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Spin labeling EPR studies of the properties of oxidized phospholipid-containing lipid vesicles

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ABSTRACT

This study aims at characterizing the structure and some properties of phospholipid multi-lamellar vesicles (MLVs) containing the oxidized species γ-palmitoyl-β-(9-hydroperoxy-10,12-octadecanedienoyl)-lecithin (HPPLPC), γ-palmitoyl-β-(9-hydroxy-10,12-octadecanedienoyl)-lecithin (HOPLPC), γ-palmitoyl-β-glutaroyl-lecithin (GlPPC) and γ-palmitoyl-β-azelaoyl-lecithin (AzPPC). Sepharose 4B gel-chromatography was used to ensure and check that only MLVs are used in EPR measurements. Gel-solid to gel-liquid transition temperature (T_m), lateral phase separation, fluidity gradient and polarity profile were studied by use of EPR spectroscopy of enclosed n-doxylstearoyl lecithin spin labels. Contrarily to conjugate dienes and normal phospholipids, pure carboxyacyl species yielded aqueous suspensions showing gel-chromatography elution profile resembling that of lysolecithin micelles. Conjugate dienes/DPPC MLVs showed lateral phase separation at room temperature and T_m value lower than pure DPPC MLVs. Pure conjugate dienes MLVs resembled more PLPC MLVs and displayed free miscibility with PLPC in mixed MLVs. Pure HPPLPC MLV bilayer appeared to be slightly more rigid, while that of HOPLPC and the polarity profile of MLVs made of the pure conjugate dienes species were similar to those of normal PLPC. It is concluded that carboxyacyl lecithins in MLVs tend to disrupt vesicle structure, while conjugated dienes lecithins are more able to affect some physical properties of the bilayer, and that DPPC in MLVs enhances these effects while PLPC shows a better compatibility with the lipoperoxides.

1. Introduction

Recently, we have described some characteristics of lipid vesicles containing oxidized lecithins, obtained by oxidative attack to PLPC with a FeII/H_2O_2 mixture (the Fenton reagent), and reported aggregates or micelle formation depending on the percent composition of DPPC/PLPC/PLPCox ternary mixture [1]. We also found that the same oxidized PLPC preparation was able to induce lateral phase separation when present up to 25% in DPPC/PLPC mixtures with DPPC in higher percentage. More recently, by use of mass spectrometry [2] and of up-to-date criteria of lipidomics [3–7], we pointed out the complex molecular heterogeneity of Fenton-oxidized PLPC, and shifted our attention to the effects brought about in the lipid bilayer by selected lipoperoxide molecular species. Defined lipoperoxides were obtained by synthetic or enzymatic reaction protocols. Consequently, disordering of the fatty acyl residue packing was attributed to the carboxyl species [2] more correctly than it was possible in early experiments using Fenton oxidized stearoyl-arachidonoyl-PC [8]. It was proposed that various lipoperoxides molecular species deserve a separate study, since their properties and their impact on the structure and the characteristics of a membrane can be widely variable.

On this basis, the present study is dedicated to the characterization of model membranes containing different lipoperoxides. To this purpose, pure phospholipoxides (PLox) or high percentage PLox-containing vesicles have been used despite their physiological concentration in natural membranes is retained to be low. While conclusive definition of this aspect in many pathologic tissues is still intensely sought for, we argue that polyunsaturated fatty acids (PUFA) rich membrane domains, possibly forming due to lateral phase separation, can give rise to high local PLox concentrations following oxidative stress. Therefore, we consider PLox-enriched artificial membranes as useful models to mimic those micro-domains and to elicit related effects to such a visible extent as to allow detailed study and characterization.

DPPC (di-palmitoyl-lecithin) and PLPC (γ-palmitoyl-β-linoleoyl-lecithin) were taken as reference normal phospholipids, while γ-palmitoyl-β-(9-hydroperoxy-10,12-octadecanedienoyl)-lecithin

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Abbreviations: DPPC, di-palmitoyl-lecithin; PLPC, γ-palmitoyl-β-linoleoyl-lecithin; EYPC, egg yolk lecithin; n-DSPPC, γ-palmitoyl-β-(n-doxylstearoyl)-lecithin HPPLPC, γ-palmitoyl-β-(9-hydroperoxy-10,12-octadecanedienoyl)-lecithin; HOPLPC, γ-palmitoyl-β-(9-hydroxy-10,12-octadecanedienoyl)-lecithin; CD-PC, conjugate dienes lecithins, the ensemble grouping HPPLPC and HOPLPC, full-chain phospholipoxides; GlPC, β-glutaroyl-lecithin; AzPC, β-azelaoyl-lecithin; CX-PC, β-carboxyacyl-lecithins, the ensemble grouping GlPC, AzPC and others, cleft-chain phospholipoxides; n-DSPPC, γ-palmitoyl-β-(n-doxylstearoyl)-lecithin (n=5, or 12, or 16); PUFA, poly unsaturated fatty acids; MLV, multilamellar vesicles; SUV, single unilamellar vesicles; ROS, reactive oxygen species; *phospholipoxides*, neologism standing for (per)oxidized phospholipids

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(HPPLPC), γ-palmitoyl-β-(9-hydroxy-10,12-octadecanedienoyl)-lecithin (HOPLPC), β-azelaoyl-lecithin (AzPC) and β-glutaroyl-lecithin (GlPC) were chosen as the oxidized species to be studied.

According to the lipoperoxidation cascade:

$$LH \rightarrow L\bullet \rightarrow \underline{LOOH\ \&\ LOH} \rightarrow L(-OOH, -OH, -O-)$$

$$\rightarrow L(CH = O) \rightarrow \mathbf{L}(\mathbf{COOH})$$

we consider the two leading species (referred to as conjugate dienes species, or CD, underlined) as the first stable intermediates of lipoxidation, while the latter (the carboxyl species, CX, bold) represent the final products of extreme oxidation. We also consider long-chain oxidized compounds (CD) to be oxidatively modified at a lesser extent, since the acyl chain still retains its full length, and representative of early oxidation stage, while carboxyacyl-containing molecules (CX) are apparently final oxidation products. At the present stage, our study focuses on the membrane behaviour of these species, in comparison – or in mixture – with normal species DPPC and PLPC (LH). In particular, the latter is taken in more consideration, being the precursor molecule of HPPLPC, HOPLPC and of AzPC. Intermediate oxidation compounds, such as aldehyde-containing and most of the mixed oxidized group-bearing fatty acid forms (italics in the above reaction scheme) also deserve attention, and will be the object of future investigation.

The main body of this study is a revisitation of many well known phospholipid bilayer properties, such as the gel-solid to gel-liquid transition temperature (T_m), the fluidity gradient, the polarity profile and the lateral phase separation, of a new version of the widely used multi-lamellar vesicles, that is MLV containing, or made of, the above cited oxidized PLPC species.

