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Effects of α-eleostearic acid on asolectin liposomes dynamics: Relevance to its antioxidant activity

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ABSTRACT

In this study, the effect of α-eleostearic acid (α-ESA) on the lipid peroxidation of soybean asolectin (ASO) liposomes was investigated. This effect was correlated to changes caused by the fatty acid in the membrane dynamics. The influence of α-ESA on the dynamic properties of liposomes, such as hydration, mobility and order, were followed by horizontal attenuated total reflection Fourier transform infrared spectroscopy (HATR-FTIR), nuclear magnetic resonance (NMR), differential scanning calorimetry (DSC) and UV–vis techniques. The α-ESA showed an *in vitro* antioxidant activity against the damage induced by hydroxyl radical (·OH) in ASO liposomes. The analysis of HATR-FTIR frequency shifts and bandwidths and ^{1}H NMR spin–lattice relaxation times, related to specific lipid groups, showed that α-ESA causes an ordering effect on the polar and interfacial regions of ASO liposomes, which may restrict the ·OH diffusion in the membrane. The DSC enthalpy variation analysis suggested that the fatty acid promoted a disordering effect on lipid hydrophobic regions, which may facilitate interactions between the reactive specie and α-ESA. Turbidity results showed that α-ESA induces a global disordering effect on ASO liposomes, which may be attributed to a change in the lipid geometry and shape. Results of this study may allow a more complete view of α-ESA antioxidant mode of action against ·OH, considering its influence on the membrane dynamics.

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1. Introduction

The α-eleostearic acid (9Z11E13E octadecatrienoic acid, α-ESA) is a conjugated fatty acid (Fig. 1), which can be obtained from tung (*Aleurites fordii*) oil [1]. Its π conjugation system may be responsible for prooxidant or antioxidant activities. On one hand, α-ESA showed a selective and considerable suppressive effect on tumor growth via induction of apoptosis by promoting lipid peroxidation in membranes [2,3]. On the other hand, α-ESA can reduce the levels of lipid peroxidation caused by oxidative stress in plasma, lipoproteins and erythrocyte membrane [4].

It is known that lipid peroxidation is related to significant modifications in the membranes, such as the rearrangement or loss of double bounds, variations in membrane fluidity degree, increase of permeability and changes in thermotropic phase properties [5–8]. Exogenous molecules which can affect lipid peroxidation levels may also cause changes in membrane properties and dynamics [9–11]. To the best of our knowledge, the effects of α-ESA in membrane physico-chemical properties have not been completely elucidated. Thus, it is important to establish a basic knowledge about the interactions concerning α-ESA and membranes in order to contribute to better understanding of its effects on the membrane dynamics and lipid peroxidation process.

The study of cellular membranes physico-chemical properties is difficult due their complex structures [12]. Thus, the α-ESA influence on membrane molecular dynamics can be investigated by models of less complexity such as the liposomes, which are suitable for *in vitro* studies of membrane structure and properties. Liposomal systems facilitate the control of phospholipid composition, structure and dynamics [13,14]. In this study, the effect of α-ESA on the *in vitro* hydroxyl radical (·OH) – induced lipid peroxidation of soybean asolectin (ASO) liposomes was studied and correlated to the fatty

Abbreviations: ASO, soybean asolectin; DSC, differential scanning calorimetry; Δ*H*, enthalpy variation; α-ESA, α-eleostearic acid; FTIR, Fourier transform infrared spectroscopy; HATR-FTIR, horizontal attenuated total reflection-Fourier transform infrared spectroscopy; ·OH, hydroxyl radical; MLV, multilamellar large vesicles; NMR, nuclear magnetic resonance; S.D., standard deviation; SPC, soybean phosphatidylcholine; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid; τ, correlation time; *ν*, stretching vibration.

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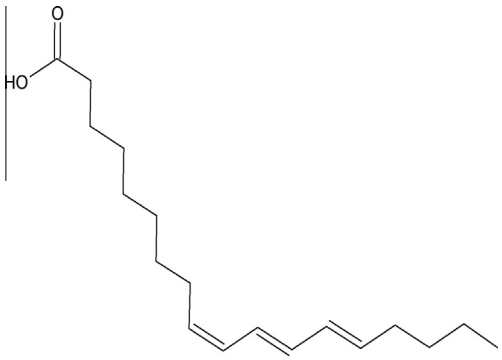

Fig. 1. Structure of α-eleostearic acid (α-ESA, 9Z11E13E octadecatrienoic acid).

acid influence on the membrane dynamics. The ASO is a mixture of unsaturated phospholipids which contain several bis-allylic methylene hydrogens. These hydrogens have weaker bond dissociation energy (75–80 kcal/mol), which facilitates their abstraction in free radical processes [15,16]. The ASO phospholipids composition includes soybean phosphatidylcholine (SPC) as a major component, phosphatidylethanolamine and phosphatidylinositol. All these lipids contain oleic (C 18:1), linoleic (C 18:2) and linolenic (C 18:3) acids [17].

The influence of α-ESA on liposome dynamic properties, such as hydration, mobility and order, were followed by horizontal attenuated total reflection Fourier transform infrared spectroscopy (HATR-FTIR), nuclear magnetic resonance (NMR), differential scanning calorimetry (DSC) techniques and turbidity essays.

