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Calorimetric evaluation of the interaction and absorption of eicosapentaenoic acid by biomembrane models

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ABSTRACT

The interaction of eicosapentaenoic acid, a polyunsaturated fatty acid belonging to the omega-3 class, with biomembrane models represented by multilamellar or unilamellar vesicles made of dimyristoylphosphatidylcholine was monitored by means of differential scanning calorimetry technique. The calorimetric analysis of vesicle prepared in the presence of increasing molar fraction of eicosapentaenoic acid was carried out to show its maximum interaction with biomembrane models evaluating the effects of eicosapentaenoic acid on the biomembrane models thermotropic parameters (transition temperature and enthalpy variation). Furthermore, in order to detect the influence of the presence of hydrophilic or lipophilic media on the entity of the compound absorption by the biomembrane models, kinetic experiments were carried out.

The results indicate that eicosapentaenoic acid strongly interacts with the biomembrane models depressing the transition temperature and the enthalpy variation. Eicosapentaenoic acid is absorbed by the biomembrane models and the absorption is affected by the used medium; in fact a bigger absorption happens in the presence of a lipophilic medium.

1. Introduction

Eicosapentaenoic acid (EPA) is an omega-3 fatty acid, its chemical structure is characterized by a 20-carbon chain, with a carboxylic and a methyl (omega) end, containing five *cis* double bonds, the first is located at the third carbon from the omega end (Scheme 1). EPA belongs to the essential fatty acids, which the human body cannot synthesized *de novo*, but it can be made from the eighteen-carbon $n-3$ fatty acid, α-linolenic acid. Synthesis of the longer $n-3$ fatty acids from linolenic acid within the body occurs competitively with the synthesis of $n-6$ analogues. Therefore, the most important source of omega-3 fatty acids is represented by food, in particular fish, fish oil and vegetables [1]. Omega-3 fatty acids are essential component of each living cell, they are not only especially important for the integrity of lipid structures of cell membranes [2], and for neurological and brain development [3], but also important sources of energy and precursors for numerous biologically active compounds. In addition, dietary intake of EPA is related to the prevention of many diseases such as breast, colon and prostate cancer [4–6], inflammatory and autoimmune disorders including rheumatoid arthritis [7], psoriasis [8], diabetes [9] and heart diseases [10].

To exert its activity EPA have to interact and cross the biological membrane, then we have studied the interaction and the absorption of EPA by biomembrane models constituted by multilamellar (MLV) or unilamellar vesicles (LUV) made of dimyristoylphosphatidylcholine (DMPC).

Phospholipid vesicles undergo, upon heating, to a transition from an ordered (gel) state to a disordered (liquid-crystalline) state (L_β to L_α transition) characterized by a well defined temperature (T_m), and an enthalpy change (ΔH). Substances interacting with phospholipid vesicles cause modifications on the T_m and ΔH [11,12]. The interaction of EPA with biomembrane models was monitored by means of the differential scanning calorimetry (DSC) technique; in particular DSC was employed to evaluate the enthalpy variation and the transition temperature depression of pure DMPC liposomes, by following the Vant'hoff temperature depression [13], due to the presence of EPA into the vesicles. We have prepared and submitted to DSC analysis DMPC MLV containing increasing amount of EPA, the interaction between EPA and the MLV was evaluated detecting the modification of the DMPC MLV calorimetric curves caused by the eventual incorporation of EPA in the ordered structure of the model biomembranes.

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Scheme 1. Structural formula of EPA.

Furthermore, the effect of aqueous and lipophilic medium on the absorption of EPA by the biomembrane models was also investigated by kinetic experiments.

2. Experimental

2.1. Chemicals

DMPC (purity: 99%) was obtained from Genzyme (Switzerland). EPA (purity: ≥98%) was purchased from Cayman Chemical (Mi, USA). 50 mM Tris(hydroxymethyl)aminomethane (TRIS) adjusted to pH 7.4 with hydrochloric acid was used to prepare liposomes. Lipid concentrations were determined by the phosphorous analysis [14].

