (>99% water weight).

Using differential scanning calorimetry (DSC), we have studied unilamellar vesicles with average radii of 50 nm made from binary mixtures composed of the lipopolymer $DC_{18}PE\text{-}EO_N$ (N = 45 or 110) and a phospholipid, 1,2-dihexadecanoyl-s,n-glycero-3-phosphotidylcholine ($DC_{16}PC$), which, compared to the lipopolymer, contains two carbon atoms less in its acyl chains. Previous DSC studies (Bedu-

Received for publication 30 December 1996 and in final form 9 June 1997.

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0006-3495/97/09/1479/13 $2.00

Addo and Huang, 1993; Blume and Cevc, 1993; Kenworthy et al., 1995a) have focused on multilamellar vesicles of phospholipids and lipopolymers, with the two components having the same number of carbon atoms in the acyl chains. Fluorescence quench experiments were used to gain insight into the lamellar and micellar phase structures of the binary mixtures of lipopolymers and phospholipids.

In a series of two papers, Kostarelos et al. (1995a,b) proposed the use of membrane-spanning tri-block copolymers to sterically stabilize liposomes instead of the lipopolymer scheme. The insertion of membrane-spanning molecules, like bola lipids (Duwe et al., 1990; Lemmich et al., 1996), transmembrane proteins, or polypeptides (Sperotto and Mouritsen, 1988; Mouritsen and Bloom, 1984, 1993; Zhang et al., 1993), is known to have considerable influence on both phase equilibria as well as the structure of lipid-bilayer membranes. In the present work we have examined a novel ABA-type (hydrophilic-hydrophobic-hydrophilic) tri-block copolymer (tri42) with 42 monomers in each segment, incorporated into $DC_{16}PC$ bilayers. Each block is therefore of the same size as the PEO part of the lipopolymer $DC_{18}PE\text{-}EO_{45}$.

Not only did we want to study the triblock copolymer sterical stabilization effect, we also wanted to test the polymer for possible use as a model membrane-spanning protein. Furthermore, we intended to set up a reference system for future systematic experimental examination of the mattress model of lipid-protein interactions in membranes (Mouritsen and Bloom, 1984) by varying the degree of polymerization within the two polymer parts, especially within the hydrophobic domain. We consider tri-block copolymers suitable for testing the hydrophobic-matching principle of the mattress model, not only because of the possibility of varying the size of the polymeric segments, but also because we expect that the hydrophilic parts of such molecules and the interactions with the hydrophilic headgroup regions of the lipid bilayer will help to force the hydrophobic part of the amphiphilic tri-block copolymer to span the membrane and match the hydrophobic acyl-chain part of the bilayer. An additional aspect of working with the polymer tri42 is the closeness in size to the hydrophilic part of the polymeric head of the lipopolymer $DC_{18}PE\text{-}EO_{45}$. This should allow for a comparison between the results of the two series of experiments, particularly because the hydrophilic part of tri42 is polyethylene oxide (PEO), as in the lipopolymers.

MATERIALS AND METHODS

Materials

$DC_{16}PC$ and $DC_{18}PE$ of a purity higher than 99% were obtained from Sigma Chemical Co. The lipopolymers used, $DC_{18}PE\text{-}EO_N$ (N = 45 and 110), were gifts from Prof. Dr. E. Sackmann (München, Germany). The ethylene oxide headgroups have polydispersities of 1.1 and molecular weights of 2000 and 5000 (Baekmark et al., 1995; Lasic, 1994), respectively. The tri-block copolymer, tri42, was a gift from Prof. Dr. G. Riess (Mulhouse, France). It consists of three polymer segments linearly connected, type ABA, with polyethylene oxide (PEO) segment A with a molecular weight of 1800, and polystyrene (PS) B, with a molecular weight of 2400. The segments contain ~42 monomers each. All chemicals were used without further purification. The headgroup labeled fluorescent lipid probe *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (NBD-PE) was obtained from Molecular Probes (Eugene, OR).

Methods

Lipid mixtures were made by mixing the phospholipids with the lipopolymers or the tri-block copolymers in chloroform. A clean N_2 stream was used to evaporate the solvent, followed by subsequent drying at low pressure for at least 12 h. Multilamellar vesicles (5 mM) were created by suspending the dry lipid powder mixtures in a solution of 50 mM KCl and 1 mM NaN_3 (pH 6.75) for a minimum period of 2 h at 60°C, during which they were shaken vigorously every 15 min.

Unilamellar vesicles with narrow size distributions were created from the multilamellar vesicle suspensions by the extrusion method as described by Hope et al. (Hope et al., 1985; Mayer et al., 1986). The lipid suspensions were extruded 10 times through two stacked polycarbonate filters with pore sizes of either 50 or 100 nm. For vesicles incorporated with the tri-block copolymer, filters with pore sizes of 100 nm were used. All other vesicles were produced using pore sizes of 50 nm. Upon preparation we found that the time necessary for the samples to pass through the extruder increased with increasing polymer content. For two of the samples it was impossible to extrude more than six times. (These samples were 10% $DC_{18}PE\text{-}EO_{110}$ and 5% tri42. The tri42 suspension was left in the extruder for 2 h at 40 atm without passing the filters, and not even a change of filters allowed for a seventh extrusion.) We were unable to prepare extruded unilamellar vesicles of $DC_{18}PE\text{-}EO_{110}$ and tri42 containing higher concentrations of polymer than 10% and 5%, respectively. No attempt was made to produce vesicles containing more than 40% $DC_{18}PE\text{-}EO_{45}$. Increasing the polymer concentration of both lipopolymers as well as of tri42 led to an increased production of foam. This is known to happen in water-polymer systems (Pradhan and Khilar, 1994), because the polymer stabilizes the foam. Thus the stated polymer (and lipid) contents can be taken only as upper limit values.

The heat capacity at constant pressure, C_p, was measured as a function of temperature with a Microcal MC-2 DSC instrument (Northampton, MA). Scans presented in this work are averages of the last three of six consecutive scans performed at a scan rate of 20°C/h. All C_p scans presented are scan-rate and concentration normalized and have had reference scans of buffer versus buffer subtracted from the raw scans.

Values for the enthalpy of transition, ΔH_T, the transition temperature, T_m, taken at maximum peak height as well as the peak width $\Delta T_{1/2}$ at half peak height, were calculated with the Microcal Origin software package.

Fluorescence measurements were made with a SLM DMX-1100 fluorometer (SLM Instruments, Urbana, IL). The fluorescence intensity of 150 μM unilamellar vesicles incorporated with 0.5% flourescent NBD-PE probes was obtained using a bandpath of 4 nm for both the emission and excitation monochromators. The excitation and emission wavelengths for NBD-PE were 470 nm and 530 nm, respectively. The lipid samples were placed in a thermostatted cuvette controlled by an external water bath. The fluorescence quench measurements were performed at 25°C by adding 40 μl of a freshly made 1 M sodium dithionite solution to 3-ml lipid samples containing 0, 2.5, and 40% $DC_{18}PE\text{-}EO_{45}$. Triton X-100 (150 μl) was added to the lipid suspension to solubilize the lipid mixture and quench any remaining fluorescence. The time development of the fluorescence signal, F, was normalized by the stable fluorescence signal, F_0, before dithionite treatment. Dithionite quenching techniques of this type used to measure bilayer packing defects and permeability have previously been used by Langner and Hui (1993).

RESULTS

Mixtures of $DC_{16}PC$ and $DC_{18}PE\text{-}EO_N$ lipopolymers

In this section we present results obtained by DSC on binary mixtures of $DC_{16}PC$ incorporated with $DC_{18}PE\text{-}EO_{45}$ and

$DC_{18}PE\text{-}EO_{110}$. For comparative purposes, we also report results for the binary lipid mixture $DC_{16}PC\text{-}DC_{18}PE$. The specific heat data, C_p, are shown for these three cases in Fig. 1, and the corresponding transition enthalpies, ΔH, are given in Fig. 2. The C_p curves of Fig. 1 *A* are characterized by two major features. A rather sharp peak ($\Delta T_{1/2} < 2°C$) appears in the C_p curves at ~41.4°C, and the peak position is found to be rather insensitive to changes in composition, whereas its intensity decreases with increasing concentration of $DC_{18}PE\text{-}EO_{45}$. Furthermore, a second peak is found to appear on the high-temperature side of the sharp peak from ~10% $DC_{18}PE\text{-}EO_{45}$. This second peak is rather broad and becomes broader as the lipopolymer concentration is increased. Furthermore, the position of the second peak moves toward higher temperatures and the intensity increases as a function of lipopolymer content. The C_p curves of Fig. 1 *B* reveal the same general trends as those of Fig. 1 *A*. This is manifested as a rather sharp low-temperature peak that decreases in intensity with increasing lipopolymer content, whereas a high-temperature broader peak appears for mixtures containing more than ~5% lipopolymer. Contrary to this, the C_p curves in Fig. 1 *C* for the $DC_{16}PC\text{-}DC_{18}PE$ mixture reveal a considerable broadening as the $DC_{18}PE$ content increases. The development of a broad shoulder at high concentrations of $DC_{18}PE$ signals the chain melting of predominantly $DC_{18}PE$ lipids. For pure $DC_{16}PC$ vesicles, the peak is, as expected for unilamellar vesicles (Inoue et al., 1993; Pedersen et al., 1996), rather broad, and the pretransition can hardly be seen.