2. Materials and methods

2.1. Chemicals

Soybean asolectin (ASO, containing 25% of SPC), salts, tricine, deuterated water/sodium 3-(trimethylsilyl)-[2,2,3,3-2H4]-1-propionate (TSP, 0.05%) and thiobarbituric acid were obtained from Sigma–Aldrich (St. Louis, MO, USA). Hydrogen peroxide, phosphate buffer (KH_2PO_4) and trichloroacetic acid (TCA) were purchased from Synth (São Paulo, SP, Brasil). Lipids were used without further purification and all the other chemicals were of analytical grade.

2.2. Preparation of α-ESA

The α-Eleostearic acid was obtained from tung oil, which was purchased from Campestre Ltda (São Paulo, SP, Brazil). It was purified by saponification of 25 g tung oil with KOH/CH_3CH_2OH and neutralized by H_2SO_4 [18]. α-ESA was isolated from the other fatty acids by recrystallization using acetone [19].

2.3. Preparation of liposomes

Multilamellar large vesicles (MLV) containing ASO 150 mg/mL were produced pure or loaded with α-ESA, through lipid co-solubilization in $CHCl_3$ followed by solvent evaporation under vacuum. The dried films were then resuspended at room temperature from the walls of a round bottom flask by vortexing in tricine/$MgCl_2$ buffer 10 mM, pH 7.4 [20]. In order to prepare liposomes which contained α-ESA, different fatty acid concentrations were added during the solvent co-solubilization process [21]. The α-ESA concentrations added to liposomes ranged from 0 to 30 mg/mL.

2.4. Lipid peroxidation essays

Peroxidation of ASO liposomes was induced by hydroxyl radical (·OH). ·OH was produced from the reaction of H_2O_2 2.8 mM, $FeCl_3$ 0.02 mM and ascorbate 0.1 mM, adapted from the study carried out by Halliwell and co-workers [22]. The ·OH was added to a dispersion of ASO liposomes pure or containing different concentrations of α-ESA (ranging from 0 to 30 mg/mL) and KH_2PO_4 10 mM at pH 7.4. The samples were then incubated for 30 min at 37 °C. The lipid peroxidation level was obtained by thiobarbituric acid reactive substances (TBARS) method [23,24]. This method is based on the quantification of the complex produced by the reaction between malondialdeyde and thiobarbituric acid, in the presence of TCA. The amount of TBARS was calculated from optical densities at 535 nm, obtained by a Shimadzu UV-2550 UV–vis spectrophotometer (Kyoto, JP), using an extinction coefficient of 1.56×10^5 - $M^{-1}\,cm^{-1}$. Results were shown as mean ± standard deviation (S.D.) of triplicates from three independent experiments. In all experiments, controls were obtained by adding reagents except lipid membranes in the reaction medium.

2.5. HATR-FTIR experiments

HATR-FTIR measurements were performed using a Shimadzu IR Prestige-21 spectrometer (Kyoto, JP) at 23 °C. The ASO liposomes 150 mg/mL, pure or loaded with α-ESA (in the concentration range from 0 to 30 mg/mL) were deposited on a ZnSe crystal support and immersed into the tricine/$MgCl_2$ buffer 10 mM, pH 7.4. Interferograms were averaged for 50 scans, with a resolution of 2 cm^{-1}, and recorded in the frequency range from 400 to 4000 cm^{-1}. The spectra were analyzed by Shimatzu IR solution software 1.5. Frequency variations were detected through the analysis of α-ESA-induced shifts of ASO vibrational bands. Changes in the bandwidth of the lipid groups stretching bands were measured relative to a straight base line at ¾ of peak height position. The following lipid group vibrations were analyzed: the phosphate antisymmetric stretching vibration, in the range of frequency from 1260 to 1220 cm^{-1} (ν_{as} PO_2^-); the choline antisymmetric stretching vibrations, in frequency values around 970 cm^{-1} (ν_{as} CN^+C) and near 3005 cm^{-1} (ν_{as} NCH); the carbonyl stretching mode, in the frequency range from 1725 to 1740 cm^{-1} (ν C=O); the symmetric and antisymmetric stretching vibrations of the acyl chains methylenes, with the frequency values around 2850 cm^{-1} (ν_s CH_2) and 2920 cm^{-1} (ν_{as} CH_2), respectively. It is worth pointing out that these vibrations are related to phosphatidylcholine groups [25,26].

2.6. NMR studies

Choline ^1H spin lattice relaxation times (T_1) of ASO liposomes, empty or loaded with α-ESA, were obtained at 60 MHz by an NMR Anasazi Instrument spectrometer (Indianapolis, USA), by inversion recovery pulse sequences. The correlation time (τ) values ranged from 0.2 to 102.4 s. Temperature was held constant at 23 °C. Chemical shifts were referenced to TSP (0 ppm) [9]. Relaxation time values and relative intensities were obtained by fitting the exponential data to the NUTS code.

2.7. DSC measurements

The DSC measurements of ASO liposomes, pure or loaded with α-ESA, were performed by a TA Instrument 2010 DSC cell (New Castle, DE). The heating rate was set to 1 °C/min in a temperature range from 10 to 60 °C, under nitrogen flow (50/50 mL min^{-1}). An empty aluminum pan was used as reference [9]. The ΔH was obtained by integrating the area under the DSC peak using the Software Universal 4.0 C from TA Instruments.