2.2. Liposomes preparation

Stock solutions of DMPC and EPA were prepared in chloroform–methanol (1:1, v:v), then appropriate aliquots were mixed in glass flasks to obtain the same amount of DMPC (0.010325 mmol) and increasing molar fractions (X) (0.0, 0.015, 0.03, 0.045, 0.06, 0.09, 0.12, 0.15, 0.18) of EPA with respect to the DMPC. The solvents were removed under a nitrogen flow and the resulting films were freeze-dried under vacuum to remove the residual solvents. Lipid films were suspended with 168 μl of Tris 50 mM, pH 7.4 and the multilamellar liposomes were prepared by heating to 37 °C (temperature above the gel–liquid crystalline phase transition) and vortexing three times for 1 min. The samples were kept for 1 h in a water bath at 37 °C to homogenize the liposomes and to reach a complete distribution of EPA between lipid and aqueous phases.

To obtain LUV, MLV were repetitively (19 times) passed under moderate pressure at a temperature at least 5 °C above the T_m through polycarbonate membranes (pores diameter 100 nm) in an extruder system (LiposoFastTM Basic, Avenstin Inc.) [15,16]. The membrane pores are almost cylindrical, and vesicles (unilamellar or multilamellar) that are larger than the mean pore diameter are reduced in size and lamellarity during the passage through the pores, resulting in final vesicle size that corresponds to the mean size of the pores [17,18].

2.3. DSC analysis of EPA/vesicles interaction

DSC measurements were performed using a Mettler Toledo Star^e System equipped with a DSC 822^e calorimetric cell and a Mettler TA Star^e software. The sensitivity was automatically chosen as the maximum possible by the calorimetric system. DSC was calibrated using palmitic acid. Aliquots of 120 μl (0.007375 mmol) of MLV were transferred into a 160 μl DSC aluminium pan and hermetically sealed. The sample were submitted, at least four times to check the reproducibility of results, to the following procedure: a heating scan between 5 and 37 °C (heating rate 2 °C/min) and a cooling scan between 37 and 5 °C (cooling rate 4 °C/min) with the aim to study the interaction EPA/vesicles. The reference pan was filled with Tris buffer solution (50 mM, pH 7.4). Each experiment was carried out in triplicate. All the samples, after the calorimetric scans, were extracted from the pan and aliquots were used to determine the exact amount of phospholipids by the phosphorous assay [14].

2.4. Permeation kinetic experiments

To follow the uptake of the poor water soluble EPA by the biomembrane models in a hydrophilic medium, 120 μl of DMPC aqueous suspension (MLV or LUV) were left in contact with an exact amount of EPA in the bottom of a DSC pan, in order to obtain a 0.09 molar fraction of EPA with respect to the phospholipids dispersed in the aqueous medium. A 0.09 molar fraction was chosen as the calorimetric curve of the MLV prepared in the presence of EPA at 0.09 molar fraction (in which the most homogeneous distribution of EPA among phospholipid molecules is obtained) was modified with respect to that of the pure DMPC, but retaining a well defined shape without phase separation. The aluminium pan was hermetically closed and the sample was submitted to the following calorimetric analysis:

(1) a heating scan was performed from 5 to 37 °C, at the rate of 2 °C/min, to detect the eventual interaction of EPA with the model biomembranes due to the dissolution and the successive migration of the compound through the aqueous medium and the absorption by the phospholipid layer/s of LUV or MLV;
(2) an isothermal period (1 h) at 37 °C, when the liposomal system is in a disordered state, was then used to permit the substance, eventually dissolved in the medium, to further migrate towards the MLV/LUV surface, but also to permeate the phospholipid layer (LUV) or the inner phospholipid layers (MLV) and to deeply interact with them;
(3) a cooling scan between 37 and 5 °C, at the rate of 4 °C/min, was used to bring the phospholipid system back to the ordered state before restarting the process.

This procedure was run at least eight times, to follow any variation in the calorimetric curves at increasing incubation times. Each experiment was carried out in triplicate.