The precursor for the development of a second peak in terms of a shoulder on the C_p scans is analyzed in more detail in Fig. 3, which compares the pure $DC_{16}PC$ unilamellar behavior with that for $DC_{16}PC$ bilayers incorporated with 5% $DC_{18}PE\text{-}EO_{45}$, $DC_{18}PE\text{-}EO_{110}$, or $DC_{18}PE$. A strong effect on the bilayer phase behavior is observed for the lipopolymer-containing samples. From the C_p scans we have extracted values for the transition enthalpy, ΔH, shown in Fig. 2; the temperature, T_m, at maximum peak-height, shown in Fig. 4; and the half-width, $\Delta T_{1/2}$, tabulated in Table 1. In the case of more than one peak appearing in the scans, we use the indices "low" and "high" to denote, respectively, the lowest and highest peaks with respect to temperature.

In Fig. 5 are shown the results from adding the irreversible sodium dithionite quencher to fluorescent labeled NBD-PE lipid samples incorporated with 0, 2.5, and 40% of the $DC_{18}PE\text{-}EO_{45}$ lipopolymers. Immediately after adding the dithionite quencher, a fast reduction of more than half of the fluorescence signal takes place. This behavior corresponds to an irreversible quenching of the fluorescent NBD-PE probes positioned in the outer leaflet of the bilay-

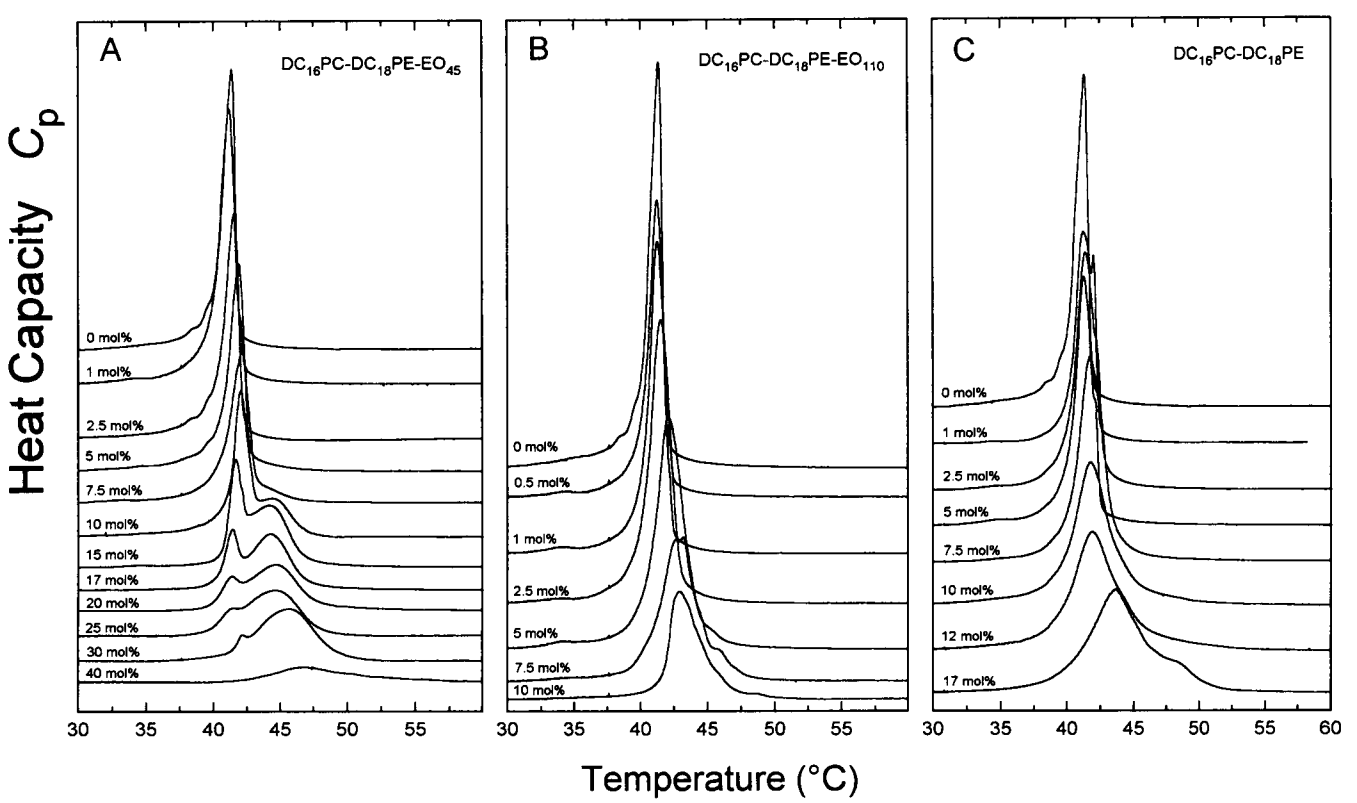

FIGURE 1 Specific heat (arbitrary units) of unilamellar vesicles of $DC_{16}PC$ mixed with (*A*) $DC_{18}PE\text{-}EO_{45}$, (*B*) $DC_{18}PE\text{-}EO_{110}$, and (*C*) $DC_{18}PE$. Percentages refer to mol% of total lipid content.

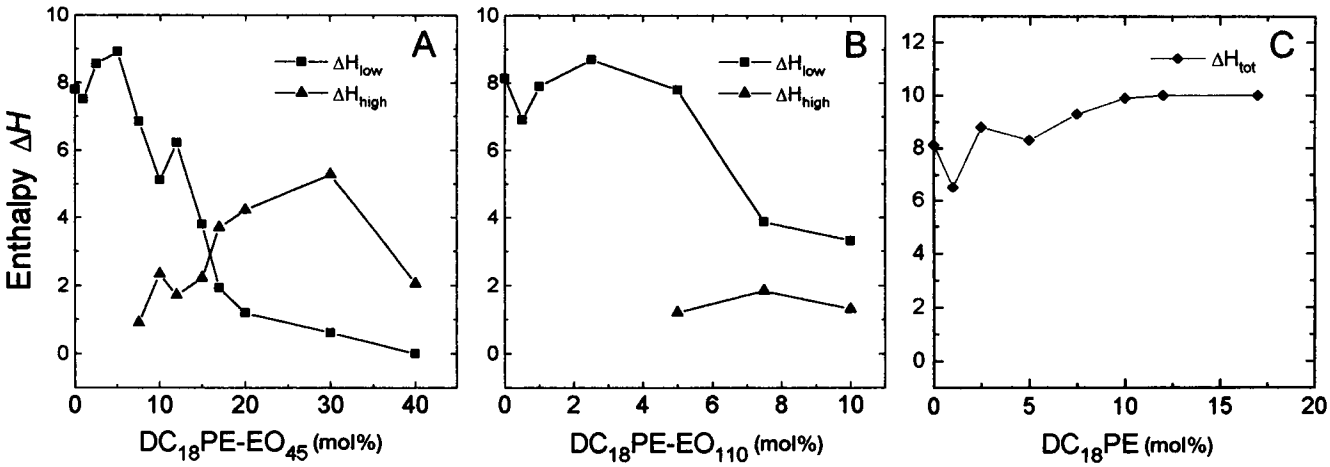

FIGURE 2 Measured transition enthalpy (kcal/mol) for mixtures (cf. Table 1) of $DC_{16}PC$ with (*A*) $DC_{18}PE\text{-}EO_{45}$, (*B*) $DC_{18}PE\text{-}EO_{110}$, and (*C*) $DC_{18}PE$.

ers. The interplay between bilayer curvature and molecular packing constraints favors a higher concentration of the bulky headgroup labeled NBD-PE fluorophores in the outer leaflet of the lipid bilayer, leading to a dithionite quenching of more than half of the fluorescence signal. The quenching results show that at 25°C, the vesicles, although created at 60°C, are present mainly as unilamellar vesicles. When Triton X-100 is added to the samples containing 0 and 2.5% of the $DC_{18}PE\text{-}EO_{45}$ lipopolymer, the remaining fluorescence is quenched because of solubilization of the lipid systems. Addition of dithionite to lipid samples containing 40% of the $DC_{18}PE\text{-}EO_{45}$ lipopolymer leads to a complete quenching of the fluorescence signal. This behavior indicates that all of the headgroup labeled NBD-PE probes are accessible to the dithionite quenching agent, as is the case when the lipid mixture adopts a micellar structure with the NBD fluorescent moities oriented toward the outer aqueous phase (Langner and Hui, 1993).