In consideration of the novelty of the phospholipid material under study, our first issue was to check the structure of oxidized MLVs by the widely used Sepharose 4B gel-chromatography method [9–14]. This method has been the key in determining structural properties of SUV [12], and we routinely used it preliminarily to any other vesicle study. Based on an already used criterion [1], only vesicle preparations yielding an elution profile similar to normal phospholipid (PLPC, egg yolk PC, DPPC) MLVs were forwarded to further characterization.

The above-cited membrane features were studied by use of spin labeling EPR spectroscopy in MLVs probed with γ-palmitoyl-β -(n-doxylstearoyl)-lecithin (n-DSPPC). It will be shown that the previously observed micelle formation with Fenton-oxidized PLPC is attributable only to CX-PC species, while CD-PC species were responsible for lateral phase separation. All oxidized species exhibited easier, or complete, miscibility with unsaturated lecithin (PLPC), while the effects of their presence were much more evident in association with saturated DPPC, which in turn appeared to be less compatible with oxidized lecithins. It will be also shown that MLV membrane fluidity and bilayer polarity are scarcely (or not at all) affected by the CD species within the experimental error range, and that these properties are more similar to those of PLPC than to DPPC vesicles.

2. Materials and methods

2.1. Materials

n-doxylstearic acid (5-DSA, 12-DSA, 16-DSA), glutaric anhydride, azelaic acid, γ-palmitoyl-β-*lyso*-lecithin, PLPC, and lipoxidase (type V from soybean, EC 1.13.11.12) were purchased from Sigma; solvents were Baker HPLC grade. Lichroprep RP18 (40–63 μm) silicagel and TLC chromatoplates (0.2-mm or 0.5-mm thick) were from Merck. Sepharose 4B was purchased from Amersham Biosciences.

2.2. Phospholipid modification

γ-palmitoyl–β-(n-doxylstearoyl)-lecithin (5-, or 12-, or 16-DSPPC) was obtained by coupling n-DSA to γ-palmitoyl-β-*lyso*-lecithin according to Boss et al. [15], and purified by reverse-phase (RP18 silicagel) flash-chromatography [2]. β-glutaroyl-lecithin and β-azelaoyl-lecithin were obtained by the method of Schlame [16] starting from lysolecithin. These products were purified by preparative TLC developed with $CHCl_3/CH_3OH/NH_4OH_{conc}$ 65:25:8 (by volume).

γ–palmitoyl-β-(9-hydroperoxy-10,12-octadecanedienoyl)-lecithin (HPPLPC) was obtained by action of lipoxidase on PLPC [17,18], and its reduction with $NaBH_4$ [19] yielded γ–palmitoyl-β-(9-hydroxy-10,12-octadecanedienoyl)-lecithin (HOPLPC). After extraction from the reaction mixture by the method of Bligh and Dyer [20], the products were purified by reverse-phase preparative column chromatography using $CH_3OH/(C_2H_5)_2/H_2O$ 95:5:2 (vol). The identity and purity of all synthetic molecular species was confirmed by mass spectrometry and by TLC. Quantitative phosphorus analysis was performed according to Nakamura [21].

2.3. Vesicle preparation

The desired phospholipid mixture (1-4 μmol lipid P) was dissolved in 0.5 ml CH_2Cl_2 and dried to a thin film under an N_2 stream in a test tube at 40 °C. After further drying for 20 to 60 min under vacuum (oil pump) the film was hydrated with 0.5 ml of a 100 mM KCl, 10 mM TRIS-HCl, pH 8 buffer for 20 to 60 min at 40 °C (50 °C for pure DPPC) [22]. 0.5-ml samples (corresponding to 4% of the bed volume) undergoing gel-chromatography were loaded as such on a Sepharose 4B column (see below). For EPR measurements, 1–2% (by mol) of the desired spin labelled n-DSPPC was included in the initial mixture and the hydrated preparation was collected as a concentrated pellet (5–10 μl) after centrifuging for 10 min (30 min for some phospholipoxides) at 10,000 RPM (9,400 *g*) in an ALC PK121R refrigerated centrifuge.

SUVs, small unilamellar vesicles, were obtained by ultrasonic irradiation (Branson sonifier) of egg yolk lecithin (EYPC) MLV preparations under an N_2 stream at 40 W output for 15 min with 60-s/20-s on/off intervals, in an ice bath.

2.4. Gel chromatography

Sepharose 4B was suspended in the vesicle buffer and loaded into a 15-cm (1-cm ID) glass column and equilibrated in the same buffer [9–11]. The column was eluted with MLV buffer at 10 ml/h flow rate, and the effluent was monitored at 300-nm wavelength [9] by letting it through a Hellma continuous-flow cuvette in a Cary 50 spectrophotometer coupled to a desktop PC. Optical dense lipid particulates (MLV, SUV, micelles) were visualized as OD peaks, and the elution pattern was plotted as a function of V_e/V_0 (elution volume, V_e, relative to the void volume, V_0). V_0 was calibrated with Dextran Blue, and MLVs always eluted at $V_e/V_0 = 1$. As an additional particle standard, SUVs (reportedly characterized by a diameter of about 20 nm [12], and eluting later) were also used.

2.5. EPR spectroscopy

Spin labelled MLV pellets were transferred to a glass capillary and inserted into the cavity of a 9-GHz Varian E-9 Century Line EPR spectrometer, at room temperature. Instrumental settings were: 12 mT scan width (3000 points, yielding a spectral resolution of 0.004 mT/point); 100 kHz and 0.2 mT field modulation frequency and amplitude, respectively; 9.5 GHz and 20 mW microwave frequency and power, respectively; 0.064 s time constant; 8 min scan time.

Controlled or variable temperature EPR scans were performed by means of a Dewar insert fitted into the cavity and connected to a Variable Temperature Controller (also from Varian). The Dewar was thermostated by means of an N_2 flow, and after dialling the desired temperature value, its stability was checked for a few minutes by means of a thermocouple inserted into the Dewar. After reaching a stable temperature reading, the sample capillary was introduced into the Dewar and allowed to thermally equilibrate for the same time interval before recording the EPR spectrum [22].

2.5.1. Transition temperature determination

The larger EPR hyperfine splitting constant $2A_{max}$ [23] was measured from EPR spectra of 5-DSPPC spin labelled MLVs as the distance between the first spectral maximum to the last minimum (in mT) and one-half this value was plotted against temperature [22]. A_{max} is the most temperature-sensitive parameter in the spectrum and its value was determined better from 5-DSPPC's than from other spin labels' EPR spectra. It was measured from spectra scanned at various temperatures, with increasing steps of 2 °C or 4 °C, according to the accuracy required by the particular experiment, in the temperature range yielding distinctly visible spectral minima and maxima. Plots of A_{max} vs. temperature allow detection of MLV bilayer gel-solid to gel-liquid transition as a steep A_{max} decrease [22], from which T_m is deduced as the abscissa of the maximum slope point (close to midpoint).