2.5. Transmembrane transfer kinetic experiments

To determinate if the lipophilic medium, where the substance can be molecularly dispersed, can improve the uptake of EPA by the lipid membranes, a transmembrane transfer kinetic was carried out. 60 μl of MLV dispersion prepared in the presence of EPA at 0.12 molar fraction (loaded MLV) and 60 μl of an equimolar dispersion of MLV made of pure DMPC (unloaded MLV) were put in contact in a 160-μl DSC pan, hermetically sealed and submitted to the DSC analysis following the same procedure reported in the previous section. The experiment was carried out in triplicate.

3. Results and discussion

MLV of pure DMPC, when submitted to heating under the ambient pressure condition, show two endothermic peaks: a smaller peak, called pretransition peak, at about 15 °C, related to the transition from the gel (L_β) to the ripple phase in which the bilayer surface is undulated and the phospholipid chains are tilted with respect to the normal layer, and a main peak at about 24.6 °C, related to the transition from the ripple phase to the liquid-crystalline phase (L_α). In the gel phase the lipid chains are ordered with all-*trans* conformation whereas the liquid-crystalline phase, occurring at higher temperatures, is characterized by rapid *trans–gauche* isomerism of the lipid chain, rotation around the long axis of the lipid molecules; the lateral packing density is considerably reduced in comparison with gel phase [19,20]. Variation of the thermodynamic parameters, transition temperature and enthalpy changes, of both phase transitions are caused by the presence of molecules dissolved in the lipid bilayer and depends on the amounts of substance interacting with the bilayer [19,21].

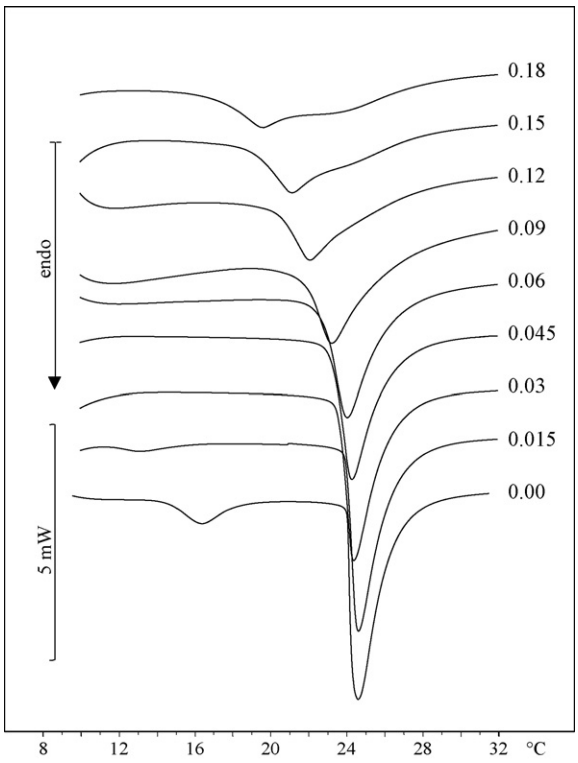

Fig. 1. Calorimetric curves, in heating mode (2 °C/min), of DMPC MLV prepared in the presence of EPA at increasing molar fractions.

MLV of DMPC in the presence of increasing molar fractions (X) of EPA were prepared and analysed by DSC; the obtained calorimetric curves are compared to that of pure DMPC MLV (Fig. 1); variations of the calorimetric curve means that the compound interacts with the MLV phospholipid bilayers. EPA interacts with the DMPC liposomes, in fact, the pretransition peak is still evident but largely shifted to lower temperature for 0.015 molar fraction then at higher molar fractions it completely disappears. As far as concern the main transition peak, EPA causes the shift towards lower temperatures; moreover the peak broadening is clearly visible. The substance, at molar fractions higher than 0.12, is not homogeneously dispersed in the phospholipidic bilayer, and poor and rich EPA regions are present as indicated by the phase separation [22].

