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Liposomal nanodelivery systems using soy and marine lecithin to encapsulate food biopreservative nisin

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ABSTRACT

Purified nisin was encapsulated in liposomes made with marine lecithin (ML) or soy lecithin (SL) using a continuous cell disruption system method of microfluidic format and compared with liposomes prepared from proliposomes. SL had higher omega-6 and polar lipids as compared to ML while proliposomes contained only saturated phospholipids. Nisin was entrapped in SL liposomes with highest encapsulation efficiency of 47% at 5% SL concentration. Average size of these liposomes ranged from 151 ± 4 to 181 ± 5 nm, without or with nisin respectively. Electrophoretic mobility was influenced by the nature and concentration of lecithin; however, incorporation of nisin reduced the negative charge of liposomes significantly. Physical stability of liposome-encapsulated nisin was demonstrated for 6 weeks at 4 °C, though transmission electron microscopic studies revealed pore-formation by nisin and fusion phenomenon after 20 weeks at 4 °C. Antimicrobial assay revealed that blend of unencapsulated/free and encapsulated nisin (1:1) exhibited a better control of *Listeria monocytogenes* CIP 82110 as compared to free or 100% encapsulated nisin alone. Thus developing liposomes formulation made from SL may provide an efficient nanodelivery system for nisin.

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1. Introduction

Preservatives are additives incorporated in food, for which controlled release can be valuable during either processing, storage, consumption, or in the human body (Luykx, Peters, Van Ruth, & Bouwmeester, 2008). Currently the customized mode of application of antimicrobial agents is direct introduction to the food system in free form (Devlieghere, Vermeiren, & Debevere, 2004). However, undesirable interactions of these active agents with food components reduce their efficacy against pathogens and thus require the addition of larger antimicrobial quantities to reduce the microbial number within limit (Were, Bruce, Davidson, & Weiss, 2003).

Natural compounds, such as nisin, chitosan or lysozyme, were investigated to replace chemical preservatives and obtain the ‘green label’ products (Devlieghere et al., 2004). Among all the antimicrobial peptides, only a very few of them are actually allowed to be used either as preservative in the food industry or as antibiotic in health care. The 34-residue-long peptide nisin is one of these few, and has been used as a food preservative for a long time (Breukink & De Kruijff, 1999). Nisin effectively inhibits Gram-positive bacteria and outgrowth spores of *Bacillus* and *Clostridium* (De Arauz, Jozala, Mazzola, & Vessoni Penna, 2009). Among bacteriocins, use of nisin has become useful in food industry due to its broad spectrum against foodborne pathogens, generally recognized as safe (GRAS) status, and its ‘bio-additive’ notion (EU, 2004; FDA, 2001). Nisin is now an efficient tool in the hurdle technology for food preservation.

Use of nisin in its free form (unpackaged or unencapsulated) is associated with loss of activity due to degradation (Benech, Kheadr, Laridi, Lacroix, & Fliss, 2002; Laridi et al., 2003). Jung et al. (Jung, Bodyfelt, & Daeschel, 1992) found significant loss of nisin activity in milk because of its interactions with milk components. Divalent cations associated with bacterial cell wall surfaces were shown to

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induce electrostatic repulsion preventing the cationic polypeptide nisin from interacting with bacterial pathogens, thus reducing its activity (Davies et al., 1999; Taylor, Gaysinsky, Davidson, Bruce, & Weiss, 2007).

Currently, some novel encapsulation methods have been introduced to overcome these limitations (Liolios, Gortzi, Lalas, Tsaknis, & Chinou, 2009; Malheiros, Daroit, & Brandelli, 2010). Liposomes are under intensive research and development by the pharmaceutical, cosmetic, and food industries as nanocarrier systems for protection and delivery of bioactive agents (Kosaraju, Tran, & Lawrence, 2006; Mozafari, Johnson, Hatziantoniou, & Demetzos, 2008). In consequence of enhanced stability and targeting at nano-scale, the quantity of bioactive required for a specific effect when encapsulated in a liposome is much less than the amount required when unencapsulated (Mozafari et al., 2006). Liposomes are particularly well suited for use in the food industry as delivery systems because they are well characterized, easily made, highly versatile in their carrier properties, highly biocompatible, and GRAS materials (Xia & Xu, 2005). As liposomes structure encloses both aqueous (core) and lipid (bilayer) phases, they can be utilized in the entrapment, delivery, and release of water-soluble, lipid-soluble, and amphiphilic materials. Because nisin is amphiphilic in nature (Breukink, Ganz, De Kruijff, & Seelig, 2000) it is entrapped simultaneously in the core and bilayers of liposome.

