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ORIGINAL PAPER

Oxidative Stability of Liposomes Composed of Docosahexaenoic Acid-Containing Phospholipids

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Abstract Oxidative stability of liposomes made of (Docosahexaenoic acid) DHA-containing phosphatidylcholine (PC) was examined during preparation and storage. After preparation of the liposomes, the concentration of primary (conjugated dienes) and secondary oxidation products (Thiobarbituric acid-reactive substances, TBARS) were significantly higher compared to the initial value. During cold storage, formation of conjugated dienes and TBARS remained more or less constant in large unilamellar vesicles (LUV), whereas in mulitilamellar vesicles (MLV) they were seen to increase over a period of 21 days. Evaporation of solvent traces from a lipid film should preferably be done under nitrogen as vacuum evaporation was found to increase oxidation of the phospholipid.

Keywords Liposomes · Phosphatidylcholine · DHA · Oxidation stability

Introduction

Phospholipids with a defined molecular structure provide excellent opportunities within liposome technology for drug delivery and the construction of anticancer prodrugs [1, 2]. The fatty acid profile and head group distribution of the phospholipid can be varied in numerous ways making it possible to optimize the biophysical membrane properties and thereby obtain liposomes that are stable with respect to drug encapsulation and circulation.

Liposomal drug delivery systems have been developed in an attempt to improve the pharmacokinetics and biodistribution of chemotherapeutic agents after systemic administration, as many of these compounds often give rise to inadequate delivery of therapeutic concentrations to the cancer tissue at doses causing severe toxic effects on normal organs [2]. To fulfill their potential as targeted microcarriers the liposomes should be tailored to accumulate and release the encapsulated drug in the tumor tissue. Secretory phospholipase A_2 ($sPLA_2$) is highly over-expressed in inflammatory and cancer tissue [3], and is considered to be an ideal tumor specific trigger enzyme that can be used to disrupt liposomes once they accumulate in cancer tissue due to the enhanced permeability and retention effect [1, 4]. $sPLA_2$ catalyzes the hydrolysis of ester-linkage in the *sn*-2 position of glycerophospholipids, producing free fatty acids and lysophospholipids. The hydrolysis products of diacylglycerophospholipids can synergistically act as locally generated permeability enhancers that increase diffusion of released drugs across the cellular membrane of the target cancer cell [5]. $sPLA_2$ trigger mechanism may furthermore prove useful in development of lipid based prodrugs, that selectively target the tumor and undergo a site-specific triggered activation and release. Attached to the *sn*-2 position of diacylglycerophospholipid could for example be docosahexaenoic acid (DHA), which has been claimed to be an effective anticancer agent [6, 7].

The long term stability or shelf-life of a drug product containing lipids can be dramatically affected by the lipid

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species used in the formulation. While saturated lipids offer the greatest stability in terms of oxidation, they also have much higher transition temperatures and thus present other difficulties in formulation. However, the more unsaturated a compound, the easier the product is oxidized, and thus the shorter the shelf life of the product. DHA is an all-*cis* polyunsaturated fatty acid with multiple double bonds, and is therefore highly prone to oxidation.

Determination of lipid oxidation poses an analytical problem as there is no single product to measure [8]. Many intermediates and products are produced during lipid oxidation reactions [9]. In the initial step, an abstraction of a hydrogen atom from the fatty acid chain occurs. The lipid radical form (R) rapidly reacts with oxygen to form a peroxy radical via a free radical chain reaction. The peroxy radical (ROO) can gain a hydrogen atom to form a lipid hydroperoxide (ROOH) (primary oxidation product). Hydroperoxides formed may be decomposed by homolytic β-scission to alkoxyl radicals. Subsequently, the alkoxyl radical may undergo further homolytic cleavage whereby secondary volatile products such as hydrocarbon, furans, alcohols, aldehydes, and ketones are formed. Additional processes can occur, such as formation of endoperoxides, and rearrangement of double bonds in the fatty acid molecule. Different analytical procedures have been developed to measure classes of compounds formed during phospholipid oxidation [8]. Some oxidation products (e.g., conjugated dienes) form early in the oxidation reaction and breakdown to immeasurable levels in later stage. Other oxidation products (e.g., volatile aldehydes) form relatively late during oxidation reaction and can only be detected after high degree of oxidation.

