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Macromolecular prodrugs interaction with mixed lipid membrane. A calorimetric study of naproxen linked to polyaspartamide interacting with phosphatidylcholine and phosphatidylcholine-phosphatidic acid vesicles

Francesco Castelli [1], Gaetano Giammona [2], Antonio Raudino [1] and Giovanni Puglisi [2]

[1] *Dipartimento di Scienze Chimiche and* [2] *Istituto di Chimica Farmaceutica e Tossicologica, Universitá di Catania, Viale A. Doria 6, 95125 Catania (Italy)*

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Summary

The thermal behaviour of pure dipalmitoylphosphatidylcholine (DPPC) liposomes or mixed liposomes of DPPC with charged dipalmitoylphosphatidic acid (DPPA) and interacting with polymeric prodrugs has been investigated by differential scanning calorimetry (DSC). The apolar drug was naproxen (NAP) covalently linked to a water-soluble polymer (α,β-poly(N-hydroxyethyl)-DL-aspartamide (PHEA)). Addition of increasing amounts of NAP to DPPC liposomes causes a decrease in the transition temperature (T_m) associated to the gel-to-liquid crystal phase transition with a small decrease in the enthalpy values (ΔH), whereas a corresponding amount of drug contained in the PHEA-adduct modifies the liposome phase transition by decreasing the ΔH and broadening the peak without T_m variations. These effects have been interpreted as a different interaction of free or polymer-bound drug with the lipid bilayer. The drug effect on mixed liposomes was also investigated, and evidence of improved interaction of the drug-PHEA adduct with two-component bilayers, which better mimic biological membranes, was found. In order to understand the prodrug-lipid interactions, we modulated the surface charge density of the mixed liposomes with Ca^{2+}, which binds strongly to the negatively charged lipid head groups. We observed lateral phase separation, induced by NAP-PHEA adduct and modulated by Ca^{2+}, and the phenomenon was explained in terms of different drug solubility in DPPC-poor and DPPC-rich microdomains. The results are indicative of different interactions of naproxen, either free or bound to a polymeric carrier, with phospholipid membranes and the ability of Ca^{2+} to influence the adsorption of the drug.

Introduction

Drug therapy has prevalently two kinds of problems: firstly, to obtain an effective drug-time specificity and secondly, to prolong drug action in the human body. One approach is the use of macromolecular carriers (Azori, 1987). To this aim, therapeutic agents have been fixed either by ionic interactions with charged compounds or by covalent bonds with a polymer backbone. The drug release rate from these prodrugs is related to enzymatic degradation or hydrolysis in vivo (Anderson and Kim, 1986).

Correspondence: F. Castelli, Dipartimento di Scienze Chimiche, Universitá di Catania, viale Andrea Doria 6, 95125 Catania, Italy.

Presently, we are studying systems in which drugs are bound to synthetic or natural polymers. Among them, α,β-poly(*N*-hydroxyethyl)-DL-aspartamide (PHEA) is a water-soluble polymer which has originally been proposed as a plasma expander (Neri et al., 1973). In a previous work, PHEA adducts with some non-steroidal anti-inflammatory agents (NSAIDs) were prepared and characterized (Giammona et al., 1989). The in vivo biological assays showed that the prodrugs present an analgesic and anti-inflammatory activity comparable to that of drugs alone.

Of these adducts, 4-biphenylacetic acid (BPAA)-PHEA was further studied to evaluate its effects upon the gel-to-liquid crystalline phase transition of a lipid membrane model (Castelli et al., 1990). In the present paper, we investigated extensively PHEA adduct with another anti-inflammatory agent, naproxen (d-2-(6-methoxy-2-naphthyl)propionic acid (NAP).

Naproxen is a commonly used drug in the treatment of rheumatic diseases (Barry et al., 1978) and for the control of phlogistic pain (Sevelius et al., 1980; Ylikorkola et al., 1980; Huskisson, 1983). Moreover, since it has a hydrophobic structure, a considerable degree of solubility within the lipid bilayer would be expected.

