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Absorption of omega-3 fatty acids by biomembrane models studied by differential scanning calorimetry

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ABSTRACT

Omega-3 fatty acids, contained in vegetables and fish, exert several beneficial effects on the human health. The interaction of three omega-3 fatty acids (linolenic acid, docosapentaenoic acid and docosahexaenoic acid) with and their absorption by biomembrane models (represented by dimyristoylphosphatidylcholine multilamellar and unilamellar vesicles) were studied to get possible information on how the absorption process of these compounds by the cell membranes after the intake of omega-3 fatty acids containing food could be. The differential scanning calorimetry technique was employed. The interaction of omega-3 fatty acids/vesicles was detected analysing phospholipid vesicles prepared in the presence of increasing amounts of the fatty acids. The absorption kinetics of omega-3 fatty acids absorption by biomembranes was investigated setting pure phospholipid vesicles in contact with omega-3 fatty acids dissolved in the aqueous medium. Docosapentaenoic acid and docosahexaenoic acid interact with biomembrane models stronger than linolenic acid. In the aqueous medium the absorption of omega-3 fatty acids by unilamellar vesicles was faster than that by multilamellar vesicles. The omega-3 fatty acids are absorbed by the biomembrane models gradually and almost completely. The results suggest that the transfer of omega-3 fatty acids from food to biomembranes could be influenced by the compounds structure and by the medium in which they are dissolved.

1. Introduction

Omega-3 fatty acids (omega-3 FA), possessing two or more double bonds in *cis* position, are incorporated into the cell membranes where they have structural and functional roles [1]; among the omega-3 FA, docosahexaenoic acid (DHA, 22:6 n-3) the longest and most unsaturated fatty acid commonly associated with membrane phospholipids, is believed to be essential in the growth and development of human and animals [2,3]. Epidemiological, clinical and biochemical studies have evidenced a protective effect of omega-3 FA against some common cancers [4], psoriasis [5], diabetes [6], rheumatoid arthritis, inflammatory and cardiovascular diseases [7]. The predominant sources of omega-3 FA are represented by vegetables oils and fish [8]. Before exerting their action, omega-3 FA have to be absorbed by the cell membrane. The precise mechanism by which fatty acids enter cells has remained controversial and two different mechanisms have been invoked. Some researchers have proposed that fatty acids can easily penetrate the outer leaflet of the cell membrane lipid bilayer, by insertion of the hydrophobic tail, then the fatty acid slowly rotates within the bilayer, transferring himself to the inner leaflet of the cell membrane. In this passive diffusion, transport is regulated by the physical properties of the membrane and by the structure (chain length and degree of unsaturation) of the fatty acid [9–11]. On the other hand, some researchers report that fatty acids transport is mediated by specific membrane proteins: plasma membrane fatty acid-binding proteins (FABPpm); fatty acid translocase (FAT/CD36) and fatty acids transport proteins (FATP1–FATP6) [12–15].

To evaluate the applicability of the passive mechanism, we studied the interaction and the absorption of three omega-3 FA, linolenic acid (LNA, 18:3 n-3), docosapentaenoic acid (DPA, 22:5 n-3) and DHA (Scheme 1) by biomembrane models represented by multilamellar (MLV) and unilamellar vesicles (LUV) made of dimyristoylphosphatidylcholine (DMPC). DPA and DHA, possessing the same carbons number but five and six double bonds, respectively, permit to evaluate the effect of the unsaturation degree on the biomembrane absorption processes. In addition, to get more information on the structural effect, the results have been compared with those relative to the previously investigated LNA [16], having 18 carbons and three double bounds.