The objective of this work was to study the influence of liposome preparation and storage on the oxidative degradation of phospholipids with DHA attached at the *sn*-2 position. Oxidation of phospholipids has remarkable influences on structure, and physical properties of liposomes, and should be limited during preparation steps. Even small quantities of lipid oxidation would give rise to physically unstable liposome formulations. Phospholipid oxidation was followed by measuring primary (conjugated dienes) and secondary (thiobarbituric acid-reactive substances, TBARS) products of oxidation [8].

Materials and Methods

Preparation of Liposomes

1-Palmitoyl-2-DHA-PC (16:0, 22:6-PC) (25 mg/mL chloroform) was obtained from Avanti Polar Lipids Inc., Alabaster, AL, USA. Fifty mg of PC in chloroform was transferred into two tubes with a tight screw cap. The solvent was evaporated under a stream of nitrogen until the weight was constant. To eliminate traces of solvent from the lipid film, two different approaches were tested. One of the tubes was maintained under nitrogen for 30 min after drying to constant weight, whereas the other tube was placed under vacuum for 6 h. From experience, even with extensive nitrogen flushing there are often still traces of solvent in the lipid film as determined by NMR (data not shown). To ensure complete removal of trace solvent, vacuum evaporation for several hours is usually required. MLV and LUV were subsequently prepared from the dried lipid film. Mulitilamellar vesicles (MLV) were prepared by dispersing the lipid film in a nitrogen-flushed Hepes buffer solution (10 mM Hepes, 150 mM KCl, 30 μM $CaCl_2$, 10 μM EDTA, pH7.5). The lipid suspensions (5 mM PC) were kept at 30 °C for 1 h in order to ensure complete hydration. During this period, the lipid suspensions were vortex every 15 min. Large unilamellar vesicles (LUV) were subsequently prepared by extrusion of MLV through two stacked 100 nm pore size polycarbonate filters 10 times [10]. MLV and LUV were transferred to 10 mL plastic vials with screw caps, and kept in darkness at 4 °C. Aliquots of liposomes were taken on the day of preparation and after 1, 3, 7, 14, and 21 days of storage for subsequent analysis. Vials were flushed with nitrogen for 30 s immediately after sampling.

Size Measurements of Liposomes

Liposome size was determined immediately after preparation by dynamic light scattering using a BI-200SM goniometer and a laser light scattering system from Brookhaven Instruments, Austin, TX, USA. Scattering data was collected at 90°, using a 35 mW HeNe laser at 632.8 nm. Two hundred channels were used and the diffusion coefficients, average size and size distribution were obtained using both cummulant analysis and CONTIN. Both methods gave comparable average sizes for the LUV liposomes.

Estimation of Lamellarity

A fluorescence quenching assay was used to estimate the lamellarity of the liposomes [11]. A suspension of liposomes containing 0.5 mol% of 1,2-dipalmitoyl-phosphatidylethanolamine with the fluorescent probe 7-nitrobenz-2-oxa-1,3-diazol-4-yl covalently linked to the head group (NBD-PE) (Fluka, Buchs, Switzerland) was prepared. The NBD probe molecules were excited at 465 nm and the peak of the emission wavelength was observed at 535 nm using a SLM DMX-1100 spectrofluorometer. A 2-mL aliquot of 0.1 mM freshly prepared lipid suspension was placed in a thermostated cuvette (15 °C), and equilibrated for 5 min

prior to the addition of irreversible dithionite quencher to the outer aqueous phase. Rapid sample mixing was attained in the cuvettes with a magnetic stir bar. The time dependent decay of the fluorescence intensity was monitored after the addition of 30 μL freshly prepared sodium dithionite (1 M $Na_2S_2O_4$ in 1 M Trizma buffer stock solution). Subsequent addition of 10 μl 10% Triton X-100 to the liposome preparations resulted in complete quenching of NBD-PE fluorescence within seconds.