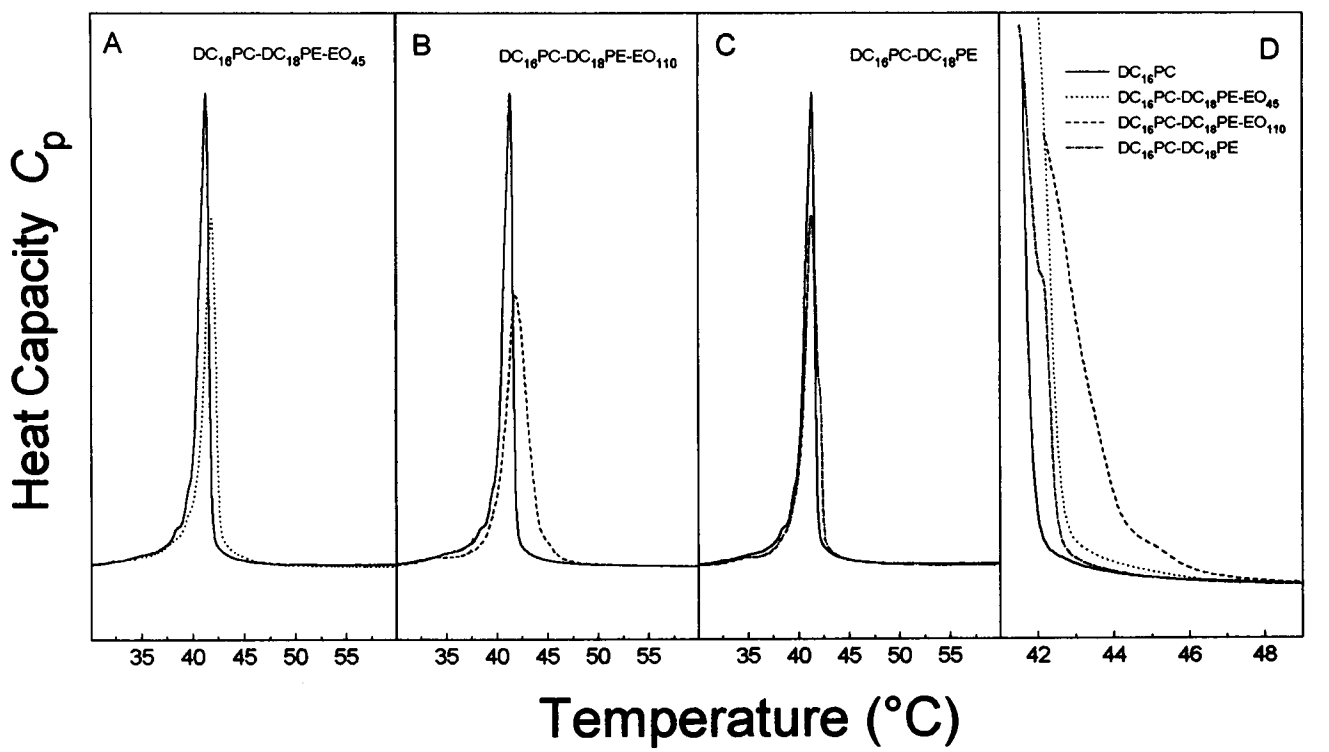

FIGURE 3 Possible development of a shoulder on the upper temperature side of the main peak in the DSC scans (arbitrary units) shown in Fig. 1 for mixtures of $DC_{16}PC$ with 5% of (*A*) $DC_{18}PE\text{-}EO_{45}$, (*B*) $DC_{18}PE\text{-}EO_{110}$, or (*C*) $DC_{18}PE$. The specific heat for the pure $DC_{16}PC$ bilayer is indicated by solid lines. (*D*) Magnification of the upper temperature side of the main peak.

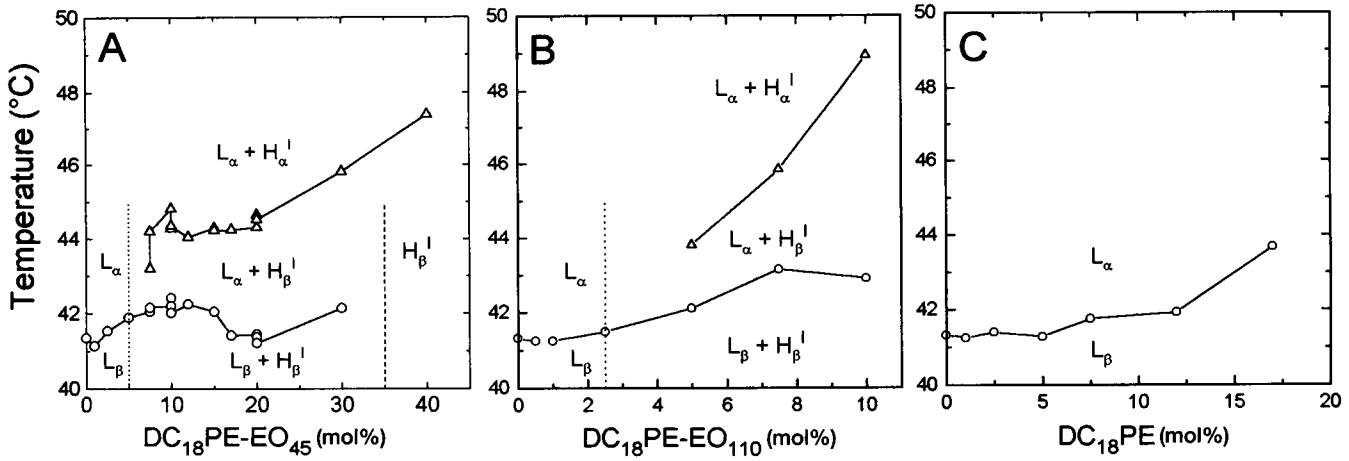

FIGURE 4 Tentative phase diagrams for unilamellar vesicles of $DC_{16}PC$ incorporated with (*A*) $DC_{18}PE\text{-}EO_{45}$, (*B*) $DC_{18}PE\text{-}EO_{110}$, or (*C*) $DC_{18}PE$. Fully drawn lines represent phase boundaries as revealed by DSC. In *A* and *B* the dotted vertical line indicates a phase boundary between an exclusively bilayer phase and a coexistence region of bilayer and micellar phases. The dashed horizontal line in *A* indicates the boundary between the coexistence region of bilayer and micellar phases and an exclusively micellar phase.

Mixtures of $DC_{16}PC$ and the amphiphilic tri-block copolymer tri42

The heat capacity curves of unilamellar vesicles of $DC_{16}PC$ with average radii of 100 nm for different contents of the tri-block copolymer tri42 are shown in Fig. 6. In Table 2 we tabulate the measured values of ΔH_T, T_m, and $\Delta T_{1/2}$, and in Figs. 7 and 8 we show, respectively, the variations in T_m and ΔH_T with copolymer content. For the sake of comparison, these figures also display the corresponding data for the two lipopolymers as well as for $DC_{18}PE$ at the same low concentrations. It is seen that both T_m and ΔH_T go through a minimum at ~1% polymer content for $DC_{18}PE\text{-}EO_{45}$ and

TABLE 1 Values for T_m, $\Delta T_{1/2}$, and ΔH_T for unilamellar vesicles incorporated with lipopolymers as derived from the DSC data shown in Fig. 1, presented together with literature values for multilamellar vesicles