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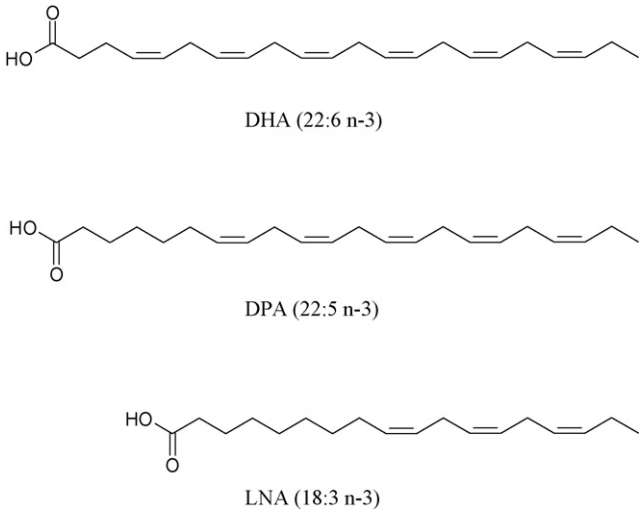

Scheme 1. Omega-3 fatty acids structure.

Phospholipid vesicles undergo a thermotropic transition from a gel (ordered) phase to a liquid crystalline (disordered) phase [17] which takes place at a well-defined temperature (T_m). To this transition is associated a characteristic enthalpy variation (ΔH). Compounds interacting with the phospholipid vesicles can influence both the T_m and the ΔH. In this research we exploited the differential scanning calorimetry (DSC) technique to detect the variations of the gel-to-liquid crystalline transition temperature and enthalpy of phospholipids due to the presence of omega-3 FA into the lipid vesicles [18,19].

2. Materials and methods

2.1. Materials

Synthetic DMPC was obtained from Genzyme (Switzerland). LNA (purity: ≥98%), DPA (purity: ≥98%) and DHA (purity: ≥98%) were purchased from Cayman Chemical (Mi, USA). Solutions of the lipids were chromatographically pure as assessed by two-dimensional thin-layer chromatography. 50 mM Tris solution pH 7.4 was used to prepare liposomes. Lipid concentrations were determined by the phosphorous analysis [20].

The absence of oxidation of omega-3 FA after each experiment was determined applying the method described by Okuda et al. [21] and Vikbjerg et al. [22].

2.2. DSC analysis

DSC was performed by using a Mettler TA Star^e System equipped with a DSC 822^e cell. The reference pan was filled with 50 mM Tris solution. Indium and palmitic acid were employed to calibrate the calorimetric system. Thermodynamic parameters of the calorimetric curves were evaluated using the Mettler STAR^e V 6.10 software.

2.3. Liposomes preparation

Multilamellar vesicles were prepared in the presence and absence of omega-3 FA as follows: chloroform–methanol (1:1, v:v) stock solutions of DMPC and omega-3 FA were prepared and aliquots were mixed in glass flasks to obtain the same amount of DMPC (0.010325 mmol) and increasing molar fractions (0.00; 0.015; 0.03; 0.045; 0.06; 0.09; 0.12; 0.15; 0.18) of omega-3 FA with respect to the DMPC. The solvents were removed under a nitrogen flow. The resulting films were freeze-dried under vacuum to remove the residual solvents. 168 μl of 50 mM Tris buffer solutions (pH 7.4) was added to the film; the samples were heated at 37 °C (temperature above the gel–liquid crystalline phase transition of the DMPC, DMPC $T_m = 24.8 \pm 0.3$ °C) [23] and vortexed for 1 min, for three times. The samples were left under gently shaken for 1 h in a water bath at 37.0 ± 0.1 °C to homogenize the liposomes.

To obtain LUV, the MLV of pure DMPC, or mixed DMPC/omega-3 FA, were repetitively (19 times) passed at moderate pressure and at 34.0 ± 0.1 °C through a polycarbonate membrane (pores diameter 100 nm) in a extruder system (Liposofast Avestin) [24,25]. The membrane pores are almost cylindrical, and vesicles (unilamellar or multilamellar) larger than the mean pore diameter are reduced in size and lamellarity during the extrusion, resulting in final vesicle size corresponding to the mean size of the pores [26,27]. Unilamellar vesicles size was checked by standard dynamic light scattering measurements.