In Fig. 2 the peak temperature shift of the calorimetric curves, caused by the presence of EPA, is expressed as $\Delta T/T_m^0$ ($\Delta T = T_m - T_m^0$, where T_m^0 and T_m, being respectively the transition temperature of pure DMPC MLV and in the presence of increasing amounts of EPA), and plotted as a function of EPA molar fraction in the lipid aqueous dispersion. The bigger the EPA molar fraction the stronger the transition temperature decrease. A quantitative description of the peak broadening is reported in Fig. 3, where the peaks width (width at peak half height, $\Delta T_{1/2}$) is plotted as a function of EPA molar fractions in the phospholipid aqueous dispersion. It is clearly evident that as the EPA molar fraction increases also the peaks width increases. Differences in ΔH are shown in Fig. 4 as $\Delta\Delta H/\Delta H^\circ$ ($\Delta\Delta H = \Delta H - \Delta H^\circ$, where ΔH is the enthalpy variation of DMPC MLV prepared in the presence of increasing molar fractions of EPA and ΔH° is the enthalpy change of pure DMPC MLV). The presence of increasing amount of EPA caused a small enthalpy change decrease.

The decrease of T_m and ΔH and the broadening of the calorimetric peak are related to the increasing amount of EPA present in the MLV aqueous dispersion. This behaviour can be justified by a fluidifying effect exerted by EPA on the phospholipid cooperativity, due to its introduction into the ordered structure of the phospholipid bilayer like several classes of compounds studied and reported

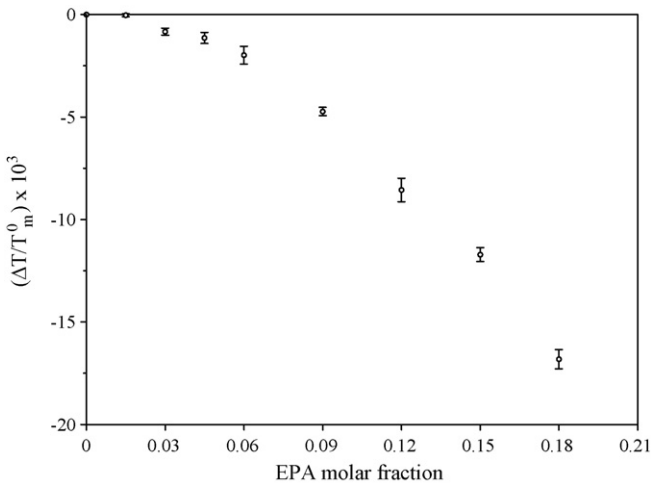

Fig. 2. Transition temperature, as $\Delta T/T_m^0$, of DMPC MLV prepared in the presence of EPA at increasing molar fractions, as a function of the molar fraction. $\Delta T = T_m - T_m^0$; T_m is the transition temperature of DMPC MLV prepared in the presence of EPA and T_m^0 is the transition temperature of pure DMPC MLV. T is expressed in K.

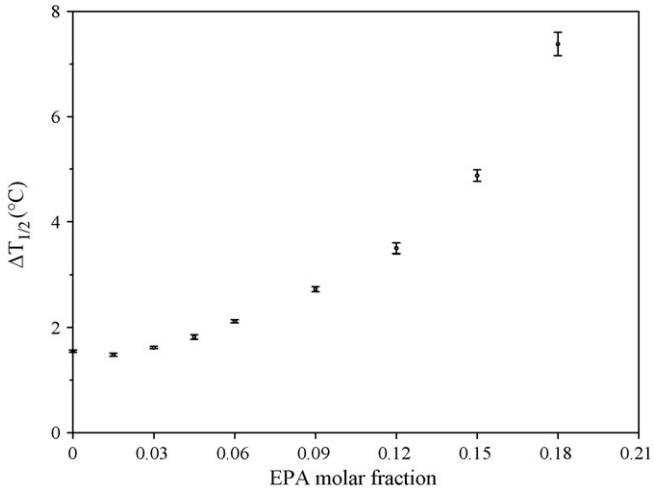

Fig. 3. Peak width (width at peak half height, $\Delta T_{1/2}$) of DMPC MLV prepared in the presence of EPA at increasing molar fractions, as a function of EPA molar fractions in the phospholipid aqueous dispersion.