Sample		T_m^{low} (°C)	$\Delta T_{1/2}^{low}$ (°C)	T_m^{high} (°C)	$\Delta T_{1/2}^{high}$ (°C)	ΔH_T^{low} (kcal/mol)	ΔH_T^{high} (kcal/mol)	ΔH_T^{tot} (kcal/mol)
$DC_{16}PC$ (Cevc, 1993)		41.5	—	—	—	8.7	—	8.7
$DC_{18}PE$ (Cevc, 1993)		74	—	—	—	10.5	—	10.5
$DC_{16}PC$		41.36	1.10	—	—	7.8	—	7.8
+ $DC_{18}PC$	1.0%	41.25	1.62	—	—	6.5	—	6.5
	2.5%	41.40	1.84	—	—	8.8	—	8.8
	5.0%	41.27	1.60	—	—	8.3	—	8.3
	7.5%	41.75	2.12	—	—	9.3	—	9.3
	10.0%	41.80	2.95	—	—	9.9	—	9.9
	12.0%	41.92	3.76	—	—	10.0	—	10.0
	17.0%	43.68	4.38	—	—	10.0	—	10.0
+ $DC_{18}PC\text{-}EO_{45}$	1.0%	41.14	1.24	—	—	7.4	—	7.4
	2.5%	41.54	1.18	—	—	8.7	—	8.7
	5.0%	41.89	1.29	—	—	9.1	—	9.1
	7.5%	42.16	1.54	44.2	1.6	6.4	1.2	7.6
	10.0%	42.02	1.35	44.36	2.7	6.2	3.4	9.6
	15.0%	41.85	1.3	44.3	2.72	3.46	3.13	6.64
	17.0%	41.42	1.6	44.2	3.51	2.0	3.88	5.87
	20.0%	41.37	1.7	44.64	4.4	1.2	3.8	5.0
	25.0%	41.46	1.52	44.70	4.8	0.9	3.9	4.8
	30.0%	42.14	—	45.82	4.9	0.6	5.3	5.9
	40.0%	—	—	47.4	6.36	0	2.04	2.04
+ $DC_{18}PC\text{-}EO_{110}$	0.5%	41.24	1.24	—	—	6.9	—	6.9
	1.0%	41.25	1.32	—	—	7.9	—	7.9
	2.5%	41.50	1.74	—	—	8.7	—	8.7
	5.0%	42.11	2.31	43.80	—	7.8	1.2	9.0
	7.5%	43.16	2.34	45.83	—	3.85	1.9	7.1
	10.0%	42.91	2.41	48.92	—	3.3	1.3	4.6

Percentages relate to mol% of a given component in $DC_{16}PC$. The estimated error in ΔH_T is ±0.5 kcal/mol and that in T_m is ±0.05°C.

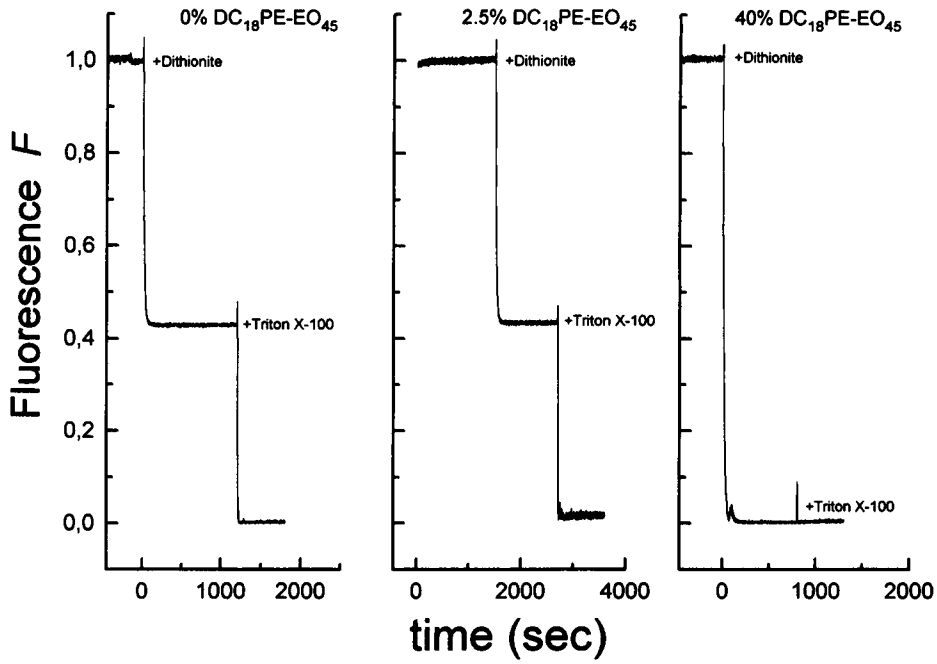

FIGURE 5 Fluorescence quench measurements of lipid samples incorporated with 0.5% NBD-PE fluorescence probes as obtained after adding the dithionite irreversible quencher $DC_{16}PC$ lipid samples incorporated with 0, 2.5, and 40% $DC_{18}PE-EO_{45}$ lipopolymer. The excitation and emission wavelengths for NBD-PE were 470 mm and 530 mm, respectively.

tri42, in contrast to the behavior of the longer $DC_{18}PE-EO_{110}$ lipopolymer. Below we shall discuss this minimum in more detail. Furthermore, we observe, as was the case for the lipopolymers, the appearance of a shoulder (a second peak) on the high-temperature side of the main peak (cf. Fig. 6). Because a weak shoulder is present in the scan of pure $DC_{16}PC$, it is possible that the extrusions using the 100-nm filters did not produce a fully homogeneous vesicle distribution (cf. the discussion by Biltonen and Lichtenberg, 1993).

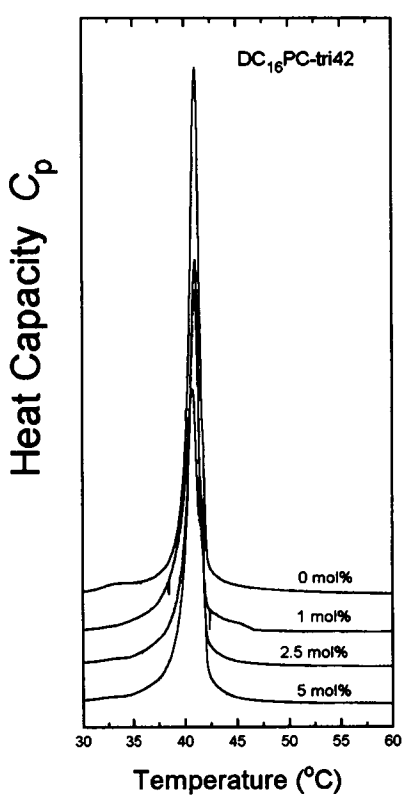

FIGURE 6 Specific heat (arbitrary units) of $DC_{16}PC$ vesicles mixed with the tri-block copolymer tri42. The shoulder appearing on the upper temperature side of all four scans is most likely due to a preparational artifact, as discussed in the text. Percentages refer to amounts of tri42 in $DC_{16}PC$.

DISCUSSION

Mixtures of $DC_{16}PC$ and polyethylene oxide-containing lipopolymers

As described above and shown in Fig. 1 *A*, a second peak in the DSC scans of the $DC_{16}PC$-$DC_{18}PE-EO_{45}$ mixture can be discerned when the concentration of lipopolymer is between 7.5% and 30%. The presence of two peaks in a DSC scan of a lipid binary mixture is indicative of lateral phase separation within the lipid bilayer (Mabrey and Sturtevant, 1976), and it is reasonable to assume that the phase equilibrium in the mixtures studied here can be inferred from the

TABLE 2 Values for T_m, $\Delta T_{1/2}$, and ΔH_T as calculated from the DSC data shown in Fig. 6 for unilamellar vesicles of $DC_{16}PC$ incorporated with the tri-block copolymer tri42, displayed together with selected literature values for multilamellar vesicles

Sample		T_m (°C)	$\Delta T_{1/2}$ (°C)	ΔH_T^{tot} (kcal/mol)
$DC_{16}PC$ (Cevc, 1993)		41.5		8.7
$DC_{16}PC$		41.06	1.07	6.44
+ tri42	1.0%	40.78	1.59	4.73
	2.5%	41.07	1.07	5.46
	5.0%	41.04	1.31	6.01

Percentages relate to mol% of copolymer in $DC_{16}PC$.

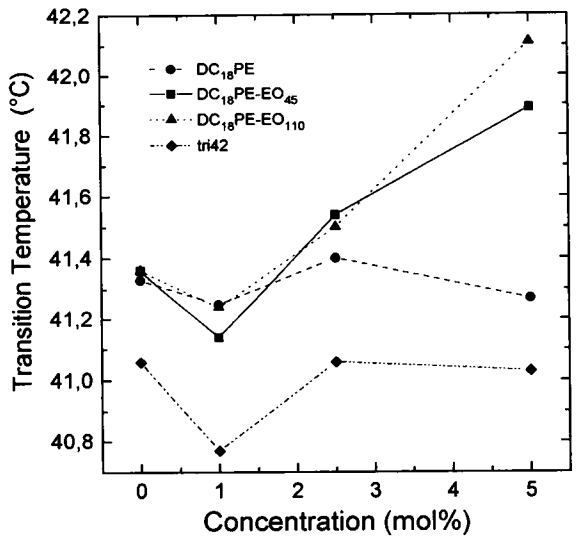

FIGURE 7 Transition temperature, T_m, for $DC_{16}PC$ lipid bilayers incorporated with $DC_{18}PE$-EO_{45}, $DC_{18}PE$-EO_{110}, or $DC_{18}PE$ in different concentrations in the low-concentration regime.

form of the specific heat function in Fig. 1 *A*. It follows from this that the lower peak of our scans may be attributed to the $\beta \rightarrow \alpha$ chain melting transition of the $DC_{16}PC$ chains, whereas the upper peak may be attributed to the same transition but predominantly related to melting of the $DC_{18}PE$ chains. From the phase rule it follows that in cases where the system exhibits lateral separation into two phases in the solid state, we must observe isothermal melting of at least one of the lipid components. This does seem to be the case in the concentration range from 7.5% to 20% $DC_{18}PE$-EO_{45} (cf. Fig. 4) for the $DC_{18}PE$ transition. Furthermore, in the case of 30% $DC_{18}PE$-EO_{45}, we still measure two separate peaks (cf. Fig. 1), but the requirement of isothermal melting no longer seems to be fulfilled (cf. Fig. 4).