2.4. Omega-3 FA/liposomes interaction

Aliquots of 120 μl (0.007375 mmol) of MLV or LUV were transferred into a 160 μl DSC aluminium pan, hermetically sealed under nitrogen atmosphere and submitted to DSC analysis as follows: (1) a heating scan from 2.0 to 37.0 °C (2.0 °C/min) and (2) a cooling scan from 37.0 to 2.0 °C (4.0 °C/min), for at least three times to achieve the reproducibility of the results. Each experiment was carried out in triplicate. After the calorimetric scans, the amount of phospholipids of the samples was determined by the phosphorous assay [20].

2.5. Permeation kinetic experiments

The absorption of omega-3 FA by the cell membranes can be studied by carrying out a permeation experiment employing membrane models. A fixed amount of DMPC aqueous dispersion (MLV or LUV) was left in contact with oily omega-3 FA (to obtain a 0.09 molar fraction with respect to the DMPC) in a DSC pan. A 0.09 molar fraction was used as, in the Omega-3 FA/liposomes interaction experiments, it showed a high variation of the thermodynamic parameters associated with well-defined peak of DMPC vesicles. The samples, hermetically sealed under nitrogen atmosphere in the calorimetric pan, were gently shaken for 10 s, submitted to calorimetric scans as follows: (1) a scan from 2.0 to 37.0 °C, at 2.0 °C/min, to detect the eventual interaction of omega-3 FA with the membrane model; (2) an isothermal period of 1 h at 37.0 °C, to permit the compound to dissolve, migrate, and permeate the phospholipid bilayer which is in a disordered state at temperatures over the T_m; and (3) a cooling scan from 37.0 to 2.0 °C, at 4.0 °C/min. This procedure was run, at least eight times, to detect the variations caused by the interaction of increasing amounts of omega-3 FA with the membrane model. Each experiment was carried out in triplicate. No thermal degradation of omega-3 FA occurred during the calorimetric scans, as observed when an equal amount of omega-3 FA was incubated under nitrogen in the same experimental conditions and the accumulation of hydroperoxides was detected by reverse phase HPLC with UV detection (data not shown).

2.6. Transmembrane transfer kinetics

To evaluate the cellular absorption of omega-3 FA in the presence of a lipophilic medium where omega-3 FA are molecularly dispersed, a transmembrane transfer kinetic was carried out. 60 μl of DMPC MLV prepared in the presence of omega-3 FA (at 0.12 molar fraction) (loaded MLV) and an equimolar amount of pure DMPC MLV (unloaded MLV) were put in contact in a 160 μl DSC pan, her-