Oxidation Stability

Oxidation of phospholipids was monitored by analysis of conjugated dienes and cyclic peroxides according to methods described by New [8]. Conjugated dienes were measured directly by diluting liposomes in absolute ethanol. The absorption spectrum (190 nm < λ < 350 nm) of the diluted liposome suspension was recorded with a Lambda2 UV/VIS Spectrophotometer (Perkin-Elmer & Co, Überlingen, Germany) using 1-cm quartz cuvettes. Conjugated dienes were expressed as absorbance at 233 nm per mg/mL of phosphatidylcholine (A233 per mg/mL PC). Ethanol was used as the blank. Cyclic peroxides (or endoperoxides) were detected by reaction of their breakdown product at elevated temperatures (malondialdehyde) with thiobarbituric acid (TBA) giving a pink chromophore which absorb at 532 nm. TBA reactivity was determined from the standard curve of 1,1,3,3-tetraethoxypropane (TEP). Aliquots of diluted liposome suspension or TEP solution were transferred into test tubes with screw caps and mixed with 0.1 mL ferric chloride solution (2.7 mg/mL), 0.1 mL butylated hydroxytoluene solution (2.2 mg/mL), 1.5 mL 0.2 M glycine buffer (pH 3.6), and 1.5 mL TBA reagent (5 mg/mL TBA and 3 mg/mL sodium dodecyl sulphate). Tubes were capped and placed in boiling water for 15 min. Tubes were then cooled to room temperature, and to each tube 1 mL acetic acid and 2 mL chloroform were added. Samples were mixed well and subsequently centrifuged at 2,879×*g* for 2 min to separate the two phases. The upper phase was taken off and absorbance at 532 nm was measured with a Lambda2 UV/VIS Spectrophotometer (Perkin-Elmer & Co). TBARS were expressed as μmol of TEP equivalent per μmol PC. Samples were taken continually during storage. Double determinations were performed for each sample.

Statistical Analysis

Significance of the results was established at $P \leq 0.05$. Differences in the results were determined by either one- or two-way analysis of variance, where 95% confidence intervals were calculated from pooled standard deviations (Microsoft Office Excel 2003, Microsoft Corporation, Redmond, WA).

Results and Discussion

Size Measurements of Liposomes

It was difficult to obtain consistent DLS results for the MLV liposomes due to polydispersity and the large size distribution of these particles. The DLS showed that the MLVs prepared from both vacuum dried lipid film and nitrogen flushed lipid film were larger than 1 μm with a polydispersity >0.5. The average size of LUV prepared from vacuum dried lipid film was 126 nm (size range, 118–141 nm), and average size of LUV from nitrogen flushed lipid film was 123 nm (size range, 111–138 nm). The size distribution of the LUV formulations was thus in a similar range.

Lamellarity of Liposomes

NBD probe was quenched to a larger extent in LUV in comparison with MLV as expected since a larger surface area would be exposed to the dithionite quencher when added to outer aqueous phase (Fig. 1). Liposomes prepared from nitrogen flushed lipid film resulted in slightly higher degree of quenching in comparison to liposomes prepared from vacuum dried lipid film. Fluorescence intensities for LUVs were similar to those reported by Armstrong et al. [11] using the fluorescence quenching assay for 1-stearyl-2-DHA-PC (18:0, 22:6-PC) LUVs under similar conditions.

Lipid Oxidation in Freshly Prepared Liposomes

The UV absorption spectra of PC immediately after opening the vial did not present any peak at 233 nm, and did therefore not contain appreciable quantities of conjugated dienes (Fig. 2). Figure 3 shows the initial concentration of conjugated dienes and TBARS in the PC, and in the freshly prepared liposomes (MLV and LUV).

After preparation of the liposomes, the concentration of conjugated dienes and TBARS were significantly higher compared to the initial value. Especially when vacuum evaporation was used for the removal of solvent traces from the lipid film, the concentration of conjugated dienes and TBARS significantly increased, as compared to lipid film dried under nitrogen ($P < 0.001$). Flushing with nitrogen for solvent evaporation seems to minimize the oxidation, however further work needs to be done to determine if the solvent traces are below an acceptable threshold limit.

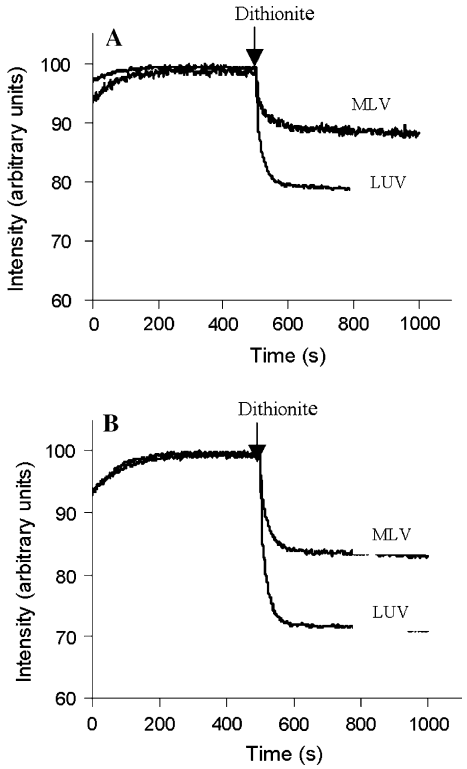

Fig. 1 The time-dependence of fluorescence intensity of 1-palmitoyl-2-DHA-PC/NBD-PE MLV and LUV at 15ºC. *Arrow* indicates the addition of dithionite. **a** Liposomes prepared from vacuum evaporated lipid film, **b** liposomes prepared from lipid film dried by nitrogen flushing. Abbreviations: DHA, Dexahexaenoic acid; NBD-PE, 7-nitrobenz-2-oxa-1,3-diazol-4-yl labeled phoshatidylethanolamine; PC phosphatidylcholine

