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Interaction of macromolecular pro-drugs with lipid model membrane: calorimetric study of 4-biphenylacetic acid linked to α,β-poly(*N*-hydroxyethyl)-DL-aspartamide interacting with phosphatidylcholine vesicles

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Summary

The effect of 4-biphenylacetic acid (BPAA) covalently linked to α,β-poly(*N*-hydroxyethyl)-DL-aspartamide (PHEA) on the thermotropic properties of dipalmitoylphosphatidylcholine (DPPC) liposomes was investigated by differential scanning calorimetry (DSC). Addition of increasing amounts of PHEA-BPAA adduct to a suspension of phospholipid vesicles modified the thermotropic gel-to-liquid crystalline phase transition by decreasing the enthalpy changes ΔH with concomitant broadening of the peak without variations in the transition temperature (T_m). These effects are interpreted in terms of a deep interaction of BPAA bound to the polymer with the apolar moiety of the lipid bilayer. The amount of drug able to suppress the phase transition was estimated by plotting the enthalpy changes of the transition vs mole ratio of added drug, and extrapolating to $\Delta H = 0$. The trend in the ΔH values yields a possible 1 : 1 stoichiometry for the interaction between drug and phospholipid. Experiments carried out at different pH values provided information about the species involved in the interaction with DPPC liposomes. Similar results can be expected in the case of the cell membrane.

Introduction

In recent years, increasing interest has been focussed on the use of natural or synthetic polymers in prolonged action, controlled release and targeting pharmaceutical systems (Kydonieus, 1980; Anderson and Kim, 1986). Polymers can thus form an inert matrix in which drugs are dissolved, adsorbed or dispersed, or surround the active compounds as a diffusion barrier. Finally, drugs can be chemically linked to the polymer backbone; the release of drug from the resulting macromolecular prodrug is resolved by the rate of cleavage, chemically or biologically induced, of

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the linkages between the covalently bound conjugates (Wan Kim et al., 1980).

In this field, we have already studied the properties of drugs linked to polymeric carrier (Giammona et al., 1987, 1989a, b). Among them, α,β-poly(*N*-hydroxyethyl)-DL-aspartamide (PHEA) has been chosen as a non-toxic, non-antigenic, water-soluble and biodegradable compound (Neri et al., 1973).

In a recent work, PHEA adducts with some non-steroidal anti-inflammatory agents (NSAIDs) were prepared and characterized. The relative 'in vivo' pharmacological tests showed that the polymeric prodrugs have an analgesic and anti-inflammatory activity similar to that of drugs alone (Giammona et al., 1989c).

Among the PHEA-linked NSAIDs, 4-biphenylacetic acid (BPAA) is a biologically active molecule that we have previously studied as a β-cyclodextrin inclusion compound, in order to improve its water solubility and bioavailability (Puglisi et al., 1989). BPAA is a strong cyclooxygenase inhibitor (Tolman and Partridge, 1975; Tolman et al., 1976a), with a potency greater than that of aspirin and phenylbutazone (Tolman et al., 1976b). Its maximum activity has been found in inflammatory pain states such as in reducing UV irradiation skin erythema on guinea pig (Sloboda and Osterberg, 1976).

In order to gain greater insights into the interaction between polymer-linked drugs and biological membranes, we investigated the effects of BPAA, covalently linked to PHEA, on the gel-to-liquid crystalline phase transition of DPPC liposomes selected as a model membrane.

In fact, phospholipids are major lipid components of biological membranes and provide a matrix for functional membrane proteins and incorporation of biological molecules besides maintaining a permeability barrier between external and internal cell environments. Even if biological membranes are a complex mixture of phospholipid species which vary with respect to both their lipid head group and associated acyl chains, synthetic DPPC liposomes usually represent a good membrane model.

On the other hand, differential scanning calorimetry is a powerful and non-perturbing thermodynamic technique for characterizing the thermotropic phase behaviour of lipid bilayers in model and biological membranes. The presence of drugs in the ordered bilayer structure could affect the packing of lipid chains depending on their amphipathic nature as well as lipophilicity, causing variation in the transition temperature of the pure lipid and/or changes in the enthalpy of chain melting (Bach, 1984).