Lipid membrane-drug interactions have been examined by DSC, a non-perturbing thermodynamic technique, and the gel-to-liquid crystalline phase transition of pure DPPC or mixed DPPA/DPPC liposomes interacting either with the drug, the polymer and the drug-polymer adduct have been investigated.

The presence of drug molecules in the ordered bilayer structure could affect the packing of lipid chains, depending on their amphipatic or lipophilic nature, causing variations in the transition temperature of the pure lipid and/or changes in enthalpy of chain melting (Lee, 1983; Bach, 1984). From these variations it is possible to obtain information on lipid-drug interactions.

The aim of this study is two-fold: (i) to explore the nature of the interaction between the drug and neutral or charged lipids and (ii) to evaluate how the polymeric drug carrier could enhance drug solubility in model membranes.

Phospholipids were selected because they are the major lipid components of biological membranes; moreover, the addition of a small amount of the negatively charged DPPA to the bilayer mimics quite well the electrostatic potential present on the cell membranes.

Materials and Methods

Chemicals

Synthetic L-α-dipalmitoylphosphatidylcholine was purchased from Fluka Chemical Co. (Buchs, Switzerland); synthetic L-α-dipalmitoyl phosphatidic acid (DPPA) was obtained from Sigma. Solutions of lipids were chromatographically pure as assessed by two-dimensional thin-layer chromatography (TLC). The phospholipidic phosphorus content was assayed as inorganic phosphate by the analytical procedures previously reported (Bartlett, 1959).

Naproxen was obtained from Sigma Chemical Co. (St Louis, U.S.A.). DL-Aspartic acid, ethanolamine and N,N-dimethylformamide were purchased from Fluka Chemical Co. (Buchs, Switzerland). α,β-Poly(*N*-hydroxyethyl)-DL-aspartamide was prepared according to previously reported methods (Neri et al., 1973; Giammona et al., 1987).

The PHEA-NAP adduct was prepared, purified and characterized following the procedure described in a preceding paper (Giammona et al., 1989) and the resulting molar ratio NAP:PHEA was 24 : 1.

A $CaCl_2$ stock solution was prepared in 50 mM Tris buffer (pH 7.4) and the Ca^{2+} content was checked by EDTA titration.

Preparation of liposomes

Aqueous dispersions of pure lipids and mixtures of NAP, PHEA and PHEA-NAP adduct with the phospholipids were prepared by the following procedure. Appropriate aliquots of DPPC and DPPA solutions in $CHCl_3$:CH_3OH (1 : 1, v/v) were mixed, resulting in homogeneous mixtures of different molar ratios (100 or 80% DPPC content). NAP solutions in CH_3OH-$CHCl_3$ (1 : 1, v/v), was added in order to obtain different drug/lipid molar ratios. The solvent was removed at 30 °C

on a rotary evaporator by a nitrogen stream and the resulting film was lyophilized for 3 h.

PHEA and PHEA-NAP adduct dispersions in DPPC, as well as in 80 : 20 (molar ratio) DPPC/DPPA, liposomes, were prepared by adding aqueous solutions of the compounds to the lyophilized lipid films.

As reference for the amount of PHEA and PHEA-NAP, the mole fraction of free or adduct-bound Naproxen was fixed; consequently, in all tables and figures the mole fraction of NAP is reported and the amount of PHEA must be referred to the PHEA present in the adduct containing the reported mole fraction of naproxen (value readily obtained from data in Materials and Methods).

Liposomes were prepared at a temperature higher than their gel-liquid crystalline phase transition in order to allow full hydration of the samples, by adding to the film 50 mM Tris buffer adjusted to pH 7.4. The samples were vortexed twice for 1 min at 75 °C and then shaken for 12 h at 75 °C in a water bath to homogenize the dispersion. Afterwards, aliquots of 120 μl of each sample were transferred and sealed in aluminium pans followed by being submitted to DSC analysis.

The experiments in the presence of Ca^{2+} were carried out by adding the appropriate amount of Ca^{2+} solution to obtain the desired ion concentration.