2.8. Turbidity essays

Turbidity measurements of liposomes containing ASO 150 mg/mL, pure or loaded with α-ESA (in the concentration range from 0 to 30 mg/mL) were performed at 400 nm using a Shimadzu UV-2550 UV–vis spectrophotometer (Kyoto, JP).

3. Theory section – instrumental techniques

The FTIR is a suitable technique to study lipid membranes at the molecular level [27,28]. Horizontal attenuated total reflection FTIR (HATR-FTIR) has been used to study liposomes since it prevents membrane damage caused by the IR laser [27,29]. In the case of phosphatidylcholine, the FTIR spectrum shows vibration bands which can be divided into three different regions corresponding to the polar, interfacial and hydrophobic moieties of the lipid [30]. Typical vibrations of covalently bonded atoms can be classified as "bending" and "stretching". The latter involves changes in the bond lengths [31]. The main stretching bands related to phosphatidylcholine groups are: the phosphate antisymmetric stretching vibration (ν_{as} PO_2^-, 1260–1220 cm^{-1}) and the choline antisymmetric stretching vibrations (ν_{as} CN^+C, ~970 cm^{-1} and ν_{as} NCH, ~3005 cm^{-1}) both located in the lipid polar region; the interfacial carbonyl stretching mode (ν C=O, 1725–1740 cm^{-1}) and the symmetric and antisymmetric stretching vibrations of the hydrophobic acyl chains methylenes (ν_s CH_2, ~2850 cm^{-1} and ν_{as} CH_2, ~2920 cm^{-1}) [25,26].

The presence of an exogenous molecule incorporated into the membrane can induce changes in the lipid FTIR bands, such as shifts and bandwidth variations. These changes can provide information about physico-chemical processes taking place in the systems [32]. With respect to the lipid polar region, ν_{as} PO_2^- is sensitive to hydration conditions. The frequency values usually decrease with increasing hydration [33]. Thus, if an exogenous molecule modifies the number and orientations of hydrogen bounded water molecules around this lipid head group, a shift in the ν_{as} PO_2^- frequency value must be observed in the FTIR spectrum. Variations of the bandwidth of specific lipid bands, such as ν_{as} PO_2^-, give information about the system dynamics since the bandwidth is affected by rotational, translational and/or collisional effects [34]. As the membrane dynamics increases, an increase in lipid peaks bandwidth will be observed [35].

The analysis of ν_{as} CN^+C and ν_{as} NCH may reflect conformation changes of the choline group due to its separation from the phosphate region related to possible intercalation of water molecules between these two charged groups [36]. The choline group is associated with water by dipole interactions which can be affected by lipid-molecule interaction [33]. In this study, HATR-FTIR information concerning the α-ESA influence on the choline group dynamics was complemented by ^1H NMR spin lattice relaxation time measurements (T_1), since the relaxation of ^1H occurs predominantly through dipolar interactions involving neighboring ^1H nuclei [37–39]. The T_1 is the time constant for the spin system to reach equilibrium with its surroundings [40]. Relaxation times are related to correlation time, which defines the time it takes for a molecule to reorient in a given membrane phase. Thus, T_1 measurements may provide dynamic information concerning membrane specific regions, which includes the influence of a molecule in lipid fast motions, such as rotation [41].

The hydration degree of the lipid interface region may be monitored by the analysis of HATR-FTIR carbonyl stretching vibration, since it is sensitive to the hydration state, polarity, degree and nature of the hydrogen bonding interactions in the lipid–water interface [33]. A decrease in the ν C=O frequency may mean the strengthening of existing hydrogen bonding or formation of new hydrogen bonding between the lipid and the interacting molecule [32,42]. Variations related to carbonyl stretching bandwidth may reflect conformational changes in the membrane interfacial region. The ν C=O bandwidth increases as the motional freedom of this lipid region enhances [29].

The analysis of acyl chains methylenes HATR-FTIR bands is very useful to the study of physical properties of lipids, since they are sensitive to the hydrophobic chain conformation [43]. Shifts in the frequency values of ν_s CH_2 and ν_{as} CH_2 are related to the conversion from trans to gauche conformers [44]. Trans-gauche isomerization is included in the gel-to-liquid crystalline lipid phase transition, as well as in a series of intermediate states which consist of mixtures of lipid molecules in both gel and liquid crystalline conformations. These states may differ in the number of lipids in the available single lipid substrates and in their lateral arrangement in the lipid matrix [45]. Changes in ν_s CH_2 and ν_{as} CH_2 FTIR bandwidths reflect alterations in the motional freedom degree related to the lipid hydrophobic region [46]. The ν CH_2 bandwidth is also sensitive to the phase transition [34]. At more ordered phase states, such as the gel one, these bands are sharp and centered at lower frequency, indicating the predominance of immobile hydrocarbon chains [47]. As the phase state becomes more disordered, these bands broaden and their peak frequencies may increase by values ranging from approximately 1.5–3 cm^{-1} for ν_s CH_2 and from 2.5 to 5 cm^{-1} for ν_{as} CH_2. These changes are related to the increasing mobility of the vibrating groups of the hydrocarbon chains [43]. The bandwidth is sensitive to the presence of gauche conformers, as well as to the restriction related to librational or torsional motion of the lipid chains [48].