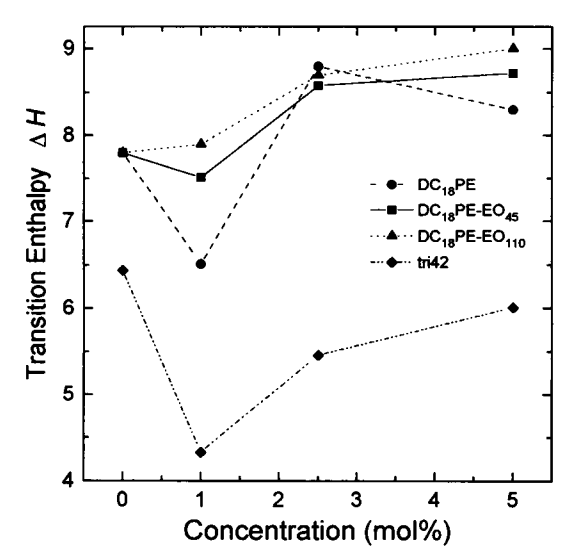

FIGURE 8 Transition enthalpy, ΔH_T (kcal/mol), for $DC_{16}PC$ lipid bilayers incorporated with $DC_{18}PE$-EO_{45}, $DC_{18}PE$-EO_{110}, or $DC_{18}PE$ in different concentrations in the low-concentration regime.

Thus the following two points must be considered when attempting to interpret the measured C_p curves: 1) Because it is known from both experimental (Blume and Cevc, 1993; Blume et al., 1993; Lasic et al., 1991; Lasic, 1994; Lasic and Papahadjopoulos, 1995; Maruyama et al., 1995; Parr et al., 1994; Torchilin et al., 1994b; Woodle and Lasic, 1992) and theoretical work (Hristova and Needham, 1994, 1995; Torchilin et al., 1994a) that the surface of the liposome is fully covered above a certain saturation value n_{sat} (Baekmark et al., 1995; Hristova and Needham, 1994, 1995; Torchilin et al., 1994a) on the order of 3–5% for $DC_{18}PE$-EO_{45} and less than 1% for $DC_{18}PE$-EO_{110} (Baekmark et al., 1995; Torchilin et al., 1994a) by the polymeric head of the lipopolymer, with the latter acting as a protective layer around the liposome, the question arises as to what influence on the physicochemical properties of the liposomes, especially the heat capacity, this polymeric cover has. (Warriner et al. (1996) estimate the saturation values to lie at 8 mol% for PEO_{45} and 3 mol% for PEO_{113}. However, the onset of the reported gel phase is found at ~2 mol% for PEO_{45} and 0.5 mol% (cf. Fig. 2 of this article). This might indicate that their estimate is too high. Most likely, they have not taken into account that water is just barely a good solvent for PEO (cf. Baekmark et al., 1995), with a solution entropy of about $-0.2\ k_BT$ per monomer. This will result in a flattening of the polymer mushroom and a fully covered surface at lower lipopolymer concentrations.) 2) The assumption of lateral phase separation at all molar fractions of lipopolymer leaves unaddressed the question of a possible transformation of the bilayer into other types of lipid aggregates upon reaching a critical lipopolymer concentration, as discussed by Hristova et al. (Hristova and Needham, 1994, 1995; Hristova et al., 1995) and Kenworthy et al. (1995a,b).

Referring to the DSC scans in Fig. 1, we notice as mentioned above the presence of two separate peaks in the scans for samples containing more than 7.5% $DC_{18}PE$-EO_{45} lipopolymer. We also notice that the presence of the lipopolymer in small concentrations (below 5%) does not seem to induce any significant reductions in the main phase transition temperature, T_m (cf. Table 1 and Figs. 4 and 7), in contrast to small hydrophobic molecules like, e.g., dibucaine or lindane (Biltonen and Lichtenberg, 1993; Sabra et al., 1995) or small amounts of proteins or polypeptides (Sperotto and Mouritsen, 1988; Zhang et al., 1993, 1995). On the contrary, the influence of the lipopolymer on the bilayer below 5% is more comparable to that of $DC_{18}PE$ in the same amounts incorporated into $DC_{16}PC$ bilayers (cf. Table 1 and Figs. 4 and 8). However, upon reaching a $DC_{18}PE$-EO_{45} concentration of ~7.5%, the DSC scans of $DC_{16}PC$ mixed with $DC_{18}PE$-EO_{45} start to deviate from those of the binary $DC_{16}PC$-$DC_{18}PE$ mixture, and become very different at concentrations higher than 10% $DC_{18}PE$-EO_{45}. The enthalpy of transition in particular is strongly influenced by the presence of the lipopolymer as seen from

Fig. 2. Furthermore, in the case of the longer lipopolymer, $DC_{18}PE\text{-}EO_{110}$, a similar behavior with respect to T_m and ΔH_T is observed (cf. Table 1 and Figs. 4 and 8), although the concentration for which deviations from that of the binary lipid mixture set in is now ~3% lipopolymer.

This leads us to conclude that lateral phase separation within the bilayer is no longer a possibility when the PEO chains start to overlap. The interchain entropic repulsion between the polymers simply forces the $DC_{18}PE$ hydrocarbon chains to be positioned in the $DC_{16}PC$ bilayer as far from each other as possible. With a forced distance between the $DC_{18}PE$ chains of 35 Å (which is the Flory radius of EO_{45}), or less if the chains have taken on a brush conformation (Baekmark et al., 1995; Kuhl et al., 1994), no room is left for lateral phase separation within the bilayer. Hence a better model is called for to interpret the C_p curves of unilamellar vesicles made from $DC_{16}PC$ and $DC_{18}PE\text{-}EO_{45}$, a model that respects the presence of the PEO head of the lipopolymers.

One such possible model has been proposed by Hristova and Needham (1994, 1995), who consider the free energy for the $DC_{18}PC\text{-}DC_{18}PE\text{-}EO_{45}$ system with contributions from two main sources, bilayer cohesion and polymer lateral pressure. In a simplified version, the equation proposed by Hristova and Needham (1995) for the free energy has the following form:

$$\frac{F}{\sigma} = \frac{F_{\text{bilayer}}}{\sigma_{\text{bilayer}}} - \frac{F_{\text{polymer}}}{\sigma_{\text{micellar}}} \quad (1)$$

where F_{bilayer} is the free energy of bilayer cohesion and F_{polymer} is the free energy arising from the lateral pressure exerted by the polymer chains. σ is the average area per lipid molecule. The subscripts "bilayer" and "micellar" are used here to indicate that the effect of $DC_{18}PC$ is to order the lipids in a bilayer structure, whereas the lipopolymer will tend to stabilize a micellar structure (Hristova and Needham, 1995).

What follows from the calculations of Hristova and Needham (1994, 1995) is the following. At high lipid concentrations (i.e., when the first term of Eq. 1 is dominant), the lipid and the lipopolymer pack into a bilayer, as is experimentally well established (Lasic et al., 1991; Lasic, 1994), but as the concentration of the lipopolymer is increased, the lamellar bilayer will become destabilized and a micellar lipid phase containing both $DC_{18}PC$ and $DC_{18}PE\text{-}EO_{45}$ is formed. The first micellar phase to form according to Hristova and Needham is H^I-type micellar rods (Hristova and Needham, 1995), with a necessary later cross-over to spherical micelles at high $DC_{18}PE\text{-}EO_{45}$ concentrations. (This is required because pure $DC_{18}PE\text{-}EO_{45}$ forms spherical micelles in water (Kenworthy et al., 1995a).) Of special interest in the present context is the prediction that the disruption of the bilayer will not take place until a certain lipopolymer threshold concentration, n_{tr}, is reached, beyond which a lipid bilayer phase coexists with a micellar phase.