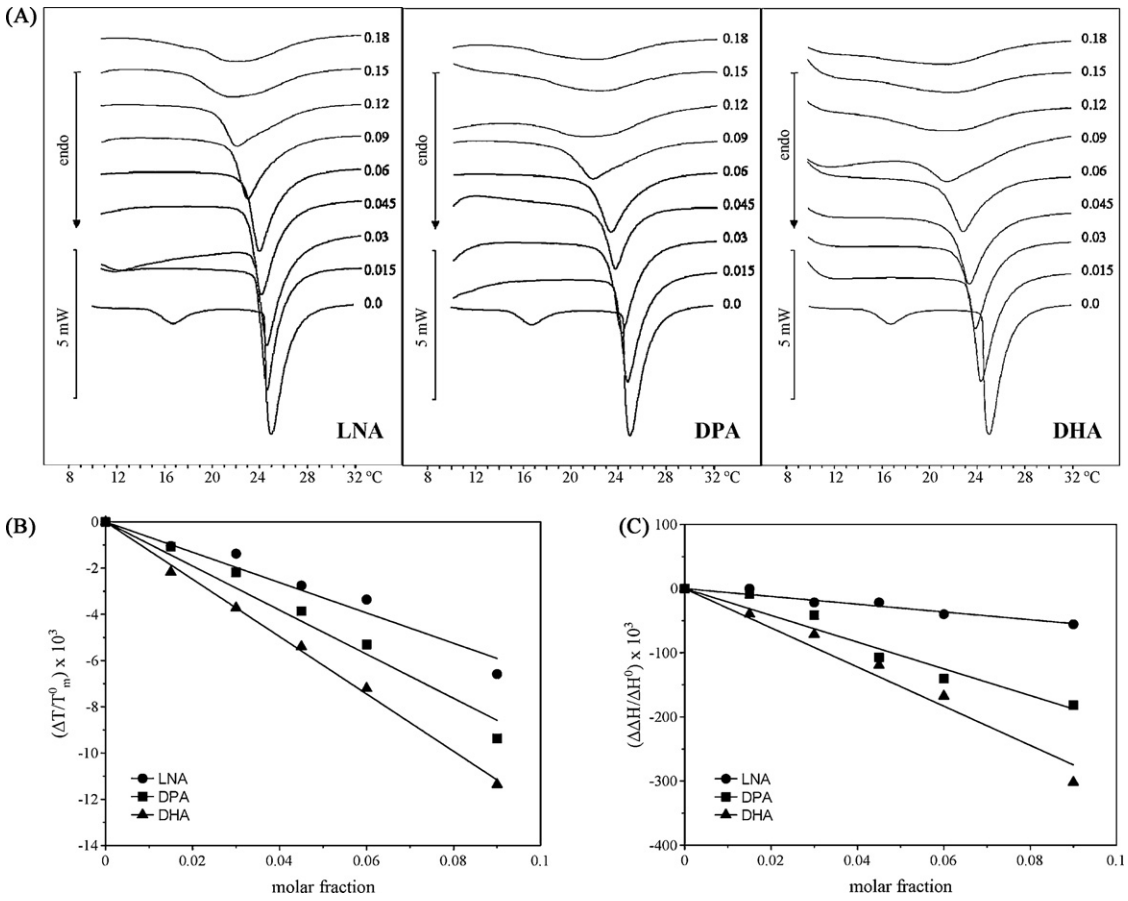

Fig. 1. (A) Calorimetric curves, in heating mode, of DMPC MLV prepared in the presence of increasing molar fractions of LNA, DPA and DHA; (B) transition temperature, as $\Delta T/T^{o}_{m}$, as a function of omega-3 fatty acids molar fractions in the MLV dispersion. $\Delta T = T_m - T^{o}_{m}$, where T^{o}_{m} is the transition temperature of pure DMPC MLV and T_m is the transition temperature of DMPC MLV prepared in the presence of omega-3 fatty acids; (C) enthalpy variations, as $\Delta\Delta H/\Delta H^0$, as a function of omega-3 fatty acids molar fractions in the MLV dispersion. $\Delta\Delta H = \Delta H - \Delta H^0$, where ΔH^0 is the enthalpy variation of pure DMPC MLV and ΔH is the enthalpy variation of DMPC MLV prepared in the presence of omega-3 fatty acids.

metically sealed and submitted to the DSC procedure described in Section 2.5.