DSC

DSC measurements were performed with a Mettler TA 3000 calorimeter, equipped with a DSC 30 cell and a TC 10 processor. Samples were analyzed by using heating and cooling rates of 2 °C/min, in the temperature range 10–80 °C, after an isothermal period of 15 min at 10 °C. The sensitivity was 1.71 mW, and the same Tris solution as in the reference pans was used.

Each sample was heated and cooled through the lipid phase transition region at least four times to ensure reproducibility of the observed behaviour. Palmitic acid was employed to calibrate the temperature scale and the ΔH. Enthalpy changes were calculated from the peak areas. After the calorimetric runs, the pan content was extracted for phosphate analysis to determine the amount of phospholipid.

Results and Discussion

Interaction with DPPC liposomes

Fig. 1a and b shows DSC curves (heating mode) of aqueous dispersions of DPPC mixed with various amounts of NAP and PHEA-NAP (Tris buffer at pH 7.4).

Differences in the thermotropic behaviour of DPPC liposomes caused by the presence of free NAP and PHEA-bound NAP are evident. In fact, while the free drug produces a shift of the transition temperature (T_m) without variations in the enthalpy values (ΔH) associated with the gel-to-liquid crystalline phase transition (see Table 1 and Fig. 1a), addition of the polymeric prodrug to DPPC bilayers induced a very different thermotropic behaviour. Indeed, DSC peaks broaden and reduce their area by increasing the prodrug mole fraction while the transition temperature remains almost constant. The total enthalpy change decreases nearly linearly until $X_{NAP} = 0.24$, beyond this value a phase separation of the bilayer components being observed because of the immiscibility of PHEA-NAP/DPPC at high prodrug mole fractions (see Fig. 1b).

The different thermotropic behaviour between free and bound NAP may provide useful information. In fact, the free NAP behaves as an inert substance which is much more soluble in the fluid liquid-crystalline phase than in the gel phase. According to the classical thermodynamic models, this phenomenon leads to a lowering and a broadening of the transition temperature (T_m) which is proportional to the drug mole fraction dissolved in the lipid 'solvent' (Lee, 1977; Sturtevant, 1982). In contrast, when the PHEA-NAP adduct is considered, the constancy of T_m and the lowering of the enthalpy changes associated with the transition might suggest a different interaction.

This behaviour could be rationalized assuming that the polymer complex is soluble in neither the gel nor liquid-crystal phase but it probably behaves as an independent phase, trapping some lipid molecules which are excluded from the main