2.5.2. Lateral phase separation

The qualitative study of this feature was performed by use of 16-DSPPC, according to Refs. [24–27], at different temperatures (not reported), similarly to our previous studies [1]. According to those criteria, superimposition of two different lineshapes with different spin label motional freedom in EPR spectra of 16-DSPPC enclosed in the vesicles was considered a confirmation of two coexisting phases with different fluidity degree [27]. Quantification of the two phases composition, as outlined in Ref. [26], was not performed and will be the object of a future study, specifically dedicated to this aspect.

2.5.3. Membrane fluidity gradient determination

MLV membrane fluidity was probed at three acyl chain positions, C-5, C-12 and C-16, by including the corresponding spin labels 5-DSPPC, 12-DSPPC and 16-DSPPC (1–2% by mol). The corrected [28] order parameter S was calculated according to Ref [23] basing on the measured distance (in mT) between the first spectral maximum to the last minimum ($2A_{max}$) and that between the first minimum to the last maximum ($2A_{min}$) of the corresponding EPR spectra, scanned at 24 °C (hereafter referred to as room temperature). The variation of resulting S-values with the spin label C-position describes the bilayer fluidity gradient [23], in which position C-16 values have more an indicative meaning due to increased time averaging of A_{max} and A_{min}.

2.5.4. Polarity measurement

The isotropic hyperfine coupling constant A_0, defined as the trace of the A-tensor, TrA, is sensitive to the spin label's environment polarity [29], owing to the contribution from the unpaired electron density at the nitroxide N-nucleus. A polar environment stabilizes the polar resonant form of the spin label, resulting in an increased A_0 value. Since this parameter is also temperature-dependent at low temperature, this dependence is minimized by performing A_0 measurements at higher temperatures, as described [30]. In practice, EPR spectra were scanned every 5 °C in the 60–80 °C range, and A_0 value was calculated from each spectrum (see below). A_0 values ranging in the narrowest variation interval (0.005–0.01 mT, as reported in Ref. [30]) were retained temperature-independent and averaged to the final temperature-independent A_0 value.

In the case of 5-DSPPC, single A_0 values were calculated by averaging A_{max} and A_{min} values (see paragraph 2.5.3) measured from each spectrum, with corrections according to Ref. [28].

A_0 values at positions C-12 and C-16 were calculated from EPR spectra of 12-DSPPC and 16-DSPPC, respectively, as one-half the distance between the low- and the-high field band, in mT [30], in the same temperature range as above.

Under these conditions, the variation of A_0 with C-position yields the bilayer polarity profile of a given MLV sample, in the considered temperature range. These measures, provided they are taken strictly under the same conditions, should be considered only for comparative purposes.

3. Results and discussion

3.1. Membrane formation

Membrane formation ability of the studied phospholipoxides was assessed by gel-chromatography analysis of MLV preparations. Gel-chromatography on Sepharose 4B column was a widely used and strongly recommended tool for obtaining or purifying MLVs and sonicated SUVs [10,14]. The method also allowed characterization and measurement of basic geometric and structural characteristics of lipid vesicles [9–12], and we adopted it as a criterion to check normal membrane formation in order to avoid forwarding artefact model membranes to EPR measurements.

Elution profiles of 100% DPPC, PLPC and EYPC are reported in Fig. 1, panel A (thin line: each lipid's elution profile was largely superimposed). All the preparations exhibited heavy turbidity and were easily collected as a thick pellet after 10 min centrifugation (10,000 RPM; 9,400 *g*). The black bold line of the same figure depicts the superimposed elution patterns of both HPPLPC and HOPLPC. These preparations were less turbid and pelleted more difficultly (30 min at 10,000 RPM) as a looser and more transparent pellet than the former. Nonetheless, they displayed the same elution pattern as normal PCs, and were considered normally shaped MLVs. The bold track reported in Fig. 1 panel B represents the superimposed elution pattern of both DPPC/HPPLPC 3:1 and DPPC/HOPLPC 3:1 MLVs. A similar behaviour was observed in MLV preparations made of PLPC/HPPLPC 1:1 and PLPC/HOPLPC 1:1, whose elution pattern is also reported in Fig. 1 panel B (thin line). Again the two profiles were almost superimposed, so that only one is reported. In general, from Fig. 1, it can be seen that CD-PC-containing MLV preparations yielded elution patterns very similar to the one of normal PCs. Therefore, all were considered normal bilayer MLVs and were subjected to EPR studies in order to detect and measure the effect of the presence of diverse phospholipoxides in the lipid bilayer.

A different picture is portrayed in Fig. 2, reporting the elution profile of β-(ω-carboxyacyl)-lecithins. As a reference, panel A reports

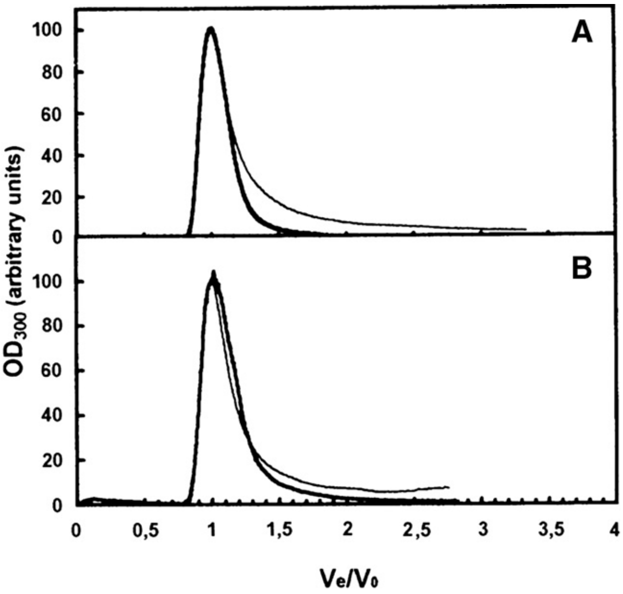

Fig. 1. Sepharose 4B chromatography elution pattern of aqueous phospholipid suspensions. Panel A: pure DPPC, or PLPC, or EYPC (thin line); pure HOPLPC or HPPLPC (bold line). Panel B: DPPC/HPPLPC 3:1, or DPPC/HOPLPC 3:1 by mol (bold line); PLPC/HPPLPC 1:1, or PLPC/HPPLPC 1:1 by mol (thin line).