3. Results

Upon heating, pure phospholipid bilayers undergo a thermotropic gel-to-liquid crystal phase transition, which is accompanied by an enthalpy change and is characterized by a sharp transition temperature [17]. This phase transition is influenced by the presence of different molecules dissolved in the lipid bilayer [17,28,29] and can be detected by DSC. DMPC vesicles, which show a sharp and reproducible thermotropic phase transition T_m, mimic the lipid structure of cell membranes and are often used as simplified biomembrane model [28,29] to study the interaction and penetration of molecules into biomembranes. Calorimetric heating curves of DMPC MLV doped with different molar fractions of LNA, DPA and DHA are reported in Fig. 1A. DMPC shows two endothermic peaks: a smaller peak at 15–16 °C and a main peak at 24.8 °C. The former is ascribed to the transition from the gel (L'_β) phase to the ripple (P'_β) phase and is called pretransition, while the latter is ascribed to the transition from the (P'_β) phase to the liquid crystalline (L_α) phase and is called main transition [29,30]. All omega-3 FA interact with DMPC liposomes causing a decrease of the pretransition peak and a broadening of the main peak accompanied by a shift towards lower temperatures. The decrease of T_m and the broadening of the calorimetric peaks depends on the omega-3 FA concentration. Similar results have been described by Inoue et al. [31] which studied the effect of oleic acid (a monounsaturated *cis* fatty acid) on the phase behaviour of dipalmitoylphosphatidylcholine bilayers. In order to compare the effects exerted by the omega-3 FA, we report in Fig. 1B the relative transition temperature variation $\Delta T/T^{o}_{m}$ (where $\Delta T = T_m - T^{o}_{m}$, T^{o}_{m} being the transition temperatures of pure DMPC and T_m, the DMPC transition temperatures in the presence of increasing amounts of omega-3 FA) against the molar fraction of omega-3 FA in the aqueous dispersion. The values relative to 0.12, 0.15 and 0.18 molar fractions are not reported because the peaks are very broad and less intense. The data were obtained in triplicate and the standard deviation was <1%. The most evident T_m decrease is caused by DHA, followed by DPA and, then, by LNA. The relative enthalpy variation $\Delta\Delta H/\Delta H^0$ is reported in Fig. 1C ($\Delta\Delta H = \Delta H - \Delta H^0$, where ΔH is the enthalpy variation of DMPC prepared in the presence of omega-3 FA and ΔH^0 is the enthalpy variation of pure DMPC) against the omega-3 FA molar fraction in the aqueous dispersion. Also the enthalpy data were obtained in triplicate and the standard deviation was <5%. All the compounds induce enthalpy decrease, the greatest effect being exerted by DHA followed by DPA and then by LNA.

In order to elicit their functions, the dietary omega-3 FA must be absorbed by the biomembranes. In order to mimic the kinetics process, empty MLV or LUV were left in contact with fixed amounts of oily omega-3 fatty FA (to have 0.09 molar fraction) for increasing incubation times and at a temperature higher than the phospholipid T_m. The temperature shift of the DMPC calorimetric peak is due to the increased absorption of such molecules in the phospholipid bilayer/s; at the end of the process a calorimetric curve similar