X-ray studies by Kenworthy et al. (1995a) and Hristova et al. (1995) of mixtures of phospholipids and ethylene oxide lipopolymers of different size, but with the same number of carbon atoms as in the lipid chains, have shown that micellar phases indeed form when the concentration of the lipopolymers is increased above a system-dependent threshold value, n_{tr}. These studies have also verified the hypothesis of coexistence of the two phases over a certain range of lipopolymer concentrations. Furthermore, these studies confirmed the finding by Lasic et al. (1991) that spherical micelles are not formed until the lipopolymer content in the $DC_{18}PC\text{-}DC_{18}PE\text{-}EO_{45}$ mixture is ~60%. For the $DC_{18}PC\text{-}DC_{18}PE\text{-}EO_{45}$ system, the calculations of Hristova and Needham (1995) predict n_{tr} to be ~7% of $DC_{18}PE\text{-}EO_{45}$, whereas Kenworthy et al. (1995a) find n_{tr} to lie between 15–20% $DC_{18}PE\text{-}EO_{45}$. This discrepancy is attributed by Hristova et al. (1995) to the crudeness of their model.

In the present case, to test whether the model of Hristova and Needham can be employed to interpret our data, we have used the transition enthalpies obtained for the $DC_{16}PC\text{-}DC_{18}PE\text{-}EO_{45}$ system listed in Table 1 to estimate the lipid content that gives rise to each of the two measured peaks, assuming for the time being that the model holds. This is done in the following way.

First we assume that only the lowest of the two peaks measured (Fig. 1 *A*) corresponds to the bilayer phase. The reasons for this are as follows. The lowest peak remains equally sharp up to at least 30% $DC_{18}PE\text{-}EO_{45}$, as seen from the $\Delta T_{1/2}$ values of Table 1. This is to be expected for vesicles with identical (as soon as n_{tr} is reached) composition. The highest lying peak does not show the same sharpness, which conforms well with the interpretation that the lipids are packed into micelles, thus having a lower lipid-chain order, and therefore a much lower cooperativity in their melting behavior. Furthermore, T_m for the lowest peak is rather constant, which is to be expected for vesicles with constant composition. We note, however, that it is not trivial to claim that we can measure a peak in C_p versus temperature for a micellar system, even though the system is more ordered than is the case for spherical micelles. Nevertheless, there is evidence in the literature for this in the case of other mixed systems of lipid and polymer (Blume and Cevc, 1993; Inoue et al., 1993; Kenworthy et al., 1995a).

Our second assumption concerns the stability of the vesicle when the temperature is changed. We shall assume that the equilibrium between micelles and vesicles is unaffected by the temperature in the measured temperature range (20–65°C), i.e., we do not measure the heat of melting of a lipid more than once during a scan. Because of the rather broad temperature window, this may seem unjustified. However, the stability of vesicles stabilized with $DC_{18}PE\text{-}EO_{45}$ within this temperature region is well established (cf., e.g., Kenworthy et al., 1995a). Therefore we feel confident that any change in the liposome/micellar equilibrium with temperature will be small, compared to the disruptive effect of having too much lipopolymer incorporated into the membrane.

By use of the two above-mentioned assumptions, we can calculate the fraction, f, of $DC_{18}PE\text{-}EO_{45}$ incorporated into a bilayer in units of the total $DC_{18}PE\text{-}EO_{45}$ content in the following way:

$$f = \frac{\Delta H_T(n_{pol})n_{tr}}{\Delta H_T^{tr} n_{pol}} \quad (2)$$

$$\simeq \frac{\Delta H_T(n_{pol})n_{app}}{\Delta H_T^{app} n_{pol}} \quad (3)$$

where $\Delta H_T(n_{pol})$ is the (measured) transition enthalpy at a given total lipopolymer concentration, n_{pol}. n_{tr} is the threshold concentration as previously explained, and n_{app} is the apparent threshold concentration obtained from experiment. ΔH_T^{tr} is the transition enthalpy at n_{tr} and ΔH_T^{app} is the transition enthalpy at the apparent threshold concentration, n_{app}. Naturally, $f = 1$ when $n_{pol} \leq n_{tr}$.

For our calculations we use a value of $n_{app} = 5\%$ $DC_{18}PE\text{-}EO_{45}$, because at $n_{pol} = 7.5\%$ the shoulder on the upper side of the C_p curve has become too large to be ignored (cf. Fig. 1), accounting for as much as 16% of the total transition enthalpy (cf. Fig. 2). The precise value of n_{tr} will lie in the concentration range from 5% to 7.5% $DC_{18}PE\text{-}EO_{45}$. Equation 2 shows that the actual values calculated for f depend critically on the value chosen for n_{app}. For example, if we choose $n_{app} = 6\%$ for $DC_{18}PE\text{-}EO_{45}$, the values calculated for f are increased by a factor of 1.2 as compared to the present results.

In Fig. 9 we plot the calculated results for f based on our measurements for $DC_{18}PE\text{-}EO_{45}$ and $DC_{18}PE\text{-}EO_{110}$. In the latter case we have used $n_{app} = 2.5\%$. The precise value of n_{tr} probably lies in the range 2.5–5%. For both lipopolymers we find that f decreases to zero very rapidly. For $DC_{18}PE\text{-}EO_{45}$, f decreases in the concentration region from 5% to 25% lipopolymer, and for $DC_{18}PE\text{-}EO_{110}$ f decreases in the concentration region from 2.5% to ~10% lipopolymer. In Fig. 9 we have also plotted the theoretical predictions by Hristova and Needham (1995). Assuming that all other bilayer properties remain the same, the theoretical result for the threshold concentration scales with polymer length as $N^{-3/5}$. It is seen that for both the $DC_{18}PE\text{-}EO_{45}$ system and the $DC_{18}PE\text{-}EO_{110}$ system there is good agreement between the theoretical predictions of Hristova and Needham and the values for f calculated from our measured C_p curves. It should be noted, however, that the interpretation of the features of the specific heat for the $DC_{18}PE\text{-}EO_{110}$ is subject to some uncertainty. For the $DC_{18}PE\text{-}EO_{45}$ system the C_p curves reveal a breakdown of the bilayer structure at lower lipopolymer concentration, in accordance with the theoretical predictions (Hristova and Needham, 1995). This lends credibility to the interpretation of the data as a result of a bilayer breakdown, because the entropic interchain repulsion is dependent on the degree of polymerization, because of which the breakdown pressure in the bilayer will be reached for lower contents of the larger polymer.

Furthermore, because the measured values of ΔH_T fluctuate strongly in the concentration region between 7.5% and 10% $DC_{18}PE\text{-}EO_{45}$ (Fig. 2 *A*), so do the calculated values of f. This supports the claim that the bilayer in this concentration regime is undergoing a change of symmetry, because even minor variations in the determination of the lipopolymer concentration would lead to large fluctuations in ΔH_T and therefore in f, similar to the effect of choosing a value for n_{tr} that is too high or too low.

The results from the fluorescence quenching experiments presented in Fig. 5 support the interpretation of the DSC measurements and the existence of a change from a bilayer structure to a micellar structure above a certain threshold

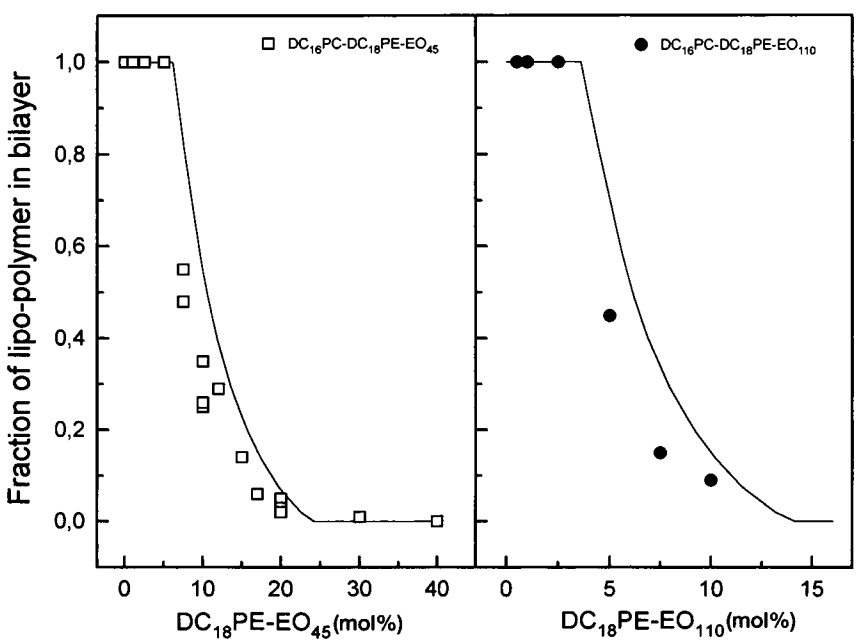

FIGURE 9 Calculated fraction f of lipopolymer in bilayer structure as a function of total concentration of lipopolymer. (□) $DC_{18}PE\text{-}EO_{45}$ in $DC_{16}PC$. ●, $DC_{18}PE\text{-}EO_{110}$ in $DC_{16}PC$. Fully drawn lines show the theoretical predictions by Hristova and Needham (1995).

concentration of $DC_{18}PE\text{-}EO_{45}$. When dithionite is added to lipid systems containing 0 and 2.5% lipopolymer, a reduction of the fluorescence signal takes place, reflecting an irreversible quenching of fluorescent NBD-PE probes positioned in the outer leaflet of the bilayer. This behavior is opposite that of the binary system containing 40% lipopolymer, where the addition of dithionite leads to a nearly complete removal of the fluorescence signal, as is to be expected when the fluorescent headgroup NBD-PE probes are located in a micellar phase.