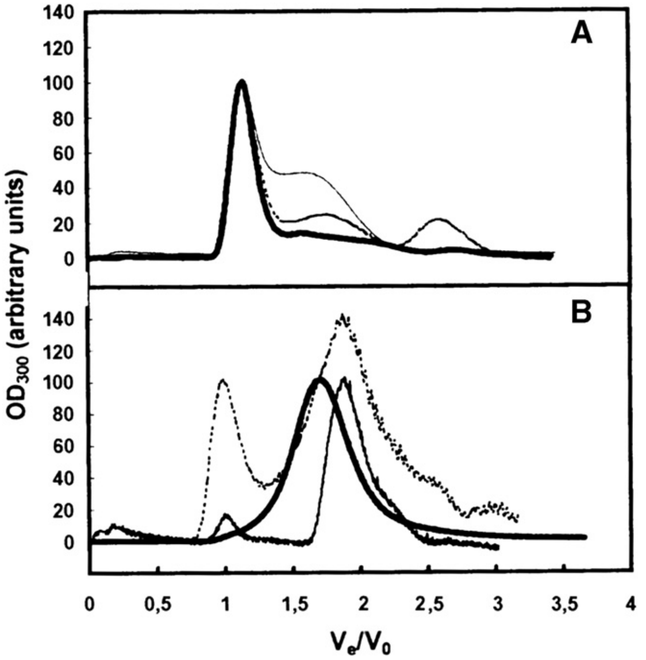

Fig. 2. Sepharose 4B chromatography elution pattern of aqueous phospholipid suspensions. Panel A: pure EYPC SUVs (thin line); DPPC/GlPC 3:1 by mol (dashed line); PLPC/GlPC 1:1 by mol (bold line). Panel B: dashed line: pure lyso-PC; thin line: pure GlPC; bold line: pure AzPC.

the elution pattern of a sonicated EYPC MLV preparation (thin black line). The sample was visually clear, and yielded no pellet after centrifugation. As previously reported, its elution profile indicates abundant SUV formation (V_e/V_0 = 1.6 ca.) [9–11] along with residual MLVs in the leading peak (V_e/V_0 = 1). This graph is useful as a low-scale vesicle size marker, since the SUV diameter was estimated to range between 20 nm and 30 nm in a number of physical studies [9–12]. This measure is also considered the minimum diameter allowable for vesicle formation, since smaller curvature radius would strongly destabilize the closed structure [12]. In panel B of Fig. 2, the dashed line represents the elution pattern of a completely clear suspension of egg lysolecithin, prepared according to the same protocol for MLVs. Keeping in mind the detergent–like properties of lyso-PC, the largest following peak, eluting at almost twice the column void volume (V_e/V_0 = 1.9) is taken as the marker for micelles or micelle-size particles, decidedly smaller than SUVs.

The thin black line in the same figure panel represents the elution profile of a 100% β-glutaroyl-lecithin aqueous suspension. The preparation was lysolecithin-like clear, and the coincidence of its principal elution peak with that of lysolecithin, indicates that it also mainly contains micelles. The bold black track also reported in this figure represents a preliminary result of an ongoing study dealing with β-azelaoyl-lecithin, and was added in support of micelle formation by CX-lecithins. Similarly to GlPC and lyso-PC, the acqueous suspension of AzPC was completely clear and showed a single elution peak beyond that of SUVs, eluting in the same range as GlPC and close to lyso-PC micelles, confirming the suggestion of a lysolecithin-like behaviour of both β-(ω-carboxyacyl)-lecithins.

GlPC appears to behave differently when mixed with normal phospholipid, depending on the companion's unsaturation degree. The micelle peak of GlPC is still present, though lower, in mixed MLV containing 75% DPPC (Fig. 2, panel A, dashed trace), suggesting a tendency of carboxyacyl-lecithins to leave (or disrupt) saturated phospholipid membranes, while it is almost suppressed in a 1:1 mixture with PLPC (panel A, bold line), hinting at better miscibility and compatibility of GlPC with polyunsaturated fatty acid-containing lecithin [13].

Micelle formation was already observed with oxidized PLPC [1]. More recently, differences in bilayer disordering ability of defined phospholipoxides was pointed out [2]. The present results allow a more appropriate attribution of micelle forming ability specifically to carboxyacyl-lecithins, as a difference from long chain lipoperoxides (CD-PCs), which in turn show normal behaviour as for membrane formation. Therefore, the inability of carboxyacyl-lecithins to form membranes turns out to be a striking peculiarity of these ROS-generated phospholipoxides. Nonetheless, these observations deserve deeper study, and we were not confident to subject CX-PC-containing mixed MLVs to further EPR measurements at this stage. By now, deeper studies aimed at determining the composition range and the proper lecithin molecular species yielding stable MLVs containing CX-lecithin have been undertaken prior to further characterization studies.

3.2. Thermal analysis and gel-solid to gel-liquid transition temperature (Tm)

Membrane transition temperature strongly depends upon the fatty acid residues of the component lecithin molecule. Its increase with the acyl residue length and saturation degree is widely confirmed and largely reported [31], and T_m is one of the most useful parameters for membrane characterization. Since lipoperoxidation affects the fatty acid chemical nature, an influence on T_m value by phospholipoxides is expected.

EPR thermal analysis of MLVs is accomplished by measuring the value of A_{max} (corresponding to one-half the distance between the first spectral maximum to the last minimum) from EPR spectra of 5-DSPC (5-DSPPC in our case) included in the MLV preparation with variable temperature [22]. Temperature intervals of 2 °C, routinely yielding 0.05 mT A_{max} variations in the shallow slope range, were considered accurate enough for measuring unknown samples T_m under the described experimental conditions (see paragraph 2.5.),