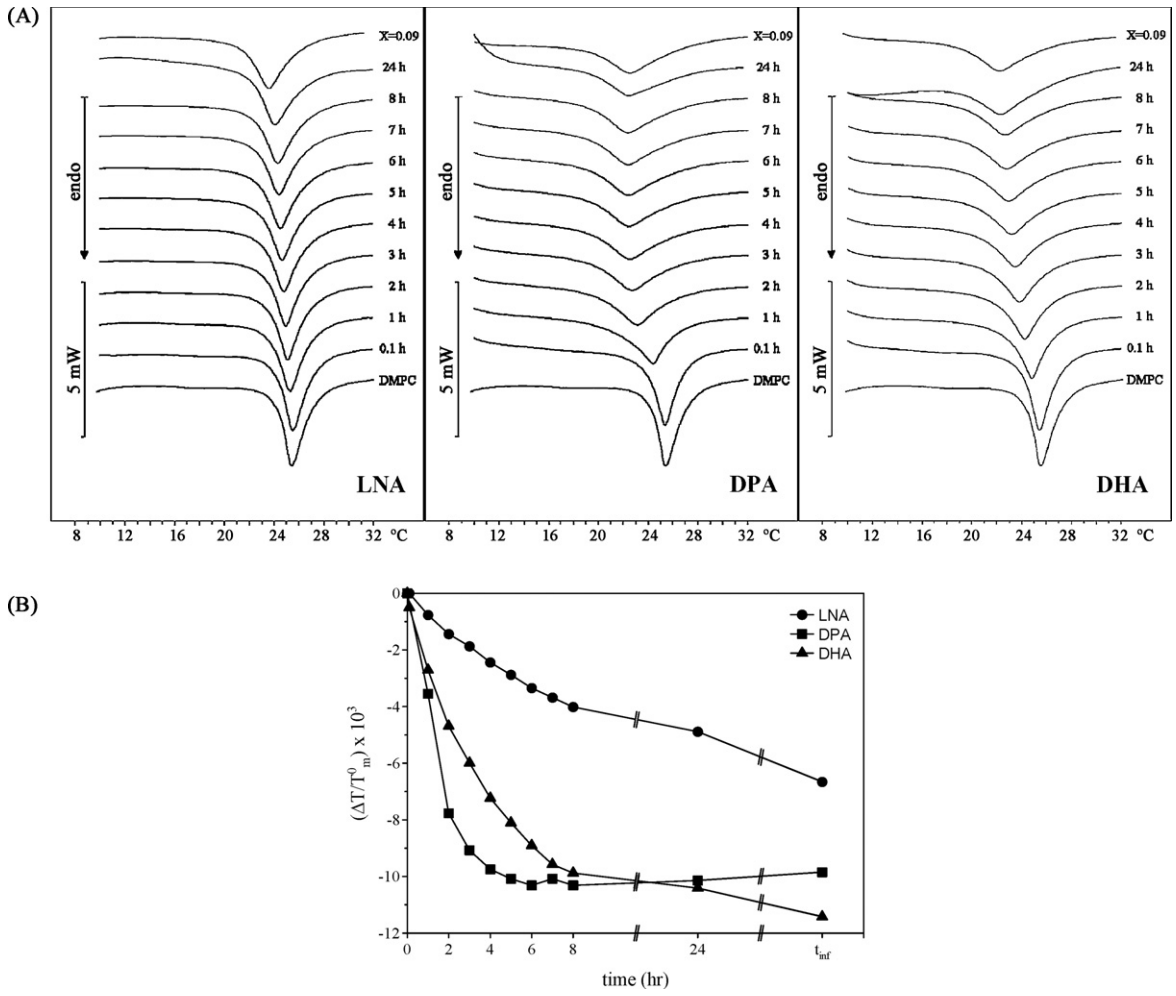

Fig. 2. (A) Calorimetric curves, in heating mode, of DMPC LUV left in contact with a 0.09 molar fraction of LNA, DPA and DHA, at increasing incubation time. Curves $X = 0.09$ belong to DMPC LUV prepared in the presence of a 0.09 molar fraction of omega-3 fatty acids; (B) transition temperature, as $\Delta T/T^{o}_{m}$, of DMPC LUV left in contact with a 0.09 molar fraction of omega-3 fatty acids, as a function of incubation time. $\Delta T = T_m - T^{o}_{m}$, T^{o}_{m} is the transition temperature of pure DMPC LUV and T_m is the transition temperature of DMPC LUV in the presence of increasing amounts of omega-3 FA. The t_{inf} values represent the transition temperature variation of DMPC LUV prepared in the presence of a 0.09 molar fraction of omega-3 fatty acids.