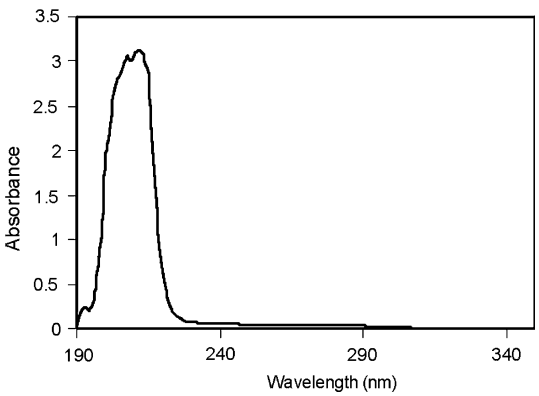

Fig. 2 UV absorption spectra of 1-palmitoyl-2-DHA-PC directly after opening vial. For abbreviations see Fig. 1

Lipid Oxidation in Liposomes During Cold Storage

Significant differences in the formation of conjugated dienes in LUV and MLV were observed during storage in dark at 4 °C (Figs. 4, 5). Concentration of conjugated dienes remained more or less constant during storage in the

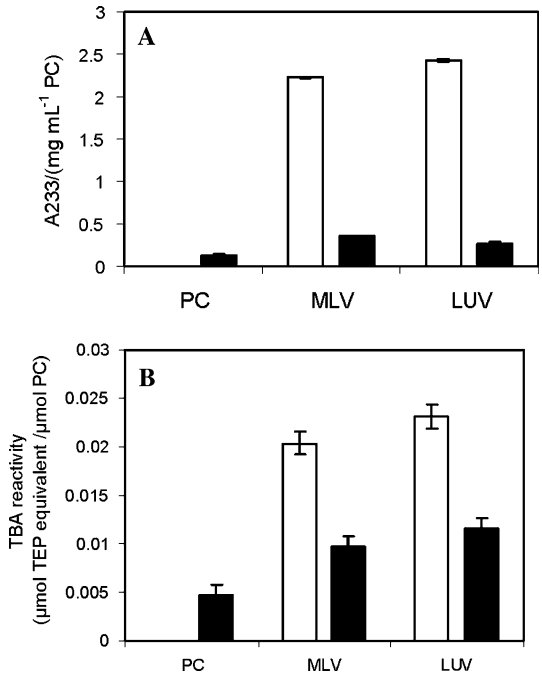

Fig. 3 Conjugated dienes (**a**) and TBARS (**b**) in 1-palmitoyl-2-DHA-PC directly after opening vial and in freshly prepared MLV and LUV. Results correspond to different procedures for removing solvent traces from lipid film (*open square*: vacuum drying; *filled square*: nitrogen flushing). *Bars* represent 95% confidence interval (n=2). Abbreviations: LUV, large unilamellar vesicles; MLV, mulitilamellar vesicles; TBARS, thiobarbituric acid-reactive substances. For other abbreviations see Fig. 1

form of LUV, whereas in form of MLV there was seen an increase. This observation was made for liposomes prepared with different methods for solvent evaporation of lipid film. Liposomes prepared from vacuum dried lipid film had significant higher initial concentration of conjugated dienes; however this apparently did not induce further oxidation in LUV. However formation of conjugated dienes was seen to increase more rapidly in MLV prepared from vacuum dried lipid film as compared to MLV prepared from nitrogen flushed lipid film. MLV prepared from nitrogen flushed lipid film showed hardly any increase in conjugated dienes for the first 7 days. Higher initial concentration of conjugated dienes in freshly prepared MLV thus seems to induce further oxidation.

Similar observations made for the formation of conjugated dienes in MLV and LUV were able to be made for the formation of TBARS. Concentration of TBARS increased more in MLV than LUV during storage (Fig. 6). Hardly any changes were observed in the TBARS concentration in LUV, whereas in MLV there was seen an increase in TBARS concentration over the 21 days of storage.