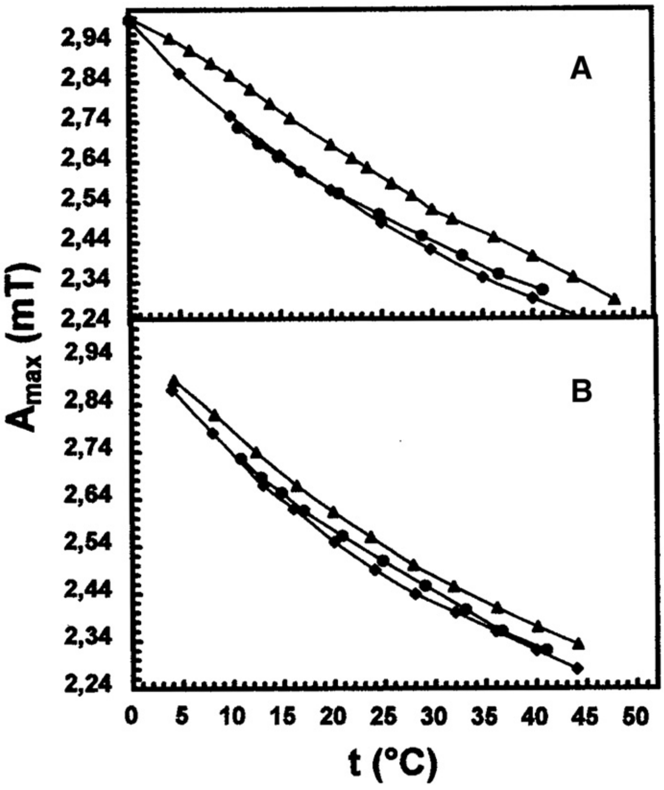

Fig. 3. EPR detection of the lipid phase transition in multibilayer vesicles (MLV). Panel A: ●, pure PLPC; ▲, pure HPPLPC; ♦, pure HOPLPC. Panel B: ●, pure PLPC; ▲, HPPLPC/PLPC 1:1 by mol; ♦, HOPLPC/PLPC 1:1.

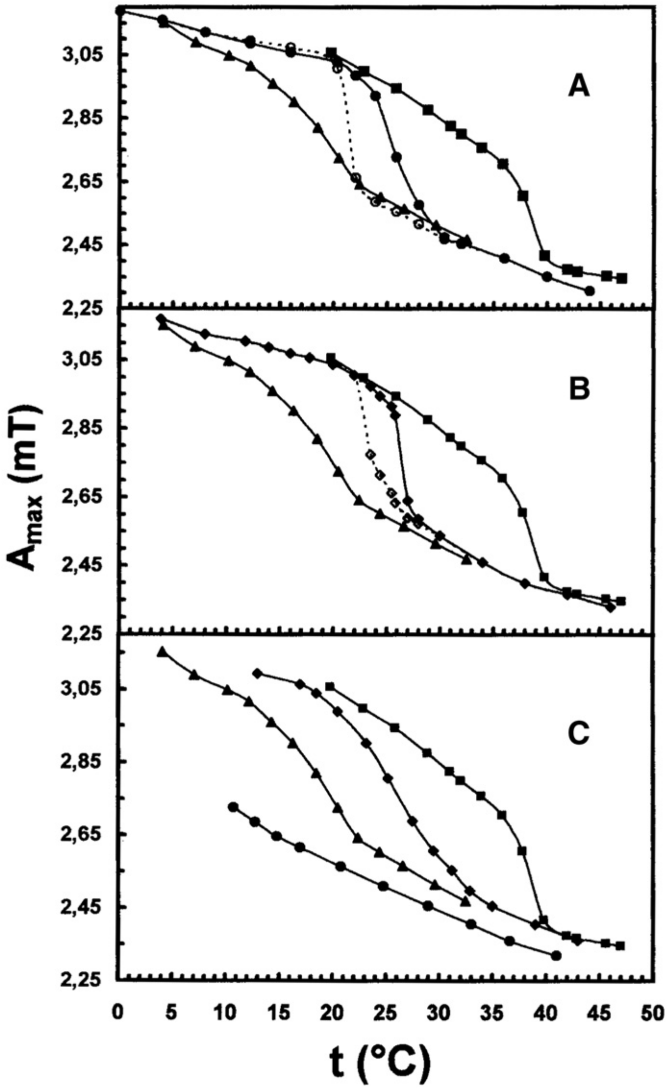

Fig. 4. EPR detection of the lipid phase transition temperature, Tm, in multibilayer vesicles (MLV). Panel A: ■ pure DPPC; ▲, DPPC/PLPC 3:1 by mol; ●, DPPC/HOPLPC 3:1 spectral component I; ○, DPPC/HOPLPC 3:1 spectral component II (see also Figs. 5 and 7). Panel B: ■ pure DPPC; ▲, DPPC/PLPC 3:1 by mol; ♦, DPPC/HPPLPC 3:1 spectral component I; ◇, DPPC/HPPLPC 3:1 spectral component II (see also Figs. 5 and 7). Panel C: ■ pure DPPC; ♦, DPPCIPLPC 9:1 by mol; ▲, DPPC/PLPC 3:1; ●, pure PLPC.

while for well-known control samples, like DPPC MLVs, 4 °C-steps were used. Plots of A_{max} vs. temperature depict the studied membrane thermal behaviour, and allow determination of T_m (if any). Care was taken of the visibility of characteristic spectral maxima and minima, so that EPR measurements have been limited to the temperature range (and phospholipid type) allowing well-resolved peaks. Thermal analysis of pure HPPLPC and HOPLPC are reported in Fig. 3, panel A, while panel B of the same figure reports the thermal analysis of 1:1 mixtures with PLPC. The thermal behaviour of pure PLPC (●) is also shown for comparison in both panels. As it can be seen from the figure, HOPLPC, both pure (♦, panel A) and in 1:1 mixture with PLPC (♦, panel B) behaves similarly to PLPC, showing a close thermal profile in both cases. Pure HPPLPC shows a similar trend (▲, panel A) though with an overall shift towards higher A_{max} values, indicating a generalized membrane rigidification not observed in HOPLPC. This effect is lower in MLVs made of HPPLPC/PLPC 1:1 by mol (▲, panel B), suggesting a moderating effect by the unsaturated lecithin.

These observations may indicate that growth of peroxidation-linked hydroperoxyl and hydroxyl groups do not overcome the overall “fluid” nature of PUFA residues, and that the fluidising ability of double bonds is able to resist structural effects from lipoperoxidation to a large extent.

The graphs reported in Fig. 4 describe the effect of PLPC oxidation in a membrane made of DPPC/PLPC 75:25.

The system's T_m variation spans a temperature interval whose upper limit is set by the thermal analysis plot of pure DPPC (■, panel A, B and C), for which we found a T_m value of 39.7 °C, in close agreement with Ref. [22], and the lower limit is represented by the thermal dependence plot of PLPC MLVs (●, panel C) (whose T_m value was not observed [31]). As additional reference plots, those of MLVs made of 3:1 (▲, panel C) and 9:1 (♦, panel C) DPPC/PLPC are also reported, displaying a T_m value of 19 °C ca. and 27 °C ca., respectively.

Thermal analysis plots of MLVs made of either HOPLPC (●, panel A) or HPPLPC (♦, panel B) in 3:1 mixture with DPPC indicate that these phospholipoxide species decrease pure DPPC T_m value less than the same molar ratio of PLPC does (▲, panel A and B) , and display an effect closer to the one of 10% by mole PLPC (♦, panel C). This observation may suggest that the presence of −OOH and −OH group represents a drawback to the fluidising effect of the two double bonds of PLPC.

