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Soy PC liposomes as CLA carriers for food applications: Preparation and physicochemical characterization

María A. Vélez [a, *], María C. Perotti [a], Paula Zanel [a], Erica R. Hynes [a], Ana M. Gennaro [b]

[a] Instituto de Lactología Industrial, Universidad Nacional del Litoral / CONICET, Facultad de Ingeniería Química, S3000AOM, Santa Fe, Argentina
[b] IFIS Litoral, Universidad Nacional del Litoral / CONICET, y Departamento de Física, Facultad de Bioquímica y Ciencias Biológicas, S3000AOM, Santa Fe, Argentina

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ABSTRACT

Soy phosphatidylcholine (PC) liposomes added with conjugated linoleic acid isomers, (CLA, 9c, 11t, and 10t, 12c) at two PC/CLA ratios, 2:0.4 and 2:1, were prepared by ethanolic injection, and followed during 30 days of storage. These systems were investigated in encapsulation efficiency and fatty acid composition by gas chromatography, size by dynamic light scattering, morphology by TEM images, and membrane fluidity by electron paramagnetic resonance (EPR). Both type of formulations showed highly significant stability and protective effect on CLA isomers with encapsulation efficiencies over 80% during storage. Liposome sizes increased at increasing CLA content. TEM showed evidence of oligolamellar vesicles formation. Regarding membrane fluidity, two behaviors were distinguished in the bilayer: CLA at both formulations disordered the outer membrane zone increasing its fluidity, but the formulation with higher CLA content caused a decreased fluidity near the center of the membrane. The method is safe and easily scalable, and formulations are suitable for future applications in food industry.

1. Introduction

Functional foods are those that generate a health benefit to the consumer beyond their basic nutritional contribution (Korhonen and Pihlanto, 2007; Korhonen, 2009; Park and Oh, 2010).

Many of them contain components known as bioactive: chemical compounds that may be naturally present, formed or added during food processing, and exert specific biochemical/physiological functions when consumed by humans. Conjugated linoleic acid (CLA) is a bioactive compound that describes a group of positional and geometric isomers of Linoleic Acid (LA, C18:2 9c12c). Mainly, their major isomers 9c,11t and 10t,12c have beneficial effects, which include the reduction of body fat content and mass muscle increment, stimulation of the immune system, reduction of plasma cholesterol, inhibition of carcinogenesis and possibly antioxidant activity (Belury, 2002; Hur et al., 2007; Kim et al., 2016).

CLA usage in food is limited because it is susceptible to spoilage, especially oxidation, leading to the loss of bioactivity and the appearance of undesired compounds. One approach to achieve foods enriched in this bioactive compound without the defects associated with its deterioration would be the addition of CLA protected by encapsulation. In this sense, liposomes have appeared as an innovative encapsulation technology. These structures are phospholipid bilayers enclosing water that favor the solubility and bioavailability of several hydrophobic or hydrophilic compounds. Biocompatibility and biodegradability make liposomes a suitable delivery system to be utilized not only in food industry but also in a variety of areas such as drug delivery, cosmetic formulations and diagnostic agents (Andhale et al., 2016; Laouini et al., 2012; Mozafari et al., 2008).

In the literature, several methods were reported for liposome preparation (Maherani et al., 2011). In particular, ethanol injection technique offers many advantages as it is easily scalable: it is simple, it does not include the use of hazardous solvents, and small liposomes are obtained without excessive technical requirements (Gharib et al., 2016; Jahn et al., 2004; Wagner et al., 2002).

The adequacy of a liposome formulation to be used as a carrier system depends on the physicochemical properties of their membranes, on the nature of their components, on their size, surface charge, and lipid organization (Bozzuto and Molinari, 2015). Indeed, liposomes are very susceptible to the type and

* Corresponding author.
E-mail addresses: mvelez@fiq.unl.edu.ar (M.A. Vélez), cperotti@fiq.unl.edu.ar (M.C. Perotti), zanelpaula@gmail.com (P. Zanel), ehynes@fiq.unl.edu.ar (E.R. Hynes), ana.gennaro@santafe-conicet.gov.ar (A.M. Gennaro).

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concentration of phospholipids, nature of the encapsulated compound, method of preparation, presence of cholesterol, ionic lipids, etc.(Sekhon, 2010). Moreover, their physical stability is an important parameter for applications, as is related with the capability of maintaining the size distribution and the encapsulation efficiency of the compound of interest (Torchilin and Weissig, 2003).

The effect of the large number of variables above mentioned on the characteristics of the vesicles and the possibility of designing structures according to the desired application is an emerging research field that has not yet been fully explored. Therefore, the aim of the present work was to study two liposomal formulations in which the proportion of phospholipid: CLA is varied, with a view to potential applications in functional foods. For this, liposomes were prepared by ethanol injection technique and characterized in size, efficiency of incorporation of CLA, and membrane fluidity, at 3 and 30 days of cold storage.