The influence of exogenous molecules on lipid acyl chains thermodynamic behavior may also be monitored by differential scanning calorimetry (DSC). Acyl chains can undergo phase transitions at specific temperatures, depending on the structure of the lipid. Important information concerning the transitions and order related to the acyl chain assembly can be obtained by DSC, by measuring the enthalpy variation of the lipid transition (ΔH) [9,49]. The ΔH is related to the actual heat required for the entire transition, and can be calculated from the integration of the area under the DSC exothermic or endothermic transition peak. The enthalpy of a membrane is given by the time or spatial average over all available phase states. The ΔH measurements enable information to be gotten on structural modifications which have occurred in lipid bilayers in the presence of an exogenous molecule [26,50].

Lipid phase transitions can also be related to changes in the system turbidity. Lipid turbidity can be defined as its "apparent absorbance" and UV–vis spectrophotometers are advisable to investigate the possibilities of observing phase transitions of lipids. For example, a decrease in the optical density related to dipalmitoylphosphatidylcholine aqueous dispersion was observed at its phase transition temperature [51]. Therefore, this decrease in the lipid turbidity is related to the transition from ordered states to disordered ones.

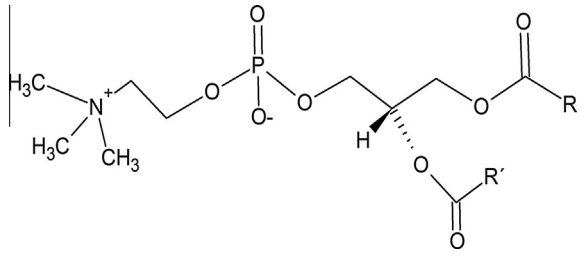

Fig. 2. Structure of soybean phosphatidylcholine (SPC). R or R′ = oleic (18:1), linoleic (18:2) or linolenic (18:3) acid.

4. Results and discussion

4.1. α-ESA influence on in vitro lipid peroxidation of ASO liposomes

To determine the α-ESA influence on the membrane *in vitro* peroxidation, ASO liposomes, pure and loaded with the fatty acid, were submitted to oxidation induced by hydroxyl radical (·OH) and the TBARS level was then determined. Fig. 3 shows the effect of increasing concentrations of α-ESA on the *in vitro* ·OH-induced peroxidation of ASO liposomes.

The TBARS quantities reported in Fig. 3 were generated by the ·OH peroxidation in ASO and are in agreement with previous studies which reported the levels of ·OH peroxidation in different kinds of membranes [17]. In Fig. 3, one can observe that α-ESA seems to have a protective activity against the damage induced by ·OH in ASO liposomes. The α-ESA half inhibition concentration (IC_{50}) was reached in 15 mg/mL (which corresponds to approximately 21.5 mol% of α-ESA with respect to ASO liposomes). At the concentration of 22.5 mg/mL (29.3 mol%), the fatty acid inhibited the ·OH-induced ASO peroxidation in approximately 77%. The α-ESA antioxidant activity corroborates with the effect reported by α-ESA in the lipid peroxidation levels of rat plasma, lipoprotein, erythrocyte membranes and non enzymatic liver tissue [4]. It is known that α-ESA may scavenge free radicals, thereby eliminating or reducing the formation of hydroperoxides [4]. The oxidation of α-ESA may result in the formation of conjugated dienoic fatty acids that also act as antioxidants [52]. These conjugated dienoic fatty acids may reduce the formation of hydroperoxides by lowering the penetration of free radicals, as well as the peroxidation of polyunsaturated fatty acids occurring in erythrocyte membranes and other lipids [4,53]. Conjugated fatty acids can also interact with free radicals to terminate the radical chain reaction or chelate transition metals to suppress the radical formation [54]. It was purposed that these conjugated acids might serve as an *in situ* defense mechanism against membrane attack by free radicals which may explain its anti-carcinogenic properties, give the possible involvement of oxygen radicals in tumor promotion and oncogene activation [55–57]. However, it is also known that, in excess amount, the α-ESA antioxidant activity may reverse and the fatty acid may act as a prooxidant [52,58]. In tumor tissues of α-ESA-treated DLD-1 human colon cancer cells, increased amounts of membrane phospholipid peroxidation and TBARS were observed. It is important to note that the content of α-ESA used in these experiments was equivalent to almost 80 mol% with respect of the tung oil tested. This concentration exceeds in almost 50 mol% the maximum concentration used in our experiments (30 mg/mL, equivalent to 35 mol% of α-ESA with respect to ASO) [2].

Lipid peroxidation is also related to changes in membrane physico-chemical properties [5–8], and the relationship between the antioxidant effects of α-ESA and the influence of this fatty acid on the molecular dynamics of lipid membrane was not establish yet. Therefore, results of the α-ESA (at the concentration range from 0 to 30 mg/mL) effects on ASO liposomes physico-chemical properties are presented below.