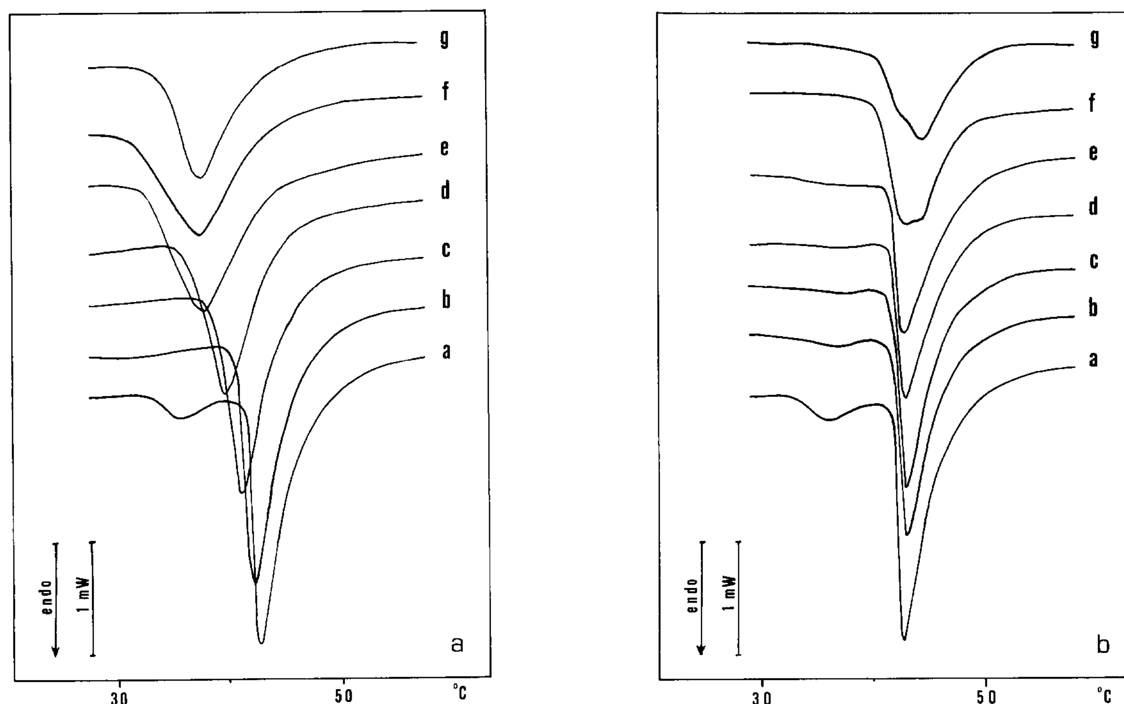

Fig. 1. Differential scanning calorimetry heating curves of hydrated DPPC containing (a) NAP and (b) PHEA-NAP at drug mole fractions of: (a) = 0; (b) = 0.06; (c) = 0.12; (d) = 0.18; (e) = 0.24; (f) = 0.37; (g) = 0.48.

lipid pool. Then, on increasing the PHEA-NAP concentration, the T_m variations are negligible, while the enthalpy decreases because more and more lipids are drained from the lipid reservoir and bound to the polymeric structure.

The same thermotropic behaviour has been observed for several hydrophobic proteins or polypeptides (see, for instance, McElhaney, 1986), suggesting that, despite the hydrophilic polymer and the low density of apolar drug bound to the poly-

TABLE 1

Main transition peak temperature (T_m expressed in °C) and main transition enthalpy changes (ΔH expressed in kcal mol^{-1}) of DPPC dispersions for different molar fractions of NAP and PHEA free or NAP content in PHEA-NAP adduct (figures are the mean values obtained from at least four DSC heating curves)

Mole fraction	DPPC + NAP		DPPC + PHEA		DPPC + PHEA-NAP	
	T_m	ΔH	T_m	ΔH	T_m	ΔH
0.00	42.2	8.2	42.2	8.2	42.2	8.2
0.06	41.6	7.8	42.2	8.2	41.8	7.0
0.12	40.6	7.5	42.2	8.2	41.5	6.6
0.18	39.2	7.0	42.2	8.2	42.1	5.3
0.24	37.2	6.5	42.2	8.2	41.8	4.8
0.37	37.3	7.0	42.2	8.2	41.7	4.9
0.48	37.3	6.1	42.2	8.2	42.1	4.5

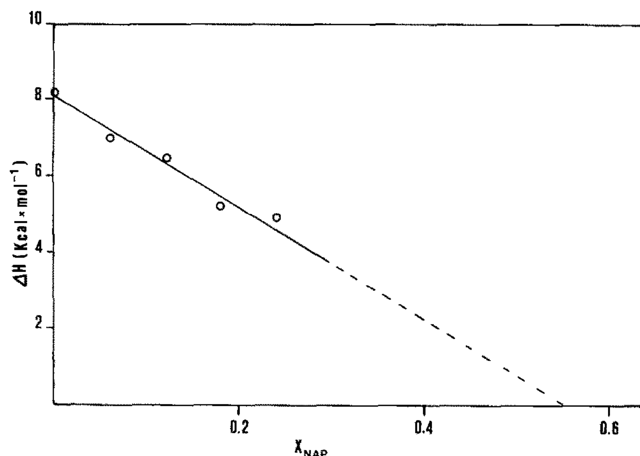

Fig. 2. Enthalpy changes, ΔH (kcal mol^{-1}), of the gel-to-liquid crystalline phase transition of DPPC as a function of the mole fraction of NAP content in PHEA-NAP adduct.

mer (about 24 mol NAP per PHEA monomer), PHEA-NAP adduct forms a quite compact structure rather than behaving as a fluctuating chain anchored to the bilayer by the hydrophobic drug residues.