Since we have already reported the characterization by DSC of the interaction of a BPAA–β-cyclodextrin inclusion complex (Castelli et al., 1989) with lipid membranes, and with the consideration that there is a scarcity of data reported in the literature on this subject, in the present paper we have extended this study to a polymeric carrier for BPAA interacting with a membrane model.

Materials and Methods

Chemicals

Synthetic L-α-dipalmitoylphosphatidylcholine was obtained from Fluka (Buchs, Switzerland). Solutions of lipids were chromatographically pure as assessed by two-dimensional thin-layer chromatography (TLC) on silica gel plates (Merck, Darmstadt, F.R.G.) using a solvent system consisting of chloroform/ methanol/ water/ acetic acid (60 : 35 : 4 : 1, by vol.).

4-Biphenylacetic acid was obtained from Janssen (Belgium; analytical grade) and was recrystallized from ethanol.

DL-Aspartic acid, ethanolamine and *N*,*N*-dimethylformamide were purchased from Fluka.

α,β-Poly(*N*-hydroxyethyl)-DL-aspartamide was prepared via polysuccinimide by polycondensation of DL-aspartic acid in the presence of H_3PO_4 at 180 °C, followed by reaction with ethanolamine in *N*,*N*-dimethylformamide solution (Neri et al., 1973). The analytical and spectral data of PHEA are in agreement with those reported in the literature. The average molecular weight of PHEA was found to be 27100, as determined viscosimetrically using the Mark Houwink equation: $[\eta] = 2.32 \times 10^{-3} \times M^{0.87} = 16.7$ ml/g (Antoni et al., 1974).

Preparation of PHEA-BPAA adduct

The adduct was prepared, purified and characterized following the procedure described in a preceding paper (Giammona et al., 1989c). The BPAA load in the polymeric prodrug, as determined by UV spectroscopy, elemental analysis and hydrolysis, amounted to about 15% (w/w).

Prodrug stability at different pH values

PHEA-BPAA adduct was dissolved in a suitable volume of pH 4 or 7.4 Tris solutions and kept at 60 °C for 48 h. The absence of the free drug was demonstrated by TLC also at the later time. A stability test at pH 10, performed as previously reported (Giammona et al., 1989c), showed that hydrolysis of the prodrug had reached completion after 30 h.

Preparation of liposomes

Aqueous dispersions of pure DPPC and mixtures of BPAA, PHEA and PHEA-BPAA adduct with the phospholipid were prepared according to the following procedure. Lipid and BPAA solutions in CH_3OH-$CHCl_3$ (1 : 1, v/v) were mixed in order to obtain different molar ratios of drug. The solvent was removed at 30 °C on a rotary evaporator using a nitrogen stream followed by overnight storage under high vacuum.

PHEA and PHEA-BPAA adduct dispersions in DPPC liposomes were prepared by adding aqueous solutions of the compounds to the lyophilized DPPC film. It should be noted that all mole fractions refer to the BPAA content in the adduct.

Liposomes were prepared at a temperature greater than that of their gel-liquid crystalline phase transition in order to allow complete hydration of the samples, by adding to the film 50 mM Tris buffer adjusted to pH 7.4. The samples were heated at 60 °C, vortex-mixed twice for 1 min and shaken for 1 h at 55 °C in a water bath in order to homogenize the lipid dispersions. 120 μl of each sample containing 5 mg DPPC were sealed in an aluminium pan and submitted to DSC analysis.

Experiments at different pH values were carried out on pure lipid alone as well as in the presence of PHEA, BPAA and PHEA-BPAA adduct by using unbuffered Tris solutions at pH 4 and 10, obtained by addition of the calculated amount of (0.1 N) HCl.

Differential scanning calorimetry

Differential scanning calorimetry was 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, within the temperature range 10–60 °C, sensitivities of 1.71 mW and the same Tris solution in the reference pans.