We therefore tentatively conclude that DSC can be used to determine the degree of micellation in mixtures of $DC_{16}PC$ and PEO-containing lipopolymers, and we reach the following conclusions based on our experiments. For $DC_{18}PE\text{-}EO_{45}$ in $DC_{16}PC$, we find that below ~5–7.5% $DC_{18}PE\text{-}EO_{45}$, only a bilayer phase is present in the aqueous solution. In the interval from 7.5 to 30% $DC_{18}PE\text{-}EO_{45}$, we find a coexistence region of a bilayer and a micellar lipid phase, and above ~30% $DC_{18}PE\text{-}EO_{45}$ only a micellar lipid phase is found. For $DC_{18}PE\text{-}EO_{110}$ in $DC_{16}PC$, we find the pure bilayer phase to lie below ~2.5%, with the coexistence region ending at ~10% $DC_{18}PE\text{-}EO_{110}$.

To illustrate the above conclusions, we have, in Fig. 4, *A* and 4 *B*, estimated the phase diagrams (based on the measured values of T_m) for the binary $DC_{16}PC\text{-}DC_{18}PE\text{-}EO_{45}$ and $DC_{16}PC\text{-}DC_{18}PE\text{-}EO_{110}$ systems. The detected lipid phases are labeled L for the lipid bilayer phase and H for the micellar phase. We have chosen to follow the labeling scheme of Hristova and Needham (1995), even though we do not possess direct evidence for the existence of a H^I rodlike micellar phase. The subscripts α and β are used to indicate the lipid intrachain transition from gel to fluid, although in the micellar case this is speculative. The dotted lines in both figures indicate the approximate phase boundary between the pure bilayer phase and the coexistence region of bilayer and micellar phases, whereas the dashed line in Fig. 4 *A* indicates the approximate phase boundary to the pure micellar phase region. Fig. 4 *C* shows the partial phase diagram for the $DC_{16}PC\text{-}DC_{18}PE$ mixture, based on the observations for T_m, in the concentration region from 0% to 17% $DC_{18}PE$. As expected, because the latter system does not contain any polymer, there is a substantial difference between the $DC_{16}PC\text{-}DC_{18}PE$ phase diagram and the phase diagrams for the $DC_{16}PC\text{-}DC_{18}PE\text{-}EO_{45}$ and $DC_{16}PC\text{-}DC_{18}PE\text{-}EO_{110}$ mixtures. Furthermore, as was found for f in the case of the smaller lipopolymer, we find that T_m also fluctuates close to the phase boundary between the pure bilayer phase and the coexistence region of bilayer and micellar phases.

It is of interest to note that our results for f in the case of the shorter of the lipopolymers closely match those found by Hristova et al. (1995) for the $DC_{16}PC\text{-}DC_{16}PE\text{-}EO_{45}$ system (n_{tr} = 8% $DC_{16}PE\text{-}EO_{45}$), even though we use lipopolymers with two additional carbon atoms in the lipid acyl chain as compared to the matrix lipid. However, Hristova et al. (1995) find that up to 20% $DC_{18}PE\text{-}EO_{45}$ can be incorporated into bilayers of $DC_{18}PC$ before the bilayer breaks down, instead of the predicted 7%. If a lysophosphatidylcholine (monooleoylphosphatidylcholine) is used as matrix lipid instead, the breakdown concentration of $DC_{18}PE\text{-}EO_{45}$ in $DC_{18}PC$ is reduced to ~8% (Hristova et al., 1995). Because incorporation of cholesterol into the lipid bilayer does not increase the maximum amount of $DC_{18}PE\text{-}EO_{45}$ that can be incorporated into $DC_{18}PC$ vesicles (Hristova et al., 1995), these authors conclude that the tensile strength of the lipid bilayer is only of minor importance for the stability of sterically stabilized vesicles. Instead, they suggest that the tendency of the matrix lipid to form micelles has larger influence on the stability of the vesicles. This points to the importance of the right choice of matrix lipid for the stability of vesicles used in drug delivery. One may possibly consider using lipids that form inverted micelles to counterbalance the disruptive effect of the lipopolymers and thereby increase the stability of lipopolymer-containing vesicles in the concentration region where these do not form sterically stabilized vesicles.

Mixtures of $DC_{16}PC$ with the amphiphilic tri-block copolymer tri42

As mentioned in the Introduction, we expect aqueous solutions of mixtures of the tri-block copolymer tri42 and lipid to interact through incorporation of the PS-part of the copolymer into the lipid bilayer. However, because tri-block copolymers are known to form micelles in solvents preferential to one of the two different polymer species (here, water to PEO; Rapoport and Caldwell, 1994; Villacampa et al., 1995; Yung-Wei et al., 1996), the possibility arises that tri42 may form micelles in water instead of incorporating into the membrane.

If tri42 forms micelles, the influence of the copolymer on the bilayer will be similar to the effect on lipid bilayers of large PEO polymers solvated in water, because the bilayer does not sense the interior of the polymeric micelles, but only its outer hydration sphere and its size (i.e., the lipid bilayer is subjected to the osmotic pressure of the polymeric micelles). It follows from this that the hydrophobic PS part of the copolymer will have no influence on the phase behavior of the bilayer, because the PS will be located in the interior of the polymeric micelle.

Large unilamellar vesicles of $DC_{16}PC$ exposed to a high osmotic pressure due to PEO have been investigated by Lehtonen and Kinnunen (1995), who used fluorescence intensity measurements. They found that dehydration of the bilayer by PEO leads to tighter packing of the lipids in the bilayer. In relation to the present work, it is interesting to note that the increase in bilayer packing order will lead to a general increase in T_m of the bilayer, which we then should be able to measure in our experiments.

As mentioned above, we observe from Figs. 7 and 8 that T_m and ΔH_T develop similarly for the lipopolymer and the tri-block copolymer for increasing polymer concentration. Specifically, we find for both types of polymer that our

measured values of T_m go through a minimum at ~1% polymer content. Because this is indicative of conventional impurity-induced freezing point depression, and because free-floating micelles of PEO do not bind to the surface of liposomes (Evans and Needham, 1988; Lehtonen and Kinnunen, 1995), we may conclude that tri42 exerts its effect on large unilamellar $DC_{16}PC$ vesicles through incorporation into the lipid bilayer, either spanning the membrane or entering and leaving the membrane on the same side of the vesicle.

Kostarelos et al. (1995a,b) examined the interaction of the triblock copolymer PEO-PPO-PEO (PPO: polypropylene oxide) with sonicated vesicles of soybean lecithin. Their investigations led them to conclude that the triblock copolymer was interacting with the lipid membrane either by spanning the membrane or by entering and leaving the membrane on the same side. Furthermore, Kostarelos et al. (1995b) found, using hydrophobic dyes to monitor the liposome bilayer microenvironment, that the bilayer environment became more apolar with increasing copolymer concentration. This was the case particularly when the copolymer was added to the lipid before sonication, which led the authors to conclude that in this case the copolymer was most likely spanning the membrane. This is in line with the results presented in the present paper, as we too mix lipid and copolymer before creation of the large unilamellar vesicles. It is also interesting that PPO is sufficiently hydrophobic, irrespective of the oxygen in the polymer chain, to span a lipid membrane. Thus the use of PS (it contains only carbon and hydrogen) as the hydrophobic part of the triblock copolymer ought to further promote the incorporation of this block into the lipid membrane.

What might speak against an incorporation of the triblock into the lipid membrane, with the two hydrophilic chains leaving the membrane on the same side, are geometrical reasons. We expect the copolymer to arrange more or less like three beads on a string, where the PEO blocks will take up more space than the PS block, because of the relatively good solubility of PEO in water. In such a triblock molecule it will be difficult to arrange the two largest beads next to each other, as is required if the two PEO blocks come out of the membrane on the same side. However, it is much more likely that these blocks will be elongated, which should favor the linear incorporation, i.e., the membrane-spanning model.