Peroxidation may be prevented by adding antioxidants to the buffer solution used for the preparation of liposomes.

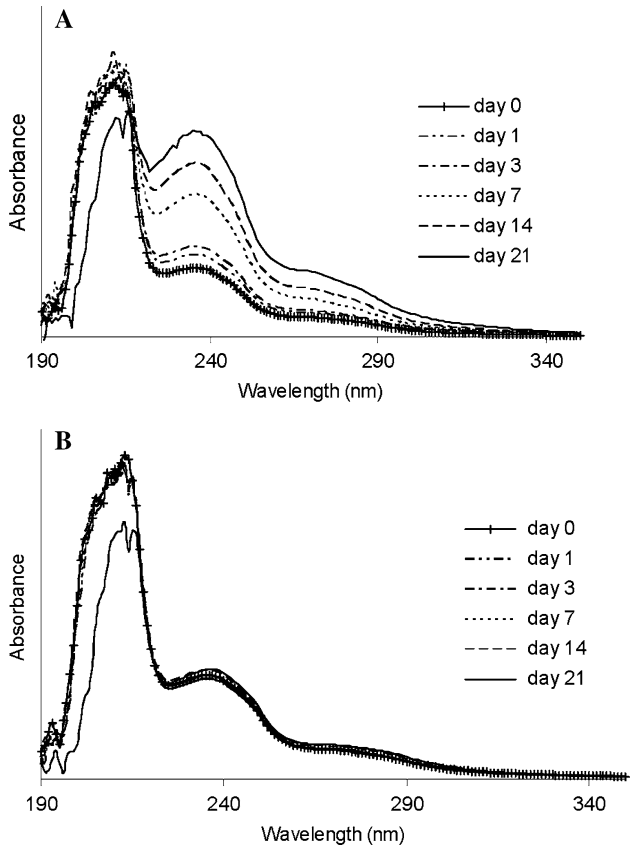

Fig. 4 UV spectra of 1-palmitoyl-2-DHA-PC liposomes (prepared from vacuum dried lipid film) during cold storage. Conjugated dienes is indicated by appearance of peak at 233 nm. **a** MLV **b** LUV. For abbreviations see Figs.1 and 3

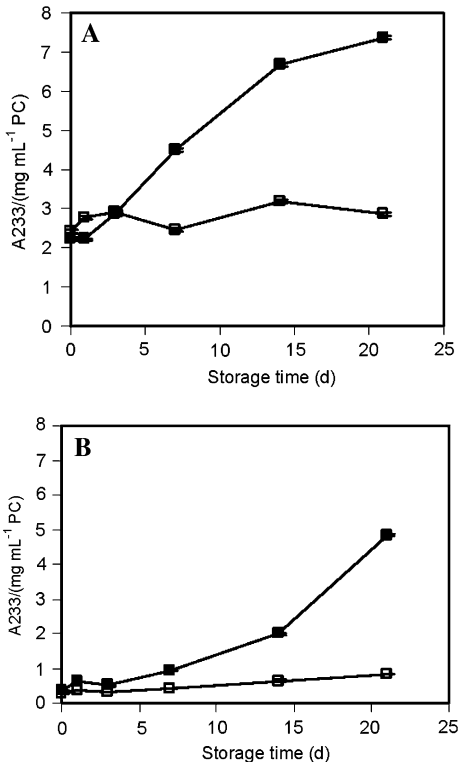

Fig. 5 Conjugated dienes in MLV (*filled square*) and LUV (*open square*) made of 1-palmitoyl-2-DHA-PC during storage in the dark at 4°C **a** Liposomes prepared from vacuum evaporated lipid film, **b** liposomes prepared from lipid film dried by nitrogen flushing. *Bars* represent 95% confidence interval (n=2). For abbreviations see Figs. 1 and 3

Hepes buffer has been reported to scavenge hydroxyl radicals [12]. The Hepes buffer used in the current study also contained EDTA, a metal chelator, which converts iron and copper ions into insoluble complexes or sterically hinder formation of the complexes between metals and lipid hydroperoxides [9]. Phospholipids themselves have been reported to be efficient antioxidants as well [13]. Even with these different antioxidants present in the buffer solution, oxidative decomposition of the DHA alkyl chain was not prevented.