4.2. α-ESA effects on ASO liposome dynamics

Characterization of the effects caused by α-ESA on the ASO liposomes dynamical properties was performed by HATR-FTIR, NMR and DSC techniques. The instrumental measurements were performed in order to compare the behavior of pure liposomes to those loaded with the fatty acid. Thus, MLVs were employed to determine details of bilayer structures since their large size (near 2 μm) makes structural analysis with techniques such as NMR more straightforward by comparison with smaller vesicles [20].

The HATR-FTIR spectra of ASO liposomes were obtained in order to understand the effects of different concentrations of α-ESA on specific lipid regions. Fig. 4 shows the spectra which correspond to ASO liposomes pure and those containing α-ESA 30 mg/mL.

For pure ASO liposomes, the following vibrational peak frequencies were detected and analyzed: (a) ν_{as} PO_2^- at 1217 cm^{-1}, (b) ν_{as} CN^+C at 974 cm^{-1}, (c) ν_{as} NCH at 3005 cm^{-1} (d) ν C=O at 1734 cm^{-1}, (e) ν_s CH_2 and ν_{as} CH_2 at 2853 cm^{-1} and 2924 cm^{-1}, respectively. These frequencies of stretching vibrations correspond to SPC groups, which represent 25% of the total lipid mixture contained in ASO [59]. The α-ESA influence on each of the SPC groups was studied by analyzing changes in the frequency values, as well as the bandwidth at ¾ of peak height position. Information obtained from HATR-FTIR essays were complemented by NMR and DSC data. All results were interpreted considering the effect of the fatty acid on the polar, interfacial and hydrophobic ASO regions.

4.2.1. α-ESA interaction with ASO polar head

It is known that phosphatidylcholine bilayers consist of an interlocking set of intermolecular electrostatic associations of the negatively charged phosphate with the positively charged choline groups of neighboring lipids. Thus, it is important to evaluate the α-ESA influence on the dynamics of these two lipid polar head

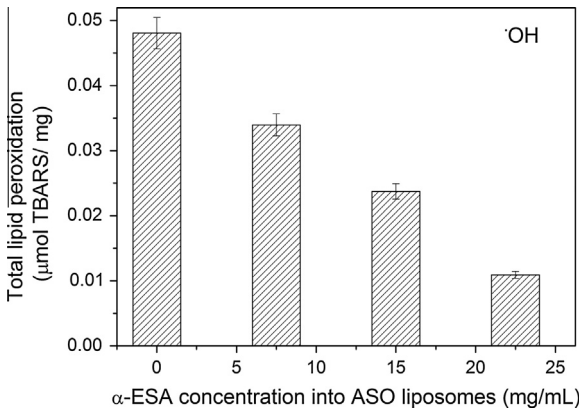

Fig. 3. Variation of total lipid peroxidation induced by hydroxyl radical (·OH) as a function of increasing concentrations of α-eleostearic acid (α-ESA) incorporated into asolectin (ASO) liposomes. Results were obtained by TBARS method and shown as mean ± S.D. of triplicates from three independent experiments.

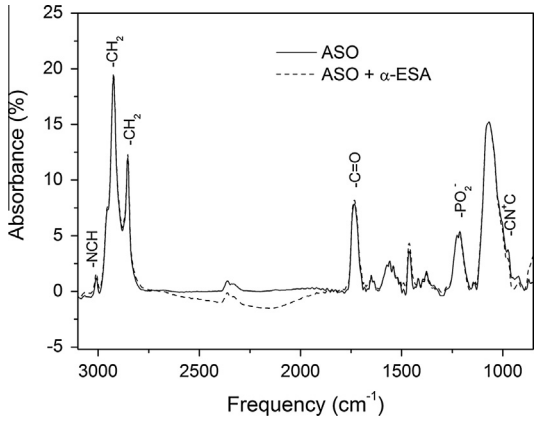

Fig. 4. FTIR spectra of asolectin (ASO) liposomes pure and in the presence of α-eleostearic acid 30 mg/mL (ASO + α-ESA). Interferograms were obtained from the average of 50 scans, with a resolution of 2 cm^{-1}, in a frequency range of 400–4000 cm^{-1}.

groups since they play an important role in the maintenance of the bilayer configuration and the barrier properties [60]. In the spectra shown in Fig. 4, all the frequency values of the groups related to pure ASO liposomes and ASO liposomes in the presence of α-ESA 30 mg/mL (equivalent to 35 mol% of α-ESA with respect to ASO) remain almost the same.

With respect to the α-ESA effect on the lipid polar region, Fig. 5 shows a zoom of Fig. 4 spectra in the region of ν_{as} PO_2^- vibrations.

In both samples, corresponding to ASO liposomes pure and in the presence of α-ESA 30 mg/mL, the ν_{as} PO_2^- band shows a small shoulder at lower frequency. This is due to the hydrogen-bounded phosphate groups, a consequence of its weak hydrogen bonding interaction with the lipid choline region [61,62]. As mentioned before, the ν_{as} PO_2^- band is useful to monitor the hydration state of the polar head groups of phospholipids [46]. An α-ESA-induced increase in the ν_{as} PO_2^- band frequency would indicate a dehydration of the phosphate group, while a decrease in the same frequency could correspond to the hydration of this region [44]. In this study, since no variation in the ν_{as} PO_2^- frequency was observed after lipid interaction with α-ESA, it can be concluded that the fatty acid does not cause changes in the quantities of hydrogen bonds among the lipid phosphate group and the hydrogen atoms of the α-ESA or water [63]. However, Fig. 5 shows that α-ESA reduced the bandwidth of ν_{as} PO_2^- bandwidth in approximately 2 cm^{-1}, suggesting that the fatty acid may cause a slight or indirect decrease in the phosphate group dynamics.