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 calorimetric runs the pan content was extracted and aliquots were taken for phosphate analysis to determine the amount of phospholipid in the pan (Bartlett, 1959).

Results and Discussion

The thermotropic behaviour of aqueous dispersions (Tris buffer at pH 7.4) of DPPC mixed with various amounts of PHEA-BPAA adduct is illustrated by a number of typical DSC runs, in the heating mode, in Fig. 1. The pretransition and main transition peaks for pure DPPC were observed to be at 36.9 and 42.2 °C, respectively. Addition of the polymeric prodrug to DPPC bilayers produced evident changes in their thermotropic behaviour. On increasing the prodrug mole fraction in the aqueous dispersion, the transition temperature (T_m) remains constant whereas the main transition peak changes shape. Broadening of the curves and a reduction in the related area, together with a decrease in the total enthalpy changes of the main phospholipid gel-to-liquid crystalline phase transition, were observed. Complete disappearance of the transition peak, corresponding theoretically to $\Delta H = 0$, did not occur, owing to the impossibility of obtaining homogeneous mixtures at PHEA-BPAA mole fractions higher than those reported in the present paper. Consequently, the total enthalpy of transition was

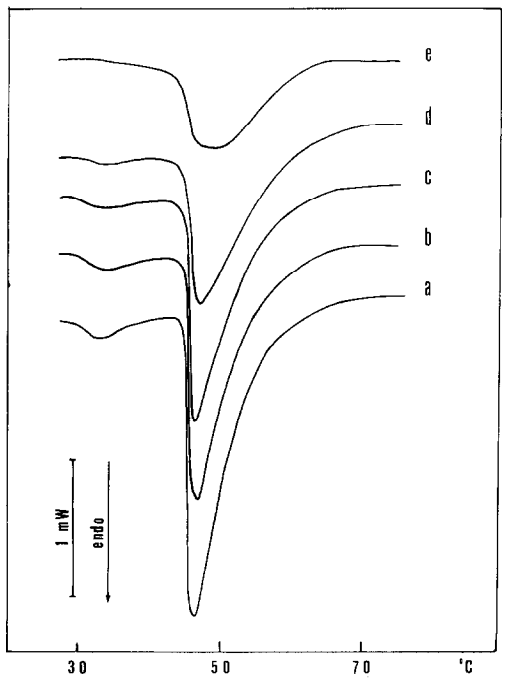

Fig. 1. Differential scanning calorimetry heating curves of hydrated DPPC containing PHEA-BPAA at BPAA mole fractions of: (a) 0, (b) 0.06, (c) 0.12, (d) 0.18, (e) 0.24.

plotted vs mole fraction of BPAA present in the macromolecular prodrug, and the BPAA concentration which leads to $\Delta H = 0$ was obtained by extrapolation (see Fig. 2). By relating the measured enthalpy changes to the mole fractions of

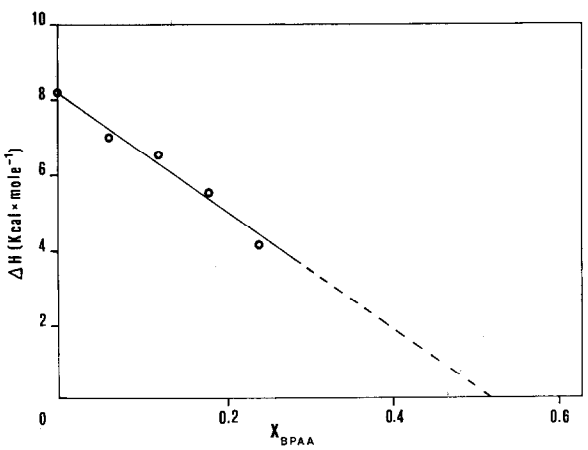

Fig. 2. Enthalpy changes, ΔH (kcal mol^{-1}), of the gel-to-liquid crystalline phase transition of DPPC as a function of mole fraction of BPAA content in PHEA-BPAA adduct.