It has been suggested in the literature (Mabrey et al., 1978; Estep et al., 1979) that by relating the enthalpy decrease to the mole fractions of the dissolved substances, and by extrapolating to $\Delta H = 0$, it is possible to gain information about the stoichiometry of the molecule/lipid complex.

In Fig. 2, a plot of the total transition enthalpy vs mole fraction of NAP present in the macromolecular prodrug is reported, and the NAP concentration which leads to $\Delta H = 0$ was obtained by extrapolation.

The extrapolation was necessary because it was not possible to obtain homogeneous mixtures at high NAP-PHEA mole fractions. The following equation was obtained by linear regression of the enthalpy data reported in Table 1:

$$\Delta H = 8.1 - 14.3 X_{NAP};\ r = -0.988$$

A 0.56 mole fraction value of NAP in PHEA-NAP adduct was obtained by extrapolating to $\Delta H = 0$, and assuming linear behaviour also at higher concentrations. This can be interpreted assuming that NAP linked to PHEA forms complexes with DPPC whose stoichiometry is 1:1. This is a realistic value, however, it should be considered only on a qualitative basis.

Finally, PHEA interaction with DPPC liposomes was also investigated, but no evident influence on DPPC thermotropic behaviour was observed (see Table 1), as reported before (Castelli et al., 1990).

Interaction with DPPC/DPPA liposomes

The interaction of NAP with mixed liposomes containing charged lipids shows interesting features. Experiments were carried out in the presence of mixed neutral and charged phospholipids (DPPC-DPPA molar ratio 80:20) in order to investigate the possibility of greater interaction between drug and membranes induced by the binding among the polymer and lipid charged residues.

Fig. 3a–c reports DSC heating curves of hydrated DPPC/DPPA containing different molar fractions of free NAP (a), PHEA-NAP adduct (b) and with the same amount of pure PHEA as in the adduct (c).

The presence of a small fraction of charged lipid in the model membrane brings about new behaviour, caused by the relevant electrostatic interactions. This phenomenon causes a lowering of the transition temperature, even in the presence of PHEA, while PHEA alone has no effect on DPPC liposomes.