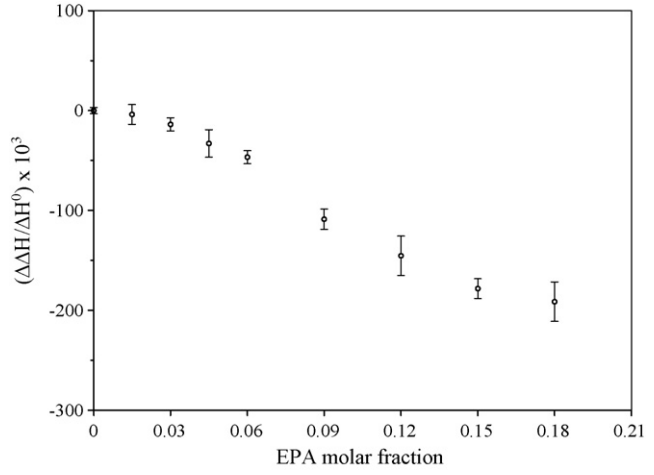

Fig. 4. Enthalpy variation as $\Delta\Delta H/\Delta H^\circ$ of DMPC MLV prepared in the presence of EPA at increasing molar fractions, as a function of the molar fraction. $\Delta\Delta H = \Delta H - \Delta H^\circ$; ΔH is the enthalpy variation of DMPC MLV prepared in the presence of EPA and ΔH° is the enthalpy variation of pure DMPC MLV.

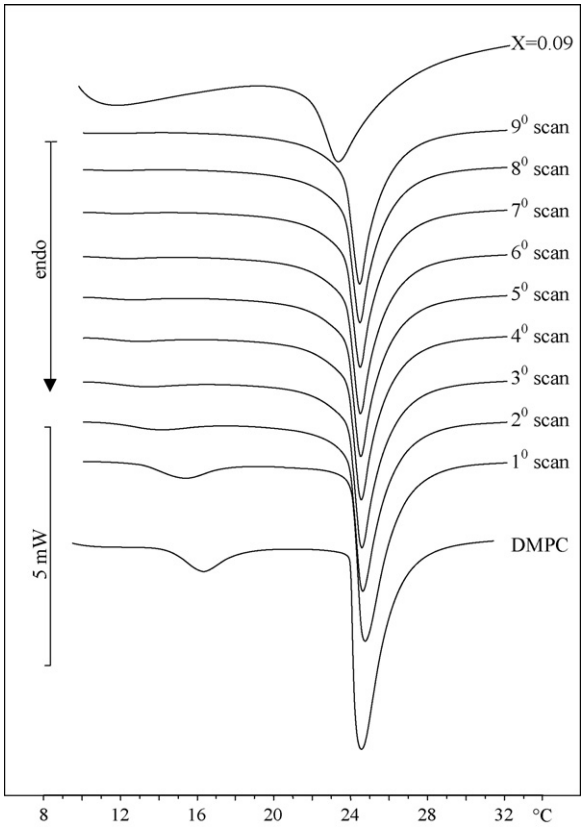

Fig. 5. Calorimetric curves, in heating mode (2 °C/min), of DMPC MLV left in contact with EPA (0.09 molar fraction), at 37 °C, at increasing incubation times. Curve $X = 0.09$ belongs to DMPC MLV prepared in the presence of EPA at 0.09 molar fraction.