TABLE 1

Main transition peak temperature (T_m, in °C) and main transition enthalpy changes (ΔH, in kcal mol^{-1}) of DPPC dispersions for different molar fractions of BPAA content in PHEA-BPAA adduct (values represent means obtained from DSC heating curves)

Mole fraction	T_m	ΔH
0.00	42.2	8.2
0.06	42.1	7.0
0.12	42.2	6.6
0.18	42.2	5.5
0.24	42.3	4.2

the prodrug present in the lipid matrix, we can obtain information on the stoichiometry of the lipid/PHEA-BPAA adduct complexes, as described by others (Mabrey et al., 1978; Estep et al., 1979).

The following equation was obtained by linear regression of the enthalpy data reported in Table 1:

$$\Delta H = 8.2 - 15.7X_{BPAA};\ r = -0.988$$

A value of 0.52 for the mole fraction of BPAA in the PHEA-BPAA adduct was obtained by extrapolation to $\Delta H = 0$, and assuming the linear behaviour to occur also at higher concentrations. This signifies that the BPAA linked to PHEA forms complexes with DPPC whose stoichiometry is 1 : 1.

With the knowledge that BPAA interacts, by itself, with DPPC liposomes at pH 7.4, decreasing the transition temperature without changes in the ΔH (Castelli et al., 1989), we attempted to determine the species that affect the thermotropic behaviour of DPPC when this drug is linked to the PHEA backbone, carrying out measurements at different pH values (range pH 4–10) of aqueous dispersions of DPPC, PHEA/DPPC, BPAA/DPPC and PHEA-BPAA/DPPC, respectively (see Fig. 3, where the calorimetric scans of DPPC at different pH values are not shown) pH effect on the thermotropic behaviour of DPPC having been observed as pointed out before). Both of the latter two systems contain the same mole fraction of BPAA ($X = 0.12$). The results can be summarized as follows:

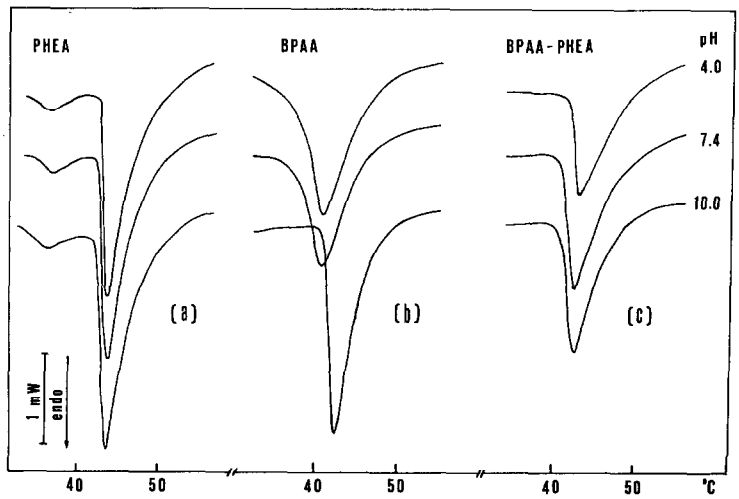

Fig. 3. Differential scanning calorimetry heating curves, at different pH values of hydrated DPPC in the presence of a 0.12 molar fraction of BPAA, free (b), contained in PHEA-BPAA adduct (c), and with the same amount of PHEA as in the preceding PHEA-BPAA adduct (a).

(i) the pH variation does not affect the phase transition of DPPC according to the literature (Trauble and Eibl, 1974);
(ii) PHEA does not interact with DPPC bilayers, demonstrating no appreciable variations in shape of the calorimetric scans (Fig. 3a);
(iii) BPAA interacts with DPPC bilayers only at neutral and acid pH values where a decrease in the T_m and a broadening of the peak occur, while at basic pH the transitional peak remains almost unaltered (Fig. 3b);
(iv) PHEA-BPAA adduct interacts at all pH values, causing broadening of the peak with a decrease in enthalpy while the T_m remains almost constant (Fig. 3c).