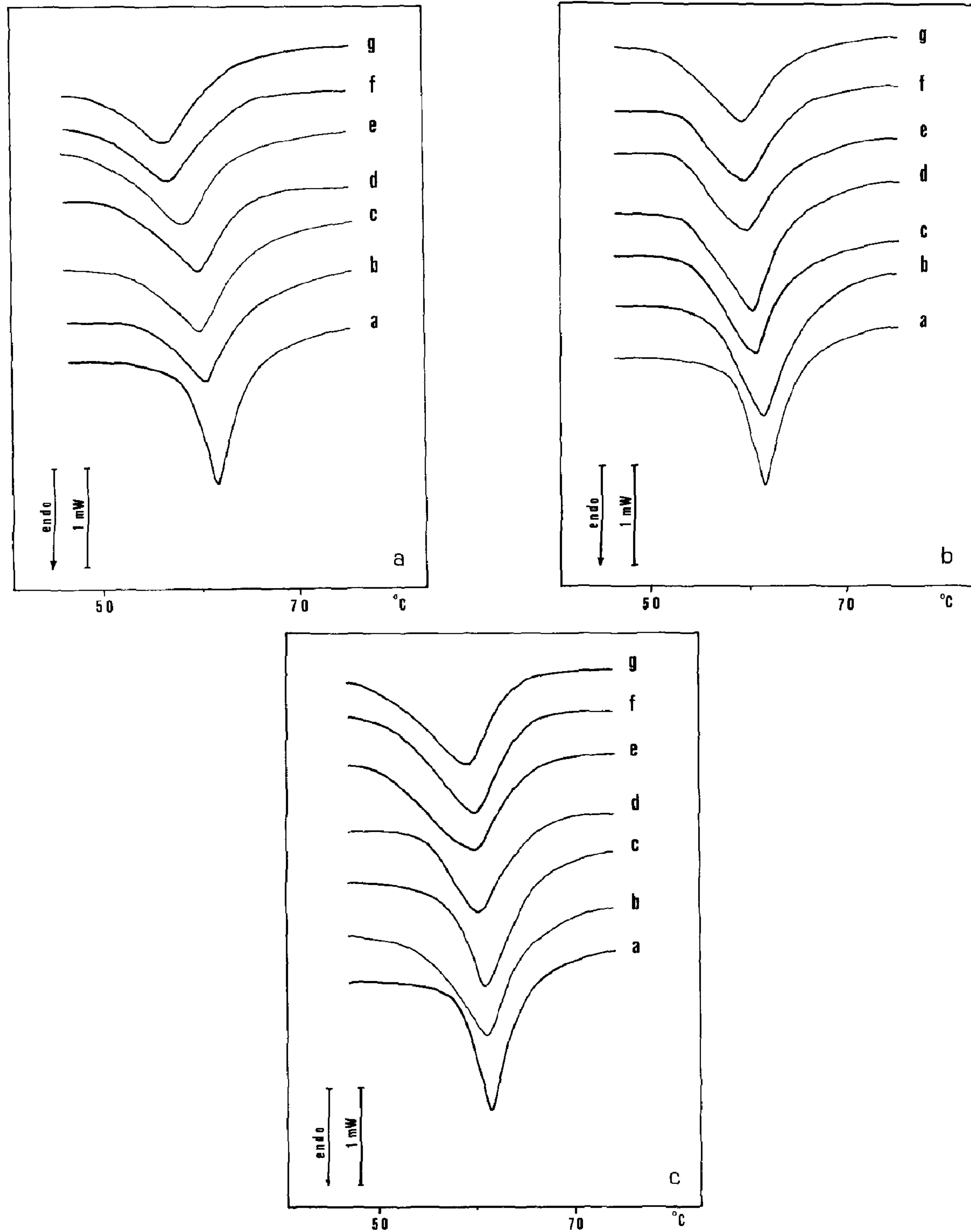

Fig. 3. Differential scanning calorimetry heating curves of hydrated DPPC/DPPA (80 : 20 molar ratio) containing (a) NAP, (b) PHEA-NAP and (c) PHEA at drug mole fractions: (a) = 0; (b) = 0.06; (c) = 0.12; (d) = 0.18; (e) = 0.24; (f) = 0.37; (g) = 0.48.

TABLE 2

Main transition peak temperature (T_m expressed in °C) and main transition enthalpy changes (ΔH expressed in kcal mol^{-1}) of DPPC/DPPA (80:20 mole ratio) dispersions for different molar fractions of free NAP or PHEA and NAP contained in PHEA-NAP adduct (PHEA values are expressed as amount of NAP present in PHEA-NAP adduct; figures are the mean values obtained from at least four DSC heating curves)

Mole fraction	DPPC/DPPA + NAP		DPPC/DPPA + PHEA		DPPC/DPPA + PHEA-NAP	
	T_m	ΔH	T_m	ΔH	T_m	ΔH
0.00	61.2	7.5	61.2	7.5	61.2	7.5
0.06	59.7	6.2	60.6	7.9	60.9	7.7
0.12	59.4	6.5	60.4	7.7	60.1	7.5
0.18	58.6	6.3	59.9	6.5	59.7	7.5
0.24	57.5	7.1	59.6	7.8	59.0	6.4
0.37	57.1	6.2	59.3	8.1	58.7	6.1
0.48	57.3	6.1	58.7	7.1	58.4	7.1

On the other hand, NAP alone has a comparable effect in interacting with both DPPC and DPPC/DPPA vesicles. Conversely, the effect of PHEA-NAP adduct is very different when it interacts with charged vesicles: T_m is shifted toward lower values (the transition temperature remains constant in the PHEA-NAP/DPPC system), whereas the enthalpy remains practically constant (ΔH decreases when the adduct interacts with DPPC vesicles).