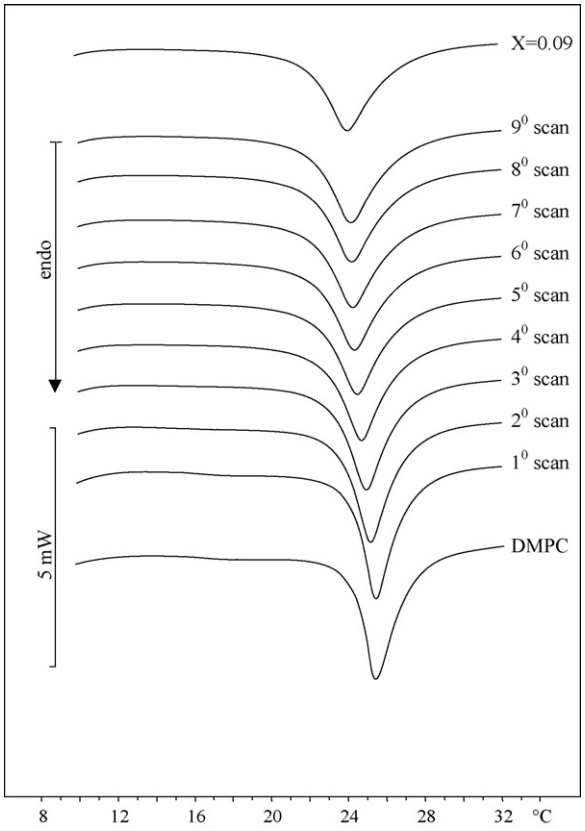

Fig. 6. Calorimetric curves, in heating mode (2 °C/min), of DMPC LUV left in contact with EPA (0.09 molar fraction), at 37 °C, at increasing incubation times. Curve $X = 0.09$ belongs to DMPC LUV prepared in the presence of EPA at 0.09 molar fraction.

in the literature [21,23–27]. EPA acts as spacer in such a structure, causing a destabilization of the lipid ordered mosaic with a decrease in the T_m of the gel-to-liquid crystal phase transition and the calorimetric peak broadening.

EPA, to perform its function after its intake, have to distribute between the aqueous medium and the biological membranes; then, to interact with the biomembranes, should be absorbed by them. In order to mimic this stage, empty MLV or LUV were left in contact with a fixed amount of EPA (to have 0.09 molar fraction in the aqueous lipid dispersion) at increasing incubation times. The calorimetric curves are reported in Figs. 5 and 6. The curve $X = 0.09$ represents the maximum interaction between EPA and lipid bilayer, and it is obtained by the vesicles prepared in the presence of 0.09 molar fraction of EPA as described in "Liposomes preparation". With regard to the experiments carried out with MLV (Fig. 5), the pretransition peak, as the incubation time increases, becomes smaller and then disappears; the main transition peak shows a small decrease and a small shift towards lower temperatures but the curve at $X = 0.09$ is not reached. EPA, because of its low water solubility, shows a very low capacity to migrate from the aqueous medium to the MLV surface and be absorbed by the phospholipid bilayers. The calorimetric curves of the kinetic experiments carried out with LUV are shown in Fig. 6. As the incubation time increases, the presence of EPA causes the calorimetric peak to gradually broaden and shift towards lower temperature reaching the curve $X = 0.09$. In Fig. 7 the transition temperature variations, as $\Delta T/T_m^0$, of DMPC MLV and LUV left in contact with a 0.09 molar fraction of compound, as a function of the calorimetric scans, are reported. The r-value is the transition temperature of DMPC vesicles prepared in the presence of EPA at 0.09 molar fraction (curve $X = 0.09$ in Fig. 5) and represents the maximum interaction between EPA and vesicles. It is the value that should be obtained if a complete absorption of EPA by the vesicles happened. A decrease of the T_m, is visible both when MLV and LUV are used. In the presence of MLV the T_m decrease is very small and the r-value is not reached whereas in the presence of LUV the decrease is gradual, and the value r is reached. EPA transfers through the aqueous medium and interacts with the lipo-