It is of interest to consider the extent to which the PS block of the copolymer must be stretched to span the membrane. For this purpose, we need to know the hydrophobic thickness of the lipid membrane. We can estimate the average hydrophobic thickness, $\bar{d}$, of a DC_mPC one-component bilayer in the following way (Sperotto and Mouritsen, 1988):

$$\bar{d} = \tfrac{1}{2}(d_L^g + d_L^f) \simeq 2.19(m - 1)\,[\text{Å}] = 32.85\ \text{Å} \quad \text{for} \quad m = 16 \tag{4}$$

where d_L^g is the hydrophobic thickness of the gel-state bilayer, d_L^f is the hydrophobic thickness of the fluid-state bilayer, and m is the number of carbon atoms in the lipid chain.

If we assume a maximum monomer length for polystyrene of 4 Å, we find that the membrane thickness is on the order of the Flory radius (≃38 Å) of the polystyrene stretch of the copolymer; i.e., the diameter of the PS stretch of tri42, if it could fluctuate freely, would be twice the size of the hydrophobic thickness of the bilayer. If the PS stretch must fit into the lipid bilayer, it must take on a conformation other than the spherical conformation of a free chain in a good solvent. This could be in the form of a compact cylinder, located mainly in the space between and parallel to the acyl chains, or alternatively, it could be a less compact, more freely fluctuating chain, located in the space between the two lipid monolayer leaflets. (If we assume the volume of the PS stretch to be constant and equal to that of a sphere with radius equal to the Flory radius ≃ 38 Å, the cylinder radius would be on the order of 47 Å.) Whereas the first possibility, a cylinder located between the lipid chains, would induce substantial disorder in the lipid chains, leading to a significant reduction of T_m (more than the 0.3°C observed at 1% tri42 content; Fig. 7), the possibility of the polymer being located between the two lipid layers might leave the bilayer less disordered because of its reduced contact with the lipid chains. This would explain why we do not observe such large reductions in T_m as predicted by the mattress model. Furthermore, if we assume that the PS part of the tri-block copolymer does not influence the bilayer significantly, then the overall similarity between the results of our DSC measurements of lipopolymers and tri42 would be explainable in a consistent and coherent way, which we shall discuss in the following paragraph.

We shall put forward the following hypothesis. When the concentration of polymer in the bilayer is sufficiently low, the properties of the bilayer are determined mainly by the conformation of the PEO part of the polymers and especially by the surface of the liposome covered by PEO. From this the following hypothesis follows. When the concentration of PEO at the surface is low, we have a situation as shown schematically in Fig. 10 *A*. The PEO polymers are not interacting or are only weakly interacting with each other, and the polymers affect the lipid bilayer as would any other impurity within the bilayer. In the case of the lipopolymers, the impurity is the $DC_{18}PE$ chain, and for tri42 it is the PS stretch of the copolymer leading to the observed reduction in T_m and ΔH_T (cf. Figs. 7 and 8). This reduction, however, is smaller than expected because of the bilayer-ordering effect of PEO, as discussed by Lehtonen and Kinnunen (1995), or as more generally expected for any adsorbed polymer, as discussed by Garel et al. (1995). When the concentration of the polymer in the bilayer is increased to the point where the PEO chains start to overlap (cf. Fig. 10 *B*), a new situation arises. This occurs for PEO_{45}, as previously noted, at a concentration of 3–5%. The bilayer surface is now fully covered with polymer, and the

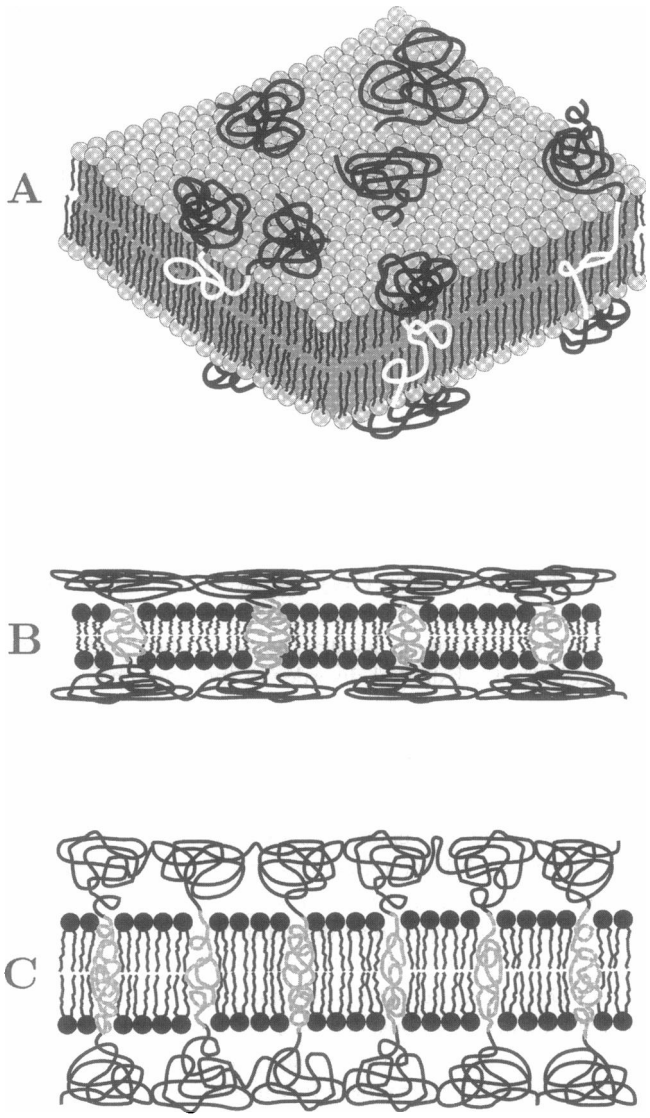

FIGURE 10 Schematic illustration of the possible ways of incorporation of the tri-block copolymer tri42 into lipid bilayers. (*A*) Situation at low tri42 concentration. (*B*) Situation at medium tri42 concentration. The PEO headgroups are still in a mushroom configuration. (*C*) Situation at high tri42 concentration. The PEO headgroups are in a brush conformation.

ordering of the lipid chains induced by the polymer is at or close to a maximum. We therefore observe increased values of T_m and ΔH_T (Figs. 7 and 8) due to the ordering effect of PEO (Lehtonen and Kinnunen, 1995; Garel et al., 1995) as compared to the situation when the surface is only partially covered with polymer. When the polymer concentration is further increased, the situation as depicted in Fig. 10 *C* arises and the PEO heads are now forced away from the surface, but this does not decrease the coverage. Therefore, a minor influence on the transition temperature and the transition enthalpy is observed (cf. Figs. 7 and 8).

The lipopolymers still have, as discussed above, the possibility of escaping into a micellar phase to relax the bilayer stress induced by the polymer-chain repulsion. This is not likely to be an option available to the tri-block copolymer, because of the symmetry of its composition. In particular, if the copolymer is spanning the membrane, micellation should be strongly inhibited. This is in accordance with the observation that bilayers containing more than 5% copolymer could not be prepared with the extruding technique, because at this polymer concentration, as our measurements with $DC_{18}PE\text{-}EO_{45}$ and $DC_{18}PE\text{-}EO_{110}$ show, micellation should set in. We are therefore unable to force more tri42 into the membrane, and the extrusion process is inhibited.

Based on the schematic model proposed in Fig. 10, it is possible to interpret the difference in behavior of the transition enthalpy for the three different polymeric systems shown in Fig. 8. The enthalpy of the long lipopolymer, $DC_{18}PE\text{-}EO_{110}$, does not go through a minimum, simply because its larger size and hence larger surface coverage imply that we are already in the situation pictured in Fig. 10 *B* at the lowest concentration studied. According to previous studies (Baekmark et al., 1995; Torchilin et al., 1994a), $DC_{18}PE\text{-}EO_{110}$ already covers the lipid surface fully at ~0.5 mol%.

Finally, it should be pointed out that the proposal of using tri-block copolymers as model systems for mimicking transmembrane proteins has in the present paper only been explored in the simplest setting. The possibilities for forming new membrane-spanning objects (e.g., by using specifically designed heteropolymers with certain folding characteristics) are abundant and should allow for novel designs in artificial liposome and membrane systems.

An illuminating discussion with Dr. Kalina Hristova is gratefully acknowledged. Dr. David Needham is thanked for providing us with a copy of Dr. Hristova's Ph.D. thesis.

This work was supported by the Danish Natural Science Research Council, the Danish Technical Research Council, and the Danish Research Academy. OGM is a Fellow of the Canadian Institute for Advanced Research.

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