This behaviour may be rationalized as follows. The addition of a macromolecule with low solubility both in the gel and liquid-crystal phases leads, as previously discussed, to a decreasing of the ΔH values and constant T_m. However, since PHEA polymer chains interact more extensively with the

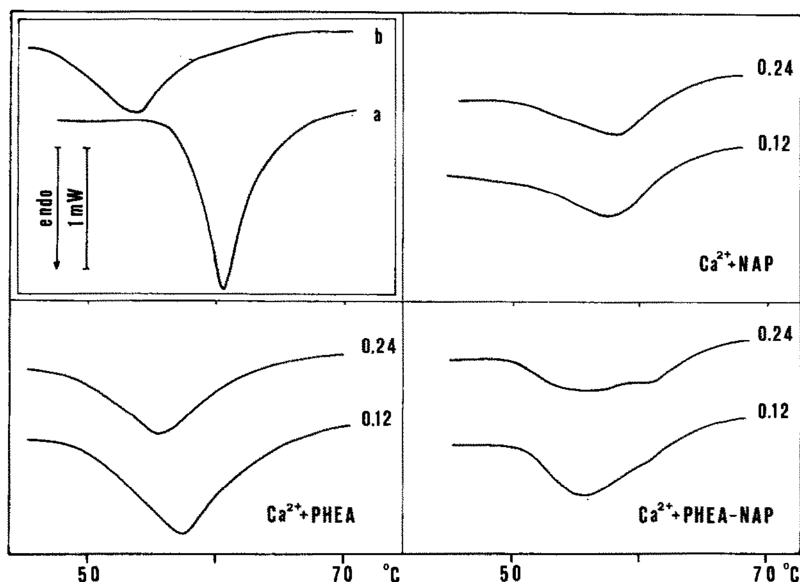

Fig. 4. Comparing effect of Ca^{2+} at 0.1 M concentration on DPPC/DPPA (80:20) mixtures alone or in the presence of NAP, PHEA and PHEA-NAP adduct at two different mole fractions (0.12, 0.24) of the drug.

charged DPPA (see Fig. 3c and Table 2), the free lipid pool enriches itself of DPPC. Since the ΔH of DPPC is greater than that of the DPPC/DPPA mixture ($\Delta H_{DPPC} = 8.2$ kcal/mol; $\Delta H_{DPPC/DPPA} = 7.5$ kcal/mol) and the T_m is lower ($T_{mDPPC} = 42.2°C$; $T_{mDPPC/DPPA} = 61.2°C$) we have a (partial) compensation of the enthalpy changes and a net lowering of the transition temperature values.

The T_m shift is larger when one considers the free drug (Fig. 3 and Table 2). The origin of this shift is probably similar to that observed for the pure DPPC liposomes and is dependent on the NAP concentration.

Calcium effect on the drug-DPPC / DPPA interaction

The adsorption of a drug in a membrane is related to the physical and chemical nature of the lipid bilayer and, among the factors influencing its fluidity and structure, one can consider bivalent ions (mainly Ca^{2+}), that are involved in the regulation of many cell functions.

DSC measurements carried out on PC/PA mixtures in the presence of Ca^{2+} are reported in Fig. 4. At 0.1 M calcium concentration a reduction of the calorimetric peak of about 9°C was observed. This shift seems to be anomalous, since it is well-known that Ca^{2+} strongly binds to the acidic phospholipid head groups causing a tighter packing of the bilayer, as demonstrated by a T_m rising towards higher values (Dzugunes and Papahadjopoulos, 1983; Graham et al., 1985). This apparent anomaly can be understood on inspection of Fig. 5, where data are shown for DPPC/DPPA (80:20) mixtures at four different calcium concentrations (1, 0.5, 0.1 and 0.0 M). At higher Ca^{2+} concentration (1 M) a very clear splitting of the peak in two components occurs, the first peak appearing at a temperature near to or higher than that observed for lipids without calcium and a second one 7–8°C lower. This splitting originates from the melting of DPPC-rich and DPPC-poor domains and the separation tends to disappear on decreasing the calcium concentration. These data can be used to elucidate the drug effects on the lipid bilayer which is largely modified by Ca^{2+}. In fact, the addition of free NAP shifts the transition temperature to higher values,