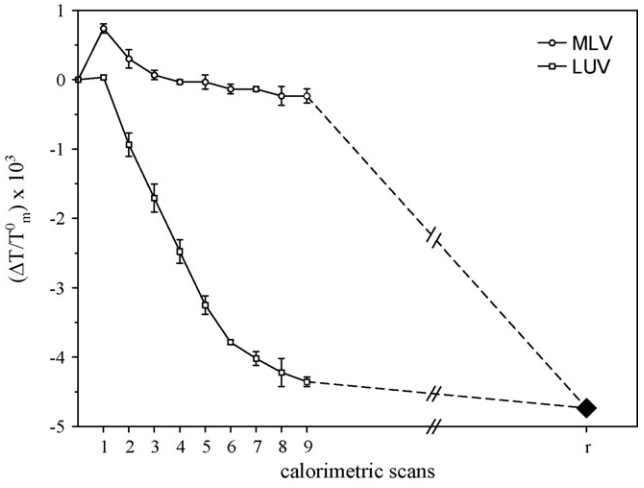

Fig. 7. Transition temperature, as $\Delta T/T_m^0$, of DMPC MLV and LUV left in contact with EPA (0.09 molar fraction) at 37 °C, as a function of the calorimetric scans. $\Delta T = T_m - T_m^0$; T_m is the transition temperature of DMPC MLV prepared in the presence of EPA and T_m^0 is the transition temperature of pure DMPC MLV. T is expressed in K. Value r is related to the transition temperature of DMPC vesicles prepared in the presence of EPA at 0.09 molar fraction and represents the maximum interaction between EPA and vesicles.

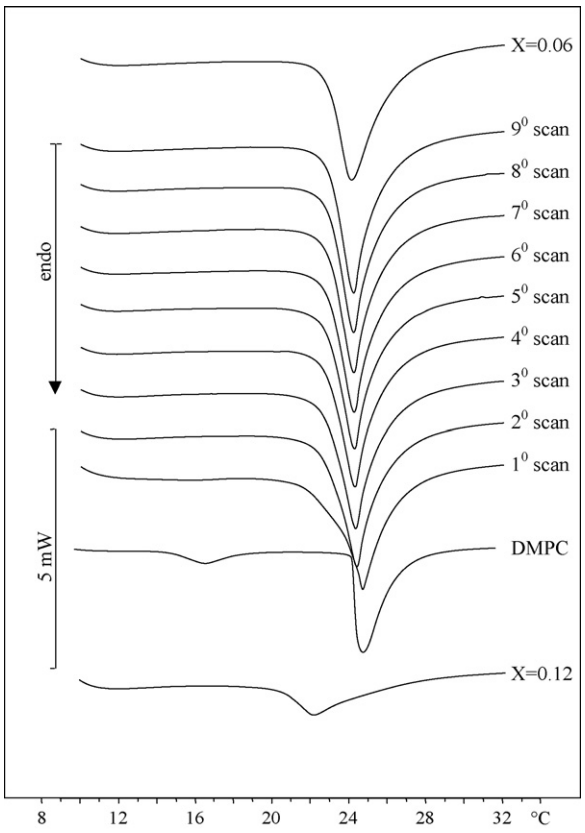

Fig. 8. Calorimetric curves, in heating mode (2 °C/min), of pure DMPC MLV left in contact (at 37 °C) with an equimolar amount of DMPC MLV prepared in the presence of EPA at 0.12 molar fraction, at increasing incubation times. Curve $X = 0.06$ is related to the transition temperature of DMPC MLV prepared in the presence of EPA at 0.06 molar fraction and represents the curve that should be obtained if EPA transfers from loaded to unloaded MLV reaching a equilibrium concentration.

somes but its absorption is faster and larger by LUV than by MLV. This result can be explained to the larger surface that LUV expose to the uptake process of EPA with respect to multilamellar vesicles, overtaking the hindered transfer in the aqueous medium.