Another conspicuous feature of HOPLPC and HPPLPC thermal analysis plot is described by the tentatively added dashed lines (panel A, ○, HOPLPC; panel B, ◇, HPPLPC) appearing in the same figure, relative to A_{max} values of a second component of EPR spectra displaying a different thermal behaviour. Explanation of it is apparent in Fig. 5, reporting EPR spectra of MLVs made of DPPC/HPPLPC (A) and DPPC/HOPLPC (B) in 3:1 molar ratio, at room temperature. When compared to the spectrum of 3:1 DPPC/PLPC MLVs (Fig. 5C) these spectra roughly display the presence of two components (see enlargements), with different fluidity at the same temperature, as previously observed with the rough oxPLPC mixture [1], suggesting possible lateral phase separation, which will be treated in more depth in the next paragraph.

3.3. Lateral phase separation

The above described two-component spectra reported in Fig. 5 suggest lateral phase separation induced by the presence of HOPLPC

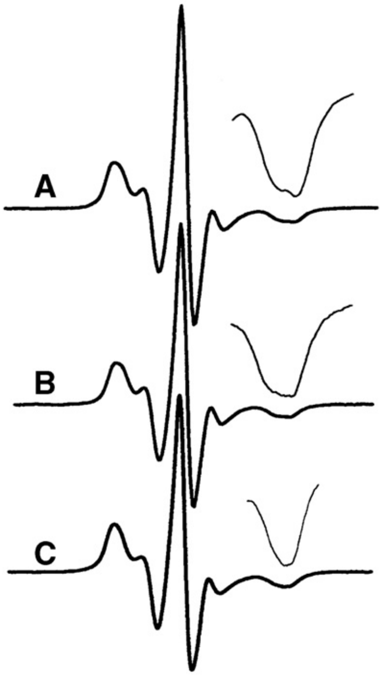

Fig. 5. EPR spectra of 5-DSPPC spin labelled MLV. (A) DPPC/HPPLPC 3:1 by mol; (B) DPPC/HOPLPC 3:1 by mol; (C) DPPC/PLPC 3:1 by mol. Spectra were recorded at 24 °C temperature. Total scan width: 12 mT. Enlargements were about ten-fold on both axes.

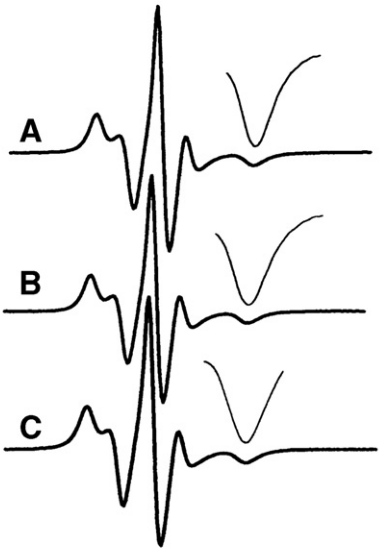

Fig. 6. EPR spectra of 5-DSPPC spin labelled MLV. (A) PLPC/HPPLPC 1:1 by mol; (B) PLPC/HOPLPC 1:1 by mol; (C) pure PLPC. Spectra were recorded at 24 °C temperature. Total scan width: 12 mT. Enlargements were about ten-fold on both axes.

and of HPPLPC in DPPC MLVs at room temperature. Two-component EPR spectra (not shown) similar to those in Fig. 5 were systematically observed in a temperature range spanning 10 °C (20–30 °C) in the case of HOPLPC and 6 °C (22–28 °C) for HPPLPC, similarly to our previous findings [1]. Interestingly, the feature was not observed in PLPC-containing MLVs, as shown in Fig. 6. In this figure, spectra of 5-DSPPC in MLVs made of either HPPLPC (A) or HOPLPC (B) in 1:1 mixture with PLPC are compared with the EPR spectrum of the same spin label in pure PLPC MLVs at room temperature (C), suggesting better miscibility of the considered phospholipoxides with unsaturated lecithin.

In a previous paper [1], dealing with the membrane properties of generically oxidized PLPC obtained by the Fenton reaction-generated ROS on PLPC, we already reported lateral phase separation. The so-obtained oxidized PLPC preparation was later demonstrated to be composed of a plethora of oxidized lecithins [2], unuseful for the characterization of the effects of defined lipoperoxides on bilayer properties. Lateral phase separation was confirmed by using 16-DSPPC spin probe, and in the present study we report similar results obtained by use of the same spin label in MLVs containing the well-defined phospholipoxides molecular species HPPLPC and HOPLPC. In fact, formation of two different-fluidity domains is clearly evident in Fig. 7, in which the EPR spectrum of 16-DSPPC in DPPC/HPPLPC 3:1 (by mol) MLVs at 20 °C is reported (A), along with the one from DPPC/HOPLPC 3:1 MLVs at 16 °C (B). The two-component nature of EPR spectra is much more readily observable than in Fig. 5, at both the high- and low-field band, closely resembling EPR spectra of 16-DSPC in membranes showing coexistence of two different fluidity domains, as those reported in Refs. [24–27]. The lower temperature range in which two components spectra are observed with the aid of this spin label, 14–24 °C for HPPLPC and 10–22 °C for HOPLPC, is possibly attributable to the higher fluidity at C-16 compared to position C-5. In the same figure, spectra C and D of 16-DSPPC spin labelled pure HPPLPC and HOPLPC MLVs, respectively, appear single-component and exhibit high motional freedom, as well as spectrum E of the same spin label in DPPC/PLPC 3:1 MLVs. On the contrary, spectrum F (16-DSPPC spin labelled pure DPPC MLVs), shows a much lower motional freedom, suggesting that the more rigid domain visible in spectra A and B could be attributed to lateral separation of oxidized species-rich PLPC domains from saturated lecithin-rich ones, possibly owing to poor miscibility with DPPC, contrarily to what observed for unsaturated PLPC (Fig. 6).

Based on well-established methods using 16-DSPC for the detection of lateral phase separation in vesicles [24–27], our data clearly indicate its occurrence in the presence of oxidized PCs. Although the EPR method we adopted was optimised to the extent of allowing quantitative determination of the different phases composition [26] and of the studied system phase diagram [27], at this stage we limit our observation to a qualitative evaluation, while being aware that the issue deserves deeper quantitative definition in a future study.