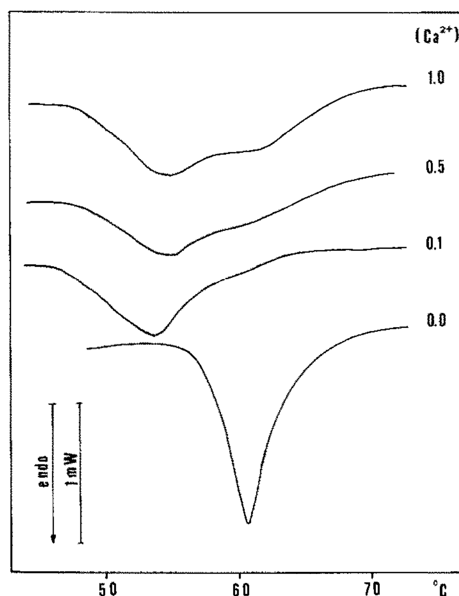

Fig. 5. Differential scanning calorimetry curves of hydrated DPPC/DPPA (80:20) mixtures at different Ca^{2+} molar concentrations.

while in the absence of Ca^{2+} the shift shows an opposite trend. On the other hand, when PHEA-bound NAP is added, Ca^{2+}-induced lateral phase separation becomes more evident.

The first result can be easily interpreted by invoking the disappearance of the microdomains caused by the free drug. This can be seen noting that the transition of DPPC/DPPA (80:20) in the absence of Ca^{2+} is higher than that observed (see Fig. 5). Therefore, the addition of a domain-dissolving agent causes an apparent shift toward higher temperature. Obviously, this effect overlaps with the usual downward shift observed for DPPC and DPPC/DPPA systems and already discussed.

The ability of some apolar substances to inhibit Ca^{2+}-induced domain formation has been observed for related systems containing mixtures of charged and neutral phospholipids and cholesterol (Tilcock, 1984). Anyway, the ability of polymer to induce lateral phase separation is also a common feature observed in several lipid-polymer systems (see, e.g. Raudino et al., 1990, and references cited therein) and it can be understood in terms of the electrostatic interactions between the negative DPPA head groups and the very polar PHEA. This interaction is further demonstrated by the

downward T_m shifts induced by PHEA alone on DPPC/DPPA and DPPC/DPPA + Ca^{2+} mixtures (see Figs. 3 and 4) which indicate the formation of microdomains.

In the present case, the ability of the charged polymer to form a cluster with the anionic lipids is modulated by the polymer hydrophobic residues (NAP) which may or may not have a different solubility in the DPPA-rich domains, favouring or hampering their formation.

All these data can be rationalized in the framework of a theory developed several years ago by Prigogine (1950). By using classical thermodynamics, the author proved that the addition of a third solute, which has a comparable solubility in both solvents forming a binary mixture, leads to a greater mixing of the solvents. In contrast, when the third substance is much more soluble in one of the solvents, the difference in their immiscibility is larger. Free NAP and PHEA-NAP adduct seem to belong to the first and second class of solutes, respectively.

Conclusions

A likely rationale for the DSC measurements can be summarized as follows:

(i) When the free drug interacts with neutral membranes its solubility is high and greater in the more fluid liquid-crystalline domains. The drug linked to the polymer carrier tends to form clusters which do not interact with the surrounding lipids.

(ii) In mixed bilayers containing acidic residues, the free drug acts as a dissolving agent for the domains richer in charged lipids. In contrast, the drug-polymer adduct favours lipid immiscibility.

(iii) The above effects are enhanced (or can be observed) by the addition of divalent cations, which induces the clustering of negatively charged lipids.

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