Lipids in bulk are more prone to oxidation than lipids incubated in solvent or aqueous solutions as a larger surface area is exposed to the air [14]. Difference in the oxidative stability of PC in bulk and organic solvent system would be due to the different rate of hydrogen abstraction by free radicals from intermolecular or intramolecular alkyl groups and not due to the phospholipid confirmation, because PC takes no packed confirmation in such systems [15]. The removal of solvent prior to hydration during liposome preparation is thus probably one of the most critical steps in terms of peroxidation. The oxidative stability of PC in bulk and organic solvent was reported to decrease with increasing degrees of unsaturation; however had little effect on the stability of PC in aqueous solution as MLV [15]. Oxidative stability of PC in liposomes seems to be affected not only by degree of unsaturation, but also the conformation of fatty acid components in PC bilayer. No correlation could be made for mean vesicle size diameter of MLV and degree of oxidation of polyunsaturated fatty acids in PC [15]; however, it was speculated to be caused by the packing degree of the PC bilayer. In aqueous solutions, DHA form a tighter intermolecular packing conformation of each bilayer, which makes it more difficult for free radicals and/or oxygen to attack [15]. Lyberg et al. [14] reported that formation of hydroperoxides in DHA was almost completely prevented by incorporating DHA into either the *sn*-1 or *sn*-2 position of PC in bulk and in chloroform solution. In contrast, PC containing DHA on both positions should be avoided, since it is highly oxidized. Similar observations have also been made by Araseki et al. [15], and were explained by the idea that an intramolecular free radical chain reaction between PUFA of esters occurred more rapidly than an intermolecular chain reaction.

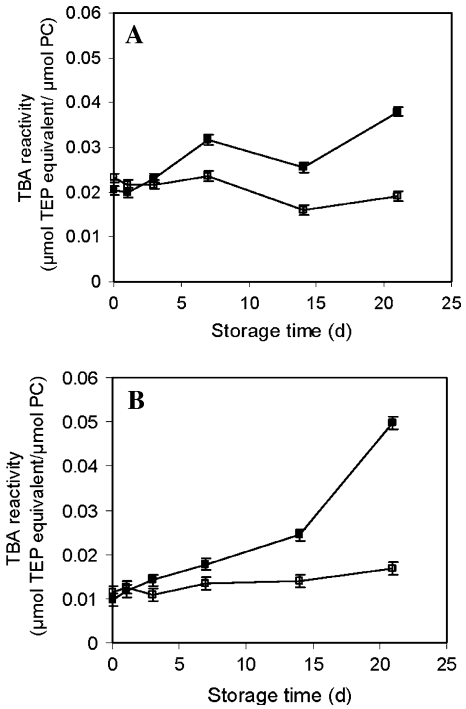

Fig. 6 TBARS in MLV (*filled square*) and LUV (*open square*) made of 1-palmitoyl-2-DHA-PC during storage in the dark at 4°C **a** liposomes prepared from vacuum evaporated lipid film, **b** liposomes prepared from lipid film dried by nitrogen flushing. *Bars* represent 95% confidence interval (n=2). For abbreviations see Figs. 1 and 3

Koga et al. [16] compared the rate of hydroperoxide formation in MLV and LUV from egg yolk PC by water-soluble and lipid-soluble radical generators. When peroxidation was induced by a water-soluble radical generator, the rate of hydroperoxides formation in LUV was larger than in MLV. Aqueous radicals seemed hardly able to penetrate the outer bilayers and reach the inner bilayers in MLV. Due to the larger surface area of LUV, it was considered that they were more likely to be attacked by aqueous radical generators, thus resulting in larger amounts of hydroperoxides during storage. An opposite trend was observed when a lipid-soluble radical generator was used [16]. LUV was more resistant to the formation of hydroperoxides than its MLV, when initiating radicals were generated within the membranes. These radicals can react not only within one membrane layer but also with another neighboring membrane layer in MLV.

The results obtained in this study clearly demonstrate that packing conformation of DHA-containing phospholipid bilayers and lamellarities have a significant effect on the oxidative stability. As LUV, in contrast to MLV, was hardly oxidized in the liposome formulation prepared in the current study, it seems that oxidation of PC is generated by radicals within membranes, and not caused by radicals present in the aqueous environment. Solvent evaporation of the lipid film should preferably be done under nitrogen as vacuum evaporation causes oxidation of PC. With an increase in primary oxidation products (conjugated dienes), there was also seen an increase in secondary oxidation products (TBARS). Information from this study can help in the further pursuit of using polyunsaturated fatty acids in liposome formulations. For future work, the issue of solvent evaporation needs to be examined more closely. Possibilities of using antioxidants for the prevention of oxidation during preparation and storage could be examined if liposomes are desired in the MLV form.

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