3.4. Membrane fluidity gradient

The trend of order parameter S calculated from EPR spectra of either 5-, or 12-, or 16-DSPPC in MLVs made of pure HPPLPC and of pure HOPLPC, scanned at room temperature, is reported in Fig. 8. In the same figure, S values from MLVs made of pure DPPC and of pure

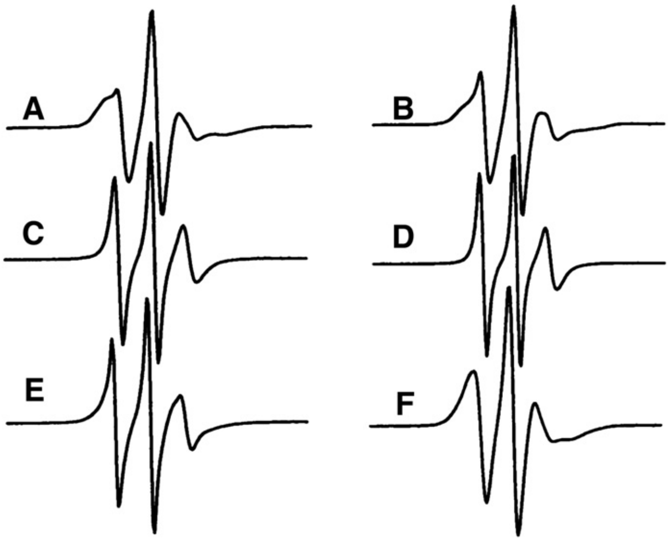

Fig. 7. EPR spectra of 16-DSPPC spin labelled MLV. (A) DPPC/HPPLPC 3:1 by mol; (B) DPPC/HOPLPC 3:1 by mol; (C) pure HPPLPC; (D) pure HOPLPC; (E) DPPC/PLPC 3:1 by mol; (F) pure DPPC. All spectra were scanned at 20 °C, apart from spectrum B (16 °C). Total scan width: 12 mT.

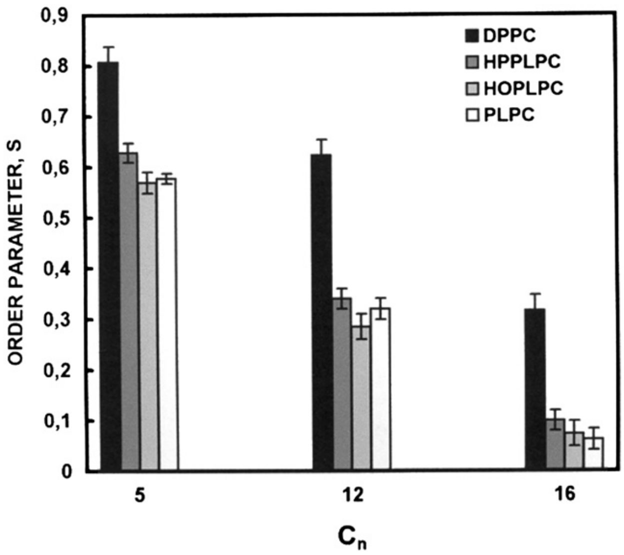

Fig. 8. Order parameter S (see Materials and Methods) value of 5-, 12-, 16-DSPPC in MLV made of pure phospholipids as indicated. Each measure's standard deviation (from 3–5 different samples) is reported on top of the bars (±SD).

PLPC were also reported for comparison. As it can be seen, inner bilayer fluidity of both CD-lecithins resembles more that of PLPC than that of DPPC at all C-positions, within the experimental error range, indicating that the unsaturation degree keeps the overall membrane fluidity gradient under control in spite of oxidative modifications, as already inferred by the previously described thermal behaviour. Nonetheless, data from HPPLPC MLVs show a slightly stronger rigidity with respect to HOPLPC, as already noted in Fig. 3. It could be argued that when dealing with −OOH and −OH containing acyl chain mobility, the possibility of hydrogen bonding should be taken into account in addition to steric hindrance.

This measurement was restricted to pure CD-PC owing to the two-component nature DPPC/CD-PC EPR spectra, impairing univocal evaluation of S value. PLPC/CD-PC mixtures were also neglected since insignificant effect was expected, due to the closeness of the S-values of the two pure components. Membrane fluidity measurements in CX-lecithins containing MLVs were considered meaningless too because of micelle formation, and will be the object of future studies provided that stable mixed MLV with defined composition will be obtained.

3.5. Membrane polarity profile

MLV bilayer polarity at position C-5, C-12 and C-16 was determined by the A_0 value, measured from EPR spectra scanned at high temperature as described in the Methods section, using the method of Marsh [30]. For reasons explained at the end of the previous paragraph, also this study was restricted to HPPLPC and HOPLPC pure species only, in comparison with 100% DPPC and PLPC MLVs. In fact, even if lateral phase separation disappeared at higher temperature allowing for a univocal A_0 determination in mixed MLVs, we preferred not to consider the resulting values descriptive of two-domain membranes observed at room temperature. Nonetheless, the high-temperature method was validated by the same author confirming the previous results by use of high-frequency EPR spectroscopy performed around room temperature [34]. Therefore, while confident of our results at this stage (for comparative purposes at least), we suggest that room-temperature polarity measurements might be performed by high-frequency EPR in order to gain information better related to natural membrane physiological conditions.

Comparison between the data reported in Fig. 9, surprisingly enough and contrarily to expectations based on the polarity and water affinity of −OH and −OOH groups, reveals no polarity difference of CD-lecithins with respect to normal lecithins at any C-position. In fact, A_0 values of all lecithins range around 1.485 mT at C-5 and are comprised in the experimental error interval. A_0 values drop to about 1.435 mT at both C-12 and C-16, without any significant difference between the various samples. As a validation of the method, we note that these values are very close to the ones reported in Ref. [30] for DPPC MLVs, and to those for DMPC (dimyrisoyl-PC) MLVs obtained by high-frequency EPR [34].

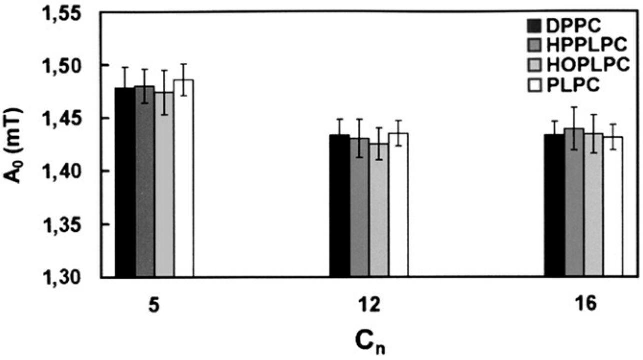

Fig. 9. EPR estimation of the bilayer polarity at various acyl positions in MLV made of pure phospholipids, as indicated. A_0 value measurement is described in the text. Each measure's standard deviation (from 3–5 different samples) is reported on top of the bars (±SD).