2. Materials and methods

2.1. Liposome preparation

Liposomes were prepared according to the ethanolic injection technique (Wagner et al., 2006). For this purpose, PC (Phospholipon 90 G, Lipoid, Switzerland, 97.2% phosphatidycholine) and CLA (conjugated linoleic acid isomers 9*c*, 11*t* and 10*t*, 12*c*, Tonalin BASF, 80% CLA, Germany) were used. Two formulations (4 mM total PC + CLA concentration) with different proportions PC: CLA were assayed: formulation A (2:0.4) and formulation B (2:1). PC and CLA were dissolved in ethanol in the desired proportions, and the ethanolic solution was injected into distilled water (0.1 mL/min at 37 °C), up to a final ethanol/water volume ratio of 0.1. In addition, controls without CLA (empty liposomes) were included for each formulation (Controls A and B: with 3.3 and 2.7 mM PC concentration, respectively). Three replicates were performed for each formulation. Liposomal suspensions were stored at 4 °C and analyzed at 3 and 30 days of storage.

2.2. Size distribution

Average particle size and size distribution (polydispersion index, PDI) of liposome preparations were measured by dynamic light scattering (DLS, Zeta Sizer Nano ZS90, Malvern Instruments) at 25 °C and 90° scattering angle. The analysis was performed in triplicate. Results were given as Z-average size, which is the mean value of the hydrodynamic diameter, and polydispersity index (PDI), which is a measure of the width of the particle size distribution.

2.3. TEM analysis

A drop of each liposome sample was placed on a formvar coated copper TEM grid. The samples were allowed to settle to the grid surface for 60 s, and excess liquid wicked away with a filter paper. The grids were then stained with a drop of aqueous 2% uranyl acetate for 30 s after which the excess stain was wicked away. The samples were observed with a JEOL-JEM 1200 EX II TEM (Laboratorio Integral de Microscopia, CICVyA, INTA) at 80 KV.

2.4. Encapsulation efficiency and fatty acid composition

The percentage of CLA incorporated into the liposomes was measured after free CLA had been separated from the liposomes by centrifugation. Approximately 2 mL of CLA liposomes were centrifuged (using Amicon Ultra filter units, 10 KDa molecular weight cutoff) at 4000×*g* for 45 min. CLA content was determined by gas chromatography (GC) analysis, both in the suspension (3 days, C_{total}), and in the retained fraction (3 and 30 days, $C_{retained}$). The procedure was as follows. Fatty acids (FA) were in situ methylated according to the Park and Goins (1994) method with some modifications. For that, 100 μl of the retained fraction, or 500 μl of the formulations were transferred into a test tube. For quantification, C17:0 was used as internal standard at a dose of 0.3 mg (Sigma-Aldrich, St. Louis, MO), and calibration curves were made for each fatty acid. Then, 200 μl of methylene chloride were added and agitated. Two mL of 14% boron trifluoride in methanol were added, and tubes were heated at 50 °C for 30 min in a water bath, with agitation. Then, tubes were cooled to 25 °C, and 1 mL of saturated NaCl solution and 1 mL of hexane were added. After that, tubes were shaken in vortex for about 1 min and left at 4 °C in refrigerator for 15 min. The aliquots were dried with anhydrous sodium sulfate; the top layer was transferred into a vial, and analyzed by GC. For that, a Perkin Elmer model GC-9000 series gas chromatograph (Perkin Elmer Corp., Waltham, MA) equipped with a flame ionization detector (FID) and with a split/splitless injector was used. FFA methyl esters were separated on a fused silica capillary column (60 m × 0.25 mm; HP-INNOWax, Agilent J&W, USA) coated with a bonded polyethylene glycol stationary phase (0.25 μm layer thickness); carrier gas H2 flow at 2 mL/min; 1 μL injection (splitless); injector and detector temperatures at 250 and 300 °C, respectively; oven temperatures running from 75 °C (1.5 min) up to 150 °C (10 min) at 8 °C/min, then increased to a final temperature of 245 °C (15 min) at 10 °C/min. Analyses were performed by duplicate.

Percent encapsulation efficiency (EE%) was determined as follows:

$$EE\% = \frac{C\ retained\ (mg/ml)}{C\ total\ (mg/ml)} \times 100 \quad (1)$$

Fatty acid composition of CLA liposomes suspensions (expressed as percentages) was also determined at 3 and 30 days of storage as described above in order to check their stability.

2.5. Lipid bilayer fluidity

The study of fluidity at different depths of the liposome membranes was carried out by electron paramagnetic resonance spectroscopy (EPR), incorporating 5-doxyl stearic acid or 16-doxyl stearic spin labels (5-SASL and 16-SASL, Sigma, USA). These labels incorporate readily to the liposomes. 5-SASL senses the outer part of the membrane, near the lipid polar heads, and 16-SASL is located in the middle part of the lipid bilayers. Liposome suspensions were labeled at a label/lipid molar ratio of 1%. The procedure was as follows: 1.7 μl of 5- or 16-SASL stock solution in ethanol was added to a plastic tube, and the solvent was evaporated under nitrogen flux. Then, 60 μl of acetate buffer, pH 5.4 were added, followed by 60 μl of a concentrated liposome suspension (25 mM lipids in distilled water). The samples were incubated 30 min at room temperature, and subsequently concentrated by centrifugation (4000×*g*, 40 min, 20 °C) in Amicon tubes (10 kDa). Concentrated samples were loaded into flame sealed capillary tubes. EPR spectra were recorded at 25 °C and X band frequency in a Bruker EMX-Plus spectrometer with temperature control by nitrogen circulation. From the spectra of both labels the order parameter S was calculated, and for 16-SASL the correlation time Tc was also calculated (Pincelli et al., 2000).

2.6. Statistical analysis

Variance analysis (ANOVA) was performed to detect significant

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