Changes induced by α-ESA in the intensity of the ν_{as} CN^+C band at 974 cm^{-1}, as well as its influence on ν_{as} NCH at 3005 cm^{-1} were also investigated. Fig. 4 shows no α-ESA influence on the shape or frequency values related to the choline bands. These results are in agreement with the unchanged behavior of the ν_{as} PO_2^- band in the presence of α-ESA, confirming that the fatty acid has no influence on the ASO polar head group hydration.

In order to obtain more information about the α-ESA effect on the polar head dynamics, NMR choline ^1H T_1 measurements were performed. Fig. 6 shows ^1H T_1 curves related to the choline region of ASO liposomes pure and loaded with α-ESA 30 mg/mL.

The presence of α-ESA in the liposomes seems to increase the T_1 of SPC choline region from 0.3 s (value corresponding to pure ASO liposomes) to 0.5 s (value corresponding to ASO liposomes loaded with α-ESA). This difference of approximately 30% may reflect a decrease in the choline motional freedom [39,64]. Since the increase of T_1 is related to an increase in τ_c values, the α-ESA induced-change in choline behavior corroborates with an ordering effect in this lipid region. Thus, the findings concerning the lipid phosphate and choline groups, obtained by FTIR and NMR techniques, suggest that α-ESA causes a discrete ordering effect on ASO head region.

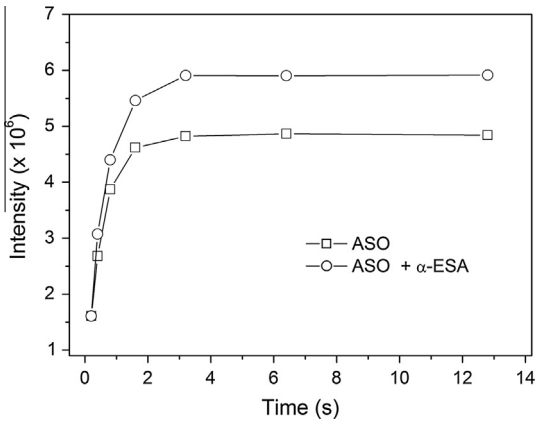

Fig. 6. Asolectin choline proton spin lattice relaxation times (^1H T_1) in the absence (ASO, open squares) and in the presence of α-eleostearic acid 30 mg/mL (ASO + α-ESA, open circles). The ^1H T_1 data were reached at 60 MHz, 23 °C, using a τ range of 0.2–102.4 s.

4.2.2. α-ESA influence on the ASO interfacial region

The α-ESA effects on the ASO interfacial region were studied by comparing the analysis of the HATR-FTIR ν C=O band in ASO liposomes, pure and in the presence of the fatty acid. As mentioned before, the ν C=O band is related to changes in its environment, such as hydrogen bonding or polarity [31]. The analysis of Fig. 4 shows no changes in the ASO ν C=O frequency after interaction with α-ESA. Thus, these results suggest that there is no hydrogen bond between the fatty acid and the ASO carbonyl group. Variations in ν C=O bandwidth as a result of increasing α-ESA concentrations are shown in Fig. 7.

In Fig. 7, one can observe that there is an α-ESA concentration dependence of ν C=O bandwidth values. Increasing concentrations of α-ESA reduced the ν C=O bandwidth values slightly. At 30 mg/mL, the fatty acid seems to be responsible for a reduction in the ν C=O bandwidth, from 15 cm^{-1} to 13.3 cm^{-1}, which corresponds to 11% of the value related to pure ASO liposomes.

The broadness of the ν C=O band seems to be attributable to the absorptions of carbonyl ester located in sn-1 and sn-2 chain, which are not equivalent [65]. A sharp ν C=O band can be attributed to the

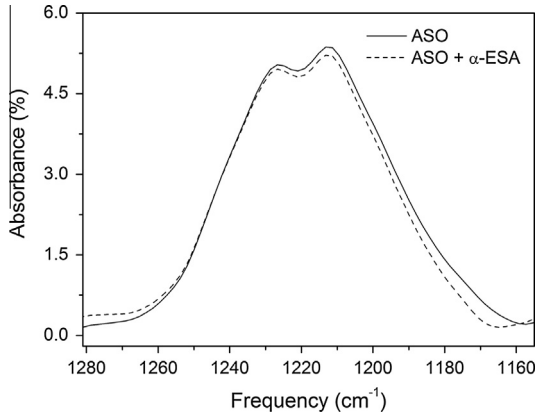

Fig. 5. Zoom of the FTIR spectra of asolectin (ASO) liposomes, pure and in the presence of α-eleostearic acid 30 mg/mL (ASO + α-ESA), in the asymmetric phosphate stretching region (ν_{as} PO_2^-). The zoom was obtained from the full spectra shown in Fig. 4. Interferograms were obtained from the average of 50 scans, with a resolution of 2 cm^{-1} in a frequency range of 400–4000 cm^{-1}.