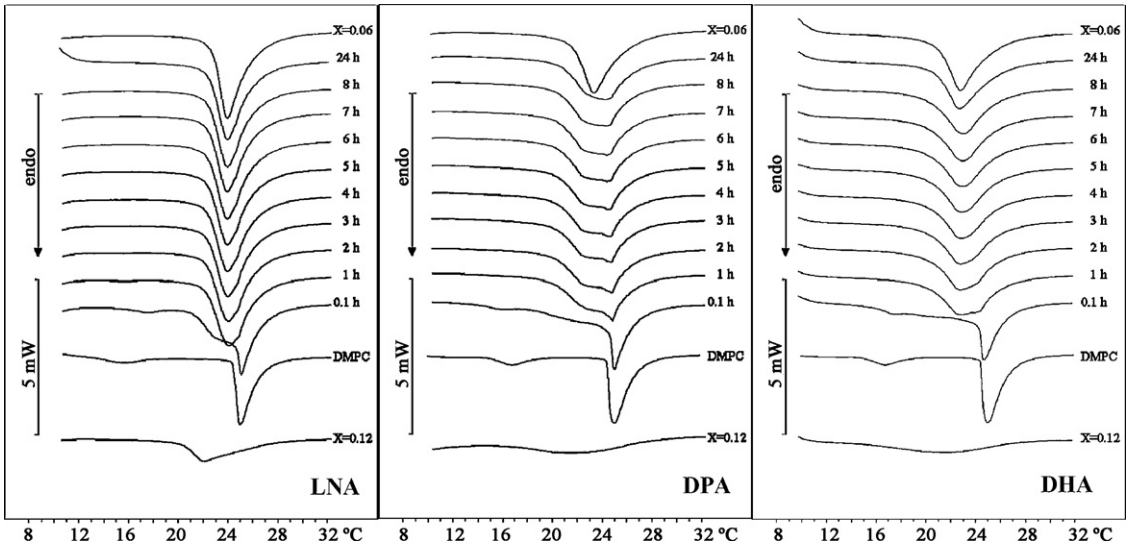

Fig. 3. Calorimetric curves, in heating mode, of DMPC MLV left in contact with DMPC MLV prepared in the presence of LNA, DPA and DHA at 0.12 molar fraction, at increasing incubation time. Curves $X = 0.06$ belong to DMPC MLV prepared in the presence of a 0.06 molar fraction of omega-3 fatty acids.

to that obtained from the liposomes prepared in the presence of a 0.09 molar fraction of compound should be present [32–35].

The calorimetric curves are compared with those obtained from vesicles prepared in the presence of 0.09 omega-3 FA molar fraction, as reported in "liposome preparation" section. With regard to experiments with MLV (curves not shown), as the incubation time t increases, all the omega-3 FA, and in particular LNA and DPA, cause modification of the pretransition peak which becomes smaller (with a consequent decrease of the enthalpy change) and moves towards lower temperatures, while the main peak does not show significant variations. The comparison of the transition temperatures indicates that only LNA causes a small decrease of T_m, whereas the other compounds do not affect this parameter. These results suggest that the omega-3 FA, probably because of their low water solubility, are not able to migrate from the aqueous medium to the MLV surface.

The calorimetric curves of the kinetic experiments carried out with LUV are shown in Fig. 2A. The curve $X = 0.09$ is relative to LUV prepared in the presence of omega-3 FA at 0.09 molar fraction and where we expect the largest omega-3 FA/phospholipid interaction. As the incubation times increase, the omega-3 FA cause a broadening and shifting of the calorimetric peak towards lower temperature on approaching the curve $X = 0.09$. In Fig. 2B we report the relative transition temperature, as $\Delta T/T_m^o$, as a function of the incubation time. The values t_{inf} refer to LUV prepared in the presence of omega-3 FA at 0.09 molar fraction. It is clearly visible that the omega-3 FA gradually decreases the lipid melting temperature T_m indicating that they are absorbed by the LUV. Comparing the results of the experiments carried out with MLV and the results of the experiments with LUV, it can be noticed that omega-3 FA are absorbed faster and to a large extent by LUV than by MLV. This result can be due to the larger surface that LUV expose to the external aqueous medium during the uptake process of omega-3 FA by the vesicles.