Phospholipid bilayer polarity profile is attributed to penetration of water molecules into the hydrophobic core, and the phenomenon was found to be enhanced by the presence of bulky cholesterol molecules [30], probably due to steric effects. In our case, hydroxyl groups' affinity to water was expected to drive water molecules into the bilayer, but this idea is invalidated by unaltered A_0 values. Displacement of polar groups to the polar region of the bilayer was also expectable as proposed for β-(ω-carboxyacyl)-PC [8,32,33], accompanied by distortion of acyl chains and bilayer disordering, but this was not observed in CD-PC-containing oriented planar bilayers [2]. Therefore, we retain that polarity differences might lay below the sensibility threshold of our EPR measurements, further suggesting that the issue should be addressed with a different method, such as high-frequency EPR [34].

As a noteworthy observation, we report high reactivity of HPPLPC towards the nitroxyl radical at high temperature, causing progressive EPR signal intensity decrease starting around 80 °C, and complete disappearance towards 90 °C. A_0 measurements on HPPLPC MLVs were only partially hindered by the observed EPR signal decrease. In fact, 80 °C was just the upper limit of the useful temperature range, and only the signal intensity (band-height) was slightly decreased while the hyperfine splitting (determining EPR band spacing useful for the measurement) remained unchanged, as shown by A_0 value remaining within the error range of values measured at lower temperatures.

The phenomenon, observed with all spin labels, was quite fast with 12-DSPPC and slowest with 5-DSPPC, while DPPC apparently slowed it down, depending upon its molar percentage in MLVs. On the contrary, PLPC showed a lower protective effect.

Though the study of the phenomenon was out of our aim, the reaction, possibly an over-oxidation effect [35] of the nitroxide moiety by the −OOH group, is novel and deserves acknowledgement as well as a study deeper than our casual, yet invariable, observation.

From our study, in general, long chain products, HPPLPC and HOPLPC, both pure and mixed with normal DPPC and PLPC (Fig. 1), appear able to form lipid vesicles. Only pure HPPLPC is seen to form slightly more rigid membrane, and shows a modest rigidification of PLPC mixed vesicles, in comparison with HOPLPC (Figs. 3 and 8), while in our hands no influence on the bilayer's polarity by either species was detected by the described method, within the experimental error range (Fig. 9). Both CD-PCs display major structural alterations in mixture with DPPC. In fact, they are able to affect MLV transition temperature and to induce lateral phase separation in 75% (by mol) DPPC-containing mixtures revealing poor miscibility with saturated lecithin (Figs. 4–7). PLPC appears to mix up better with both CD-lecithins, suggesting better resistance of PUFA-rich membranes to the consequences of lipoperoxidation. From this point of view, unsaturated PLPC seems to be better compatible with CD-PCs, and more apt to counteract lipoperoxidation-linked structural modifications. We tentatively advance the idea that the well-known beneficial effect of PUFA (ω−6 and of ω−3) at the metabolic level, might be implemented by properties similar to those observed for PLPC at the membrane level.

Between extremely oxidized lecithins, some of them, such as β-glutaroyl-PC, β-suberoyl-PC, β-succinyl-PC were already studied as standard molecules for MS identification of oxidized PL species [16]. As a new species, we previously synthesized β-azelaoyl-PC [1] (though commercially available to date) by the same method used for β-suberoyl-PC [16] since it represents the extremely oxidized form of β-linoleoyl-PC. GlPC was also studied in MLVs in a context different than lipoperoxidation, dealing with the molecular organization of PC and structural changes in liposomes [13]. In those studies, micelle-forming ability of GlPC was aknowledged and its disordering effect in vesicles was shown by EPR spectroscopy, as we also already observed in supported planar lipid bilayers [8]. Attribution of this disordering to −COOH groups moving towards the polar sheet of the bilayer was also made [33], as we later also proposed [8] and confirmed by monolayer studies [32].

In the context of the present study, micelle forming ability of CX-PC concerns us more (Fig. 2). Based on Sepharose 4B gel-chromatography of pure AzPC we suggest that micelle-forming ability might be generalized to all CX-PCs, besides GlPC. In addition, similarity of this behaviour with that of lyso-PC (Fig. 2), reveals the harm brought about after heavy membrane lipoperoxidation. In fact, micelle formation is still partially observed in GlPC/DPPC mixture, suggesting the possibility of detergent-like membrane disruption. At the same time, lesser micelle formation in PLPC/GlPC mixture is again suggestive of a possible counteracting effect of unsaturated PC. The issue is currently under deeper study, in order to verify micelle formation of more CX-PCs species, and to obtain stable MLV with known composition useful for the determination of membrane type/properties in the presence of β-carboxyacyl-lecithins. An early study by other authors pointed out variations of T_m by GlPC, attributed to micelle formation [36]. Our aim is to study such variations in well-formed membrane systems, as we did with HPPLPC and HOPLPC.

Moreover, proving the possible role of different PC molecular species in controlling the detergent-like effect of CX-PCs on vesicle membranes would be an additional goal, possibly adding valuable information about membrane stabilization/protection properties of PUFA-containing phospholipids against the damage following ROS attack.

4. Concluding remarks

The present study is far from answering the many questions raised by the mere existence of oxidized phospholipids in membranes. Evidences from up-to-date lipidomics and neurolipidomics studies increasingly focus the involvement of oxidized lipids in pathology (if not their direct responsibility), such as in Alzheimer's, atherosclerosis, type 2 diabetes and cancer, and urge addressing the issue.

Our present results are just a first step in the widely unknown field of the chemistry and physics of lipoperoxidized membranes, using models pertinent to oxidatively stressed natural membranes after ROS attack. Model membranes are also far from simulating natural oxidatively diseased membranes, yet they can be useful to mimic membrane domains in which ROS attack has occurred. Phospholipoxides-containing model membranes are also useful test-benches in which the effects of these molecules can be proved and studied, as we already stated [2].

The present results indicate that the effects exerted by phospholipoxides on the bilayer structure and properties may depend upon the degree of oxidative modification, in the sense that early oxidation products (CD species) can still be compatible with the membrane structure – especially in the presence of unsaturated acyl chains – even though altering bilayer properties, such T_m, fluidity to a limited extent, and lateral phospholipid assemblage. Terminal oxidation products (CX species) show marked incompatibility with the bilayer architecture – especially in the presence of saturated acyl chains - and tend to disrupt the membrane as they accumulate under prolonged oxidative stress conditions.

Whether these conditions actually exist or not in oxidatively injured natural membranes is still a matter of investigation. In fact, indications already exist of oxidized phospholipid repair mechanisms playing a defensive role in the living cell, suggesting that studies of lipoperoxidation in model membranes by use of peroxidized phospholipid model molecules should be complemented with the study of phospholipoxides metabolism in living systems.

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