In transmembrane kinetic experiments loaded MLV ($X = 0.12$), which mimic a lipophilic carrier, were put in contact with unloaded MLV ($X = 0.0$) (which mimic the biological membranes) to verify if the lipophilic medium can favour the EPA absorption by the biomembranes model. The transfer of EPA was monitored by DSC analysis at increasing incubation period. The obtained calorimetric curves (Fig. 8) are compared with the calorimetric curves of the samples which were put in contact each other (DMPC: unloaded MLV; $X = 0.12$: loaded MLV) and with the curve $X = 0.06$ obtained from the analysis of MLV prepared in the presence of EPA at 0.06 molar fraction. In fact, if the compound completely migrates from the loaded to the unloaded MLV at the end of the process a concentration equilibrium ($X = 0.06$) will be reached. The calorimetric curve of the first scan shows three calorimetric signals: the pretransition peak, almost visible; a large shoulder, between about 22 and 24 °C, due to the presence of loaded MLV; and a main peak, at about 24.5 °C, associated to the still unloaded MLV. In the successive scans (as the incubation time passes), the pretransition peak completely disappears, the shoulder and the main peak merge in a unique peak which moves towards lower temperature values reaching the curve $X = 0.06$. This behaviour suggests that EPA moves from loaded to unloaded MLV. This hypothesis is supported by results reported by some researchers for which the fusion of neutral lipid membrane model in the conditions employed in our experiments does not occur [28,29].

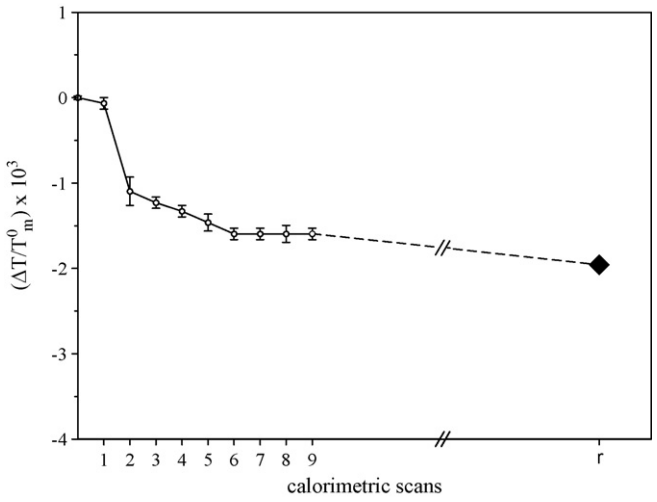

Fig. 9. Transition temperature, as $\Delta T/T_m^0$, of pure DMPC MLV left in contact (at 37 °C) with an equimolar amount of DMPC MLV prepared in the presence of EPA at 0.12 molar fraction, as a function of the calorimetric scans. $\Delta T = T_m - T_m^0$; T_m is the transition temperature of DMPC MLV prepared in the presence of EPA and T_m^0 is the transition temperature of pure DMPC MLV. T is expressed in K. Value r is related to the transition temperature of DMPC MLV prepared in the presence of EPA at 0.06 molar fraction.

In Fig. 9 the transition temperature variations, as $\Delta T/T_m^0$, of a fixed amount of pure DMPC MLV left in contact with equimolar DMPC MLV prepared in the presence of 0.12 molar fraction of EPA, as a function of the calorimetric scans are reported. The r-value is the transition temperature variation of DMPC MLV prepared in the presence of a 0.06 molar fraction of EPA and represents the maximum interaction between compound and MLV. A strong transition temperature decrease takes place within the third scan (2 h of incubation), whereas in the other scans (at higher incubation periods) the decrease is more gradual, anyway the value r is reached. These results indicate that EPA, besides transferring from loaded to unloaded MLV, reaches an equilibrium of concentration giving a population of MLV containing the same amount of EPA corresponding to a 0.06 molar fraction.

4. Conclusions

In this paper the interaction of EPA with biomembrane models was studied. The effect on the thermotropic parameter, reduction in transition temperature and enthalpy, caused by increasing molar fraction of EPA in DMPC MLV demonstrate a strong interaction of this substance with the biomembrane models. Moreover, to investigate the role of the medium on the absorption of EPA by the biomembrane models, kinetic experiments were carried out which show that the medium strongly influences the absorption process; in fact, the aqueous medium hindered the EPA absorption, probably because of its low water solubility; whereas, when carried by a lipophilic medium, EPA was strongly taken up by the biomembrane models. These results could help in the knowledge of EPA activity mechanisms.

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