The calorimetric curves of the transmembrane transport kinetic in which loaded MLV ($X = 0.12$) were left in contact with unloaded MLV ($X = 0.0$) (mean molar fraction 0.06) at different time of incubation are reported in Fig. 3 and compared with the calorimetric curves of samples which were put in contact with each other (DMPC at $X = 0.0$ and $X = 0.12$) and with the curve $X = 0.06$ obtained by preparing MLV in the presence of omega-3 FA 0.06 molar fraction. If the fatty acid completely migrated from loaded to unloaded MLV, the flux of fatty acid would stop as soon as the equilibrium concentration ($X = 0.06$) is attained. In Fig. 3 the curve 0.1 h relative to LNA shows three calorimetric signals: the pretransition peak, a large shoulder at about 22 °C associated to the loaded MLV and a main peak at about 24.5 °C relative to still unloaded MLV. As the incubation time increases, the pretransition peak disappears, while the shoulder and the main peak progressively merge; after 8 h incubation, the calorimetric peak temperature is unique and nearly identical to that of the reference curve. This behaviour clearly shows that loaded MLV are loosing LNA, whereas empty MLV are absorbing it, then a net transfer of omega-3 fatty acid from loaded to empty vesicles is happening. As far as DPA is concerned, the calorimetric curve at 0.1 h shows three distinct regions: the pretransition peak, a shoulder and a main peak. On increasing the incubation time, the pretransition peak disappears; the peak at higher temperature becomes less evident in favour of the shoulder at lower temperature with the formation of a broad peak. This behaviour indicates that DPA slowly transfers from loaded to unloaded MLV. With regard to DHA, the curve at 0.1 h presents three regions: the first one is the pretransition peak; the second, very broad, is relative to the loaded MLV; the third at about 25 °C is relative to the unloaded MLV. After 1 h of incubation, the pretransition peak disappears; the remaining two regions become less separate and, as the incubation time increases, merge in a unique broaden peak. This indicates a progressive DHA migration from loaded to unloaded MLV.

4. Discussion

The calorimetric measurements on the omega-3 FA/liposomes interactions showed that omega-3 FA interact with liposomes causing a decrease of T_m and ΔH accompanied by a broadening of the calorimetric peak. This behaviour is due to the localization of omega-3 FA inside the lipid bilayer [36] and it can be explained in terms of a fluidifying effect exerted by the omega-3 FA on the phospholipid cooperativity after their insertion into the ordered structure of the lipid bilayer, such a behaviour is typical of several classes of compounds as reported in the literature [37–39]. In the presence of omega-3 FA the phospholipid packing is influenced by steric restrictions associated with the presence of multiple rigid double bonds. As a consequence reduced intra- and inter-molecular Van der Walls interactions weakens the bilayer stability. The interaction of omega-3 FA with biomembrane models is influenced by their structure.

The only difference between DPA and DHA is that the latter possess one double bond more; this could make the molecule bulkier than DPA and give rise to the stronger destabilization of the biomembrane models. This hypothesis is supported by the weaker interaction of LNA which possess a smaller number of carbon atoms and of double bonds with respect to DPA and DHA. The strongest effect exerted by DHA is in agreement with the hypothesis for which DHA could influence brain function via the properties of the lipid matrix [40]. When the hydrophilic medium is considered, in the presence of MLV the absorption of the omega-3 FA is very low and it could be due to the low solubility of the compounds together with the small surface with which the omega-3 FA come in contact; with LUV the absorption of omega-3 FA increases and this can depend on the large surface which LUV expose to the contact with the compounds. The bigger absorption of DPA with respect to DHA could be to the stronger affinity of DPA for LUV.

In the lipophilic medium, a big transfer of omega-3 FA from the loaded to the unloaded MLV occurs. However, differently from what happens in the hydrophilic medium, the DHA absorption by the unloaded MLV is bigger than that of DPA. This behaviour could derive from the strong affinity of DPA with the vesicles phospholipid in agreement with the results of the absorption of the omega-3 FA by LUV in the hydrophilic medium. The findings of this study should be useful for the optimization of omega-3 FA formulations.

Among the compounds under study, DHA shows the strongest interaction and absorption (in a lipophilic medium) by the biomembrane model which could contribute to its numerous beneficial effects on human health [41,42].

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