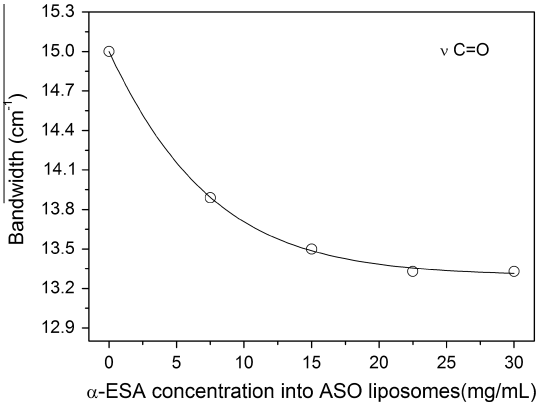

Fig. 7. Bandwidth variations related to asolectin (ASO) carbonyl stretching vibration (ν C=O) after interaction with different concentrations of α-eleostearic acid (α-ESA), The curve was obtained from HATR-FTIR interferograms, which were averaged for 50 scans at 2 cm^{-1} resolution, in frequencies ranging from 400 to 4000 cm^{-1}.

existence of highly immobilized ester carbonyl groups in the lipid chains. For lipids, it is known that the conversion of liquid crystalline states to the gel phase coincides with a change from a broader to a narrower ν C=O band [66,67]. In our study, it may suggest that an ordering effect of α-ESA occurs not only at the polar region but also affects the interfacial region of ASO membranes.

It is worth mentioning that, at physiological pH (7.4), the carboxyl group of lipids and fatty acids may be partially unprotonated, generating a molecular negative charge [68]. Thus, a dipolar interaction between the α-ESA carboxyl group and the phosphatidylcholine choline region may be reflected in changes related to the lipid choline T_1 value, as reported in this study by NMR results. This interaction may be responsible for an increase in the lipid choline region order. As a consequence of the dipolar interaction between the lipid choline and the α-ESA carboxyl group, the order of the lipid polar and interfacial regions may also be affected. It corroborates with the slighter α-ESA ordering effect observed in lipid phosphate and carbonyl groups by the HATR-FTIR technique.

4.2.3. α-ESA effects on ASO acyl chain methylenes

In Fig. 4, one can observe that no shifts in the HATR-FTIR frequencies values of ASO methylene stretching bands were induced by α-ESA. As mentioned before, changes in ν_s CH_2 and ν_{as} CH_2 band frequencies may reflect the average trans-gauche isomerization in the lipid system [32]. Thus, α-ESA does not seem to influence the trans-gauche isomerization of the lipid system. The influence of increasing concentrations of α-ESA on the bandwidth of ASO acyl chains methylenes stretching bands was also investigated (Fig. 8).

In Fig. 8, one can observe that increasing-concentrations of α-ESA were responsible for the gradual broadening of ν_s CH_2. After the incorporation of α-ESA 30 mg/mL, the ν_s CH_2 bandwidth broadened from 18.6 cm^{-1} to 21 cm^{-1}. This α-ESA-induced variation of 2.4 cm^{-1} in the ν_s CH_2 bandwidth corresponds to a broadening of approximately 13%, which is included in the range of values expected for a disordering effect on the liposomal system [43]. As no shifts in acyl chains CH_2 stretching were observed, the increase in CH_2 mobility promoted by α-ESA may not have been related to a conversion from trans to gauche conformers [44].

The DSC experiments were performed in order to determine the α-ESA influence on the ASO liposomes phase transition and order. From DSC curves (data not shown), an endothermic transition in the negative range from −40 to −18 °C was observed. This range corresponds to the typical negative range of fully hydrated unsaturated phosphatidylcholine, including those containing oleic acids in their hydrophobic region (note that Fig. 2 shows that it is one of the fatty acids present in SPC) [69]. From this transition, the enthalpy variation (ΔH) values related to ASO liposomes, in the absence and in the presence of α-ESA, were obtained. These results are shown in Table 1.

The analysis of Table 1 indicates that α-ESA reduces the ASO ΔH value in approximately 53%. Thus, the fatty acid may promote a SPC molecular rearrangement to a more disordered state, which corroborates with the FTIR findings. The disordering effect caused by α-ESA on phospholipid membranes may be attributed to the location of the fatty acid *cis* double bond [70]. The α-ESA cis double bond has a typical "kinked" conformation which can affect the packing of the hydrophobic region of the phosphatidylcholine membrane. Subbayah and co-workers [71] observed a fluidizing effect for dipalmitoylphosphatidylcholine liposomes after the insertion of phosphatidylcholine molecules containing *cis*9-*trans*11 conjugated linoleic acid in one of its acyl chain. These authors also reported that the conjugation of double bounds has a strong influence on the physical structure of the fatty acid and that it is an important factor associated to the membrane fluidizing effect.

4.2.4. Turbidity measurements

The global effect of α-ESA on ASO liposomes was investigated by turbidity studies at 400 nm. Fig. 9 shows the influence of different α-ESA concentrations on the liposomes turbidity.