From these results, information on the role of drug and polymer in the interaction of their adduct with DPPC model membrane is readily obtained. In fact, PHEA does not interact with DPPC bilayers, since it possesses no groups capable of fitting within the lipid bilayer, whereas BPAA interacts at pH 4 and 7.4, due to being in an undissociated form and because of its liposolubility which allows it to be located in the bilayer, causing a fluidizing effect on the lipid chains. Such a disordering effect on the lipid aggregate should result from the ability of BPAA to form hydrogen bonds with the polar head groups of PC, whereas the apolar biphenyl rings fit between the apolar tails of DPPC. At pH 10, BPAA is in an ionized form with greater solubility in water than the undissociated form, and therefore does not interact with the membrane (see Fig. 3).

The behaviour of PHEA-BPAA adduct interacting with DPPC liposomes appears to be independent of the pH, suggesting that BPAA bound to the polymeric chain of PHEA should be the species involved in such interactions.

Moreover, the possibility of attributing these effects to free BPAA arising from the eventual hydrolysis of the compound was also examined. In fact, we found that under our experimental conditions, partial hydrolysis occurs only at pH 10 (Giammona et al., 1989b), but not at pH 4 and 7.4 (see Experimental).

This hypothesis is inconsistent with the experimental data, since the effect of PHEA-BPAA adduct is the same at all pH values examined and the interaction also persists at pH 10, a value at which prodrug is partially hydrolyzed and BPAA alone does not fit into the bilayer. Consequently, bearing in mind the previous results, BPAA present in the adduct interacts with DPPC bilayers, because of its lipophilicity which is very similar to that of undissociated BPAA.

These results clearly demonstrate that PHEA-

TABLE 2

Main transition peak temperature (T_m, in °C) and main transition enthalpy changes (ΔH, in kcal mol^{-1}) of DPPC aqueous dispersions in the presence of a 0.12 molar fraction of free BPAA, contained in PHEA-BPAA adduct, and with the same amount of PHEA as in the preceding PHEA-BPAA adduct aliquot, at different pH values (values represent the means obtained from DSC heating curves)

pH	BPAA		PHEA		PHEA-BPAA	
	T_m	ΔH	T_m	ΔH	T_m	ΔH
4	40.8	6.9	42.8	8.0	42.5	6.8
7.4	40.6	6.8	42.6	8.1	42.2	6.6
10	42.1	8.3	42.3	7.9	42.3	6.7

BPAA adduct is able to fit within the DPPC bilayer causing profound modification in ordering of the chains mainly due to the introduction of the apolar biphenyl rings between the apolar tails of DPPC, leading to destabilization of the lipid aggregate as well as a loss in cooperativity as demonstrated by the broadening of the curves without variations in T_m.

BPAA bound to PHEA acts as a filler of the lipid structure, as demonstrated previously for other molecules such as cholesterol, which is able to suppress the transition peak without changing the T_m (Estep et al., 1978; Gallay and Kruyff, 1982; Bach, 1984). Since the polymeric structure of PHEA-BPAA adduct is known, we can assume that this compound has behaviour similar to that shown by some proteins, which in most cases exert little effect on the T_m of the DPPC phase transition, whereas the enthalpy change decreases almost linearly by increasing the protein concentration. By plotting ΔH vs mole fraction of protein, followed by extrapolation to $\Delta H = 0$, it is possible to calculate the number of phospholipid molecules withdrawn from the cooperative chain-melting process (McElhaney, 1986), and that bound to the proteins.

Thus, our system also seems to penetrate and interact with the phospholipid fatty acyl chains, removing phospholipid molecules from the cooperative chain-melting process, in a stoichiometric 1 : 1 mole ratio of drug to phospholipid.

Conclusions

The present results demonstrate that the interaction of PHEA-BPAA prodrug with phospholipids could be due to the lipophilic nature of BPAA bound to the polymer. Hence, a similar interaction with cell membranes can be expected.

The resulting modifications in membrane fluidity could improve absorption of the drug, after chemical or enzymatic hydrolysis of the polymeric adduct.

The calorimetric procedure as employed here can represent a good approach to the experimental study of physical relationships between a pharmaceutical compound and an artificial lipid membrane.

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