Increased concentrations of α-ESA cause the gradual turbidity reduction on ASO liposomes. A reduction corresponding to 20% of the initial ASO turbidity value was observed after the incorporation of α-ESA 30 mg/mL into the membrane. The reduction in the absorbance values is related to a decrease in aggregation and fusion of the vesicles; it implies in the reduction of the particle size [42,72]. The reduction of membrane turbidity can also be observed in gel to liquid–crystalline phase transitions, mainly due to changes in the lipids refractive index as a consequence of variations in lipid density during melting [51]. Thus, α-ESA seems to

Table 1
Influence of α-eleostearic acid (α-ESA) 30 mg/mL on asolectin (ASO) enthalpy variation (ΔH) values[a].

Membrane system	Δ*H* (J/g)
ASO	|1.97|
ASO + α-ESA	|0.92|
Δ*ΔH*	|1.05|

[a] Δ*H* of ASO liposomes, pure or loaded with α-eleostearic acid (ASO + α-ESA), were calculated from DSC thermograms.

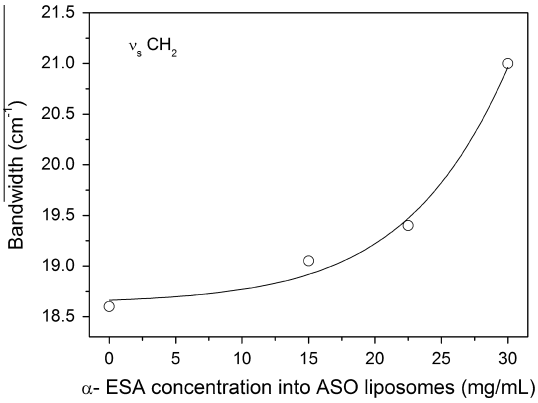

Fig. 8. Effect of different concentrations of α-eleostearic acid (α-ESA) on the bandwidths of acyl chains methylenes symmetric stretching vibrations (ν_s CH_2) of asolectin (ASO) liposomes. The curve was obtained from HATR-FTIR interferograms averaged for 50 scans at 2 cm^{-1} resolution, with frequencies ranging from 400 to 4000 cm^{-1}.

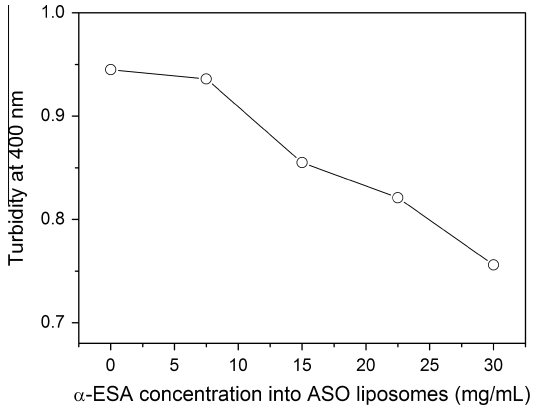

Fig. 9. Turbidity changes for asolectin (ASO) liposomes after interaction with different concentrations of α-eleostearic acid (α-ESA). The optical density values were obtained at 400 nm by a UV–vis spectrophotometer.

have a global effect related to the increase of the ASO liposomes fluidity.

Turbidity results are supported by our FTIR, NMR and DSC studies. The ordering effect caused by interaction of the unsaturated fatty acids with phosphatidylcholine polar head, as well as the influence of its *cis*-double bond in lipid acyl chains, may leads to a transition from the phosphatidylcholine typical cylindrical shape to a "cone" shaped lipid complex where the polar headgroup region has a smaller area than the hydrophobic region. It may destabilize the bilayer configuration, enhancing the membrane permeability [70,73].

5. Conclusion

Previous studies reported that membrane lipid peroxidation levels are influenced by the charge and diffusion of the reactive species, the membrane fatty acid unsaturated bonds content and order, as well as the intrinsic properties of antioxidant compounds in the system [17,74,75]. The ·OH is the most reactive among reactive oxygen species and its biological importance was discussed by other authors [74,76]. The lipid mobility can create conditions for mutual accessibility among the reactive species and the antioxidant compound [77]. Antioxidant intrinsic properties include its chemical reactivity and its distribution between the hydrophilic and hydrophobic lipid regions.

In ASO liposomes, the ordering effect promoted by α-ESA in the lipid polar head may restrict the ·OH diffusion toward this region of the membrane. The influence of membrane packing on reactive species diffusion is also related by Khairutdinov and co-workers for peroxinitrite [74]. On the other hand, the α-ESA disordering effect on phosphatidylcholine membranes, attributed to its *cis* double bound, may facilitate the interactions between the ·OH and α-ESA. It may provide a more efficient antioxidant activity of α-ESA. The relationship among the lipid peroxidation levels and the order of specific regions of the membrane was previously reported to other antioxidant molecules, such as melatonin and alpha tocopherol [39,77]. Besides the α-ESA scavenging properties, the effect of α-ESA on the asolectin membrane dynamics may contribute as an antioxidant secondary mechanism against the damage caused by reactive species.

Until we known, no correlation between the α-ESA antioxidant activity and its effects on membrane dynamics properties was established yet. Thus, findings described in this study can contribute to get a broader view of the α-ESA biological activity which can be useful to the development of new strategies for lipid peroxidation-related disease therapy.

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