Traditional liposomes preparation methods involve either organic solvent, have less production and encapsulation efficiency (EE) or result in heterogeneous and uncontrolled polydispersity in size and lamellarity. Thus additional post processing steps are required, such as solvent removal and membrane extrusion to yield homogeneous liposome populations (Jahn, Vreeland, Devoe, Locascio, & Gaitan, 2007). A simpler procedure for preparing liposomes is by using preformed bilayers, called “Pro-liposomes” (Laridi et al., 2003; Laloy, Vuillemard, Dufour, & Simard, 1998). Previous studies have indicated that soy lecithin (good source of the essential fatty acid linoleic acid) displays health benefits due to the hypocholesterolemic properties, thus helpful in reducing significant risk for cardiovascular diseases (Nicolosi, Wilson, Lawton, & Handelman, 2001) and partially purified soy lecithin can be used to encapsulate nisin (Malheiros, Daroit, da Silveira & Brandelli, 2010).

Previous investigations on nisin loaded liposomes were performed with purified phospholipids using traditional techniques (Benech et al., 2002; Laridi et al., 2003; Malheiros, Daroit, & Brandelli, 2010; Taylor, Bruce, Weiss, & Davidson, 2008). The main objective of the present study was to optimize a microfluidization approach *i.e.* continuous cell disruption system for nisin encapsulation and to compare liposome made with soy lecithin (SL) and marine lecithin (ML, extracted from salmon) to available Proliposomes. In these different types of liposomes, parameters having critical importance on their performance such as the effect of pressure and the number of passes through the homogenizer and lecithin concentration were studied. The physicochemical properties, physical stability and anti-*Listeria monocytogenes* CIP 82110 activity were characterized.

2. Materials and methods

2.1. Materials

Commercial preparation of Proliposomes H made up of hydrogenated phosphatidylcholine was obtained from Lucas Meyer (Chelles, France). Commercial SL (Sigma, Paris, France) was used, and extracted according to method describe previously by Wu and Wang (2003). ML is a mixture of different phospholipids, extracted from raw salmon head (*Salmo salar*) by an enzymatic procedure (Gbogouri, Linder, Fanni, & Parmentier, 2006). The liposomal ingredients were kept under nitrogen atmosphere at recommended storage temperatures (0–4 °C). Ammonium molybdate and all solvents e.g. chloroform (purity = 99%); methanol (purity = 99%); diethyl ether (purity > 99%) and hexane (purity = 97%) used for Iatroscan, gas chromatography and lipid extraction were purchased from Fisher Scientific (Paris, France).

Nisin Z was purchased from Honghao Chemical Co. (Shanghai, China). Nisin used in this study contained >90% pure nisin (according to the manufacturer, the formulation contains 3.84×10^6 I.U. per gram and 6.88% moisture content). Millipore nylon filters (0.2 μm) were obtained from Millipore (Cork, Ireland). Bicinchoninic acid (BCA) reagents were obtained from Sigma Chemical Co. (Lyon, France).

2.2. Fatty acid profile and lipid composition

Gas chromatography (GC) was used for analysing fatty acid composition. Fatty acid methyl esters (FAMEs) were prepared according to Ackman (1998). Separation of the FAMEs was carried out on a Perichrom TM 2000 gas chromatograph (Perichrom, Saulx-les-Chartreux, France), equipped with a flame-ionisation detector. A fused silica capillary column (30 m; 0.22 mm i.d. 0.25 mm film thickness, BPX70 SGE Australia Pty. Ltd., analytical products) was used. Injector and detector temperatures were set at 260 °C. The oven temperature was programmed as follows: 1 min at 120 °C then ramping to 220 °C at 38 °C/min, followed by a hold period of 20 min. Fatty acids were identified by comparison of their retention times with standard mixtures (PUFA1 from marine source and PUFA2 from animal source; Supelco, Sigma–Aldrich, Belfonte, PA, USA). Results were presented as triplicate analyses.

The neutral and polar lipid classes were determined by Iatroscan MK-5 TLC-FID (Iatron Laboratories Inc., Tokyo, Japan). Each sample was spotted on ten Chromarod S-III silica coated quartz rods held in a frame. The migration was done for 20 min in solution comprising hexane/diethyl ether/formic acid (80: 20: 0.2 v/v/v), then oven-dried for 1 min at 100 °C and finally scanned in the Iatroscan analyzer. The Iatroscan was operated under the following conditions: flow rate of hydrogen, 160 ml min^{-1}; flow rate of air, 2 L min^{-1}. The recording and integration of the peaks were carried out by ChromStar internal software.

2.3. Optimization of nanovesicles production

Liposomes were prepared from proliposome mixture following the procedure of Dufour, Vuillemard, Laloy, and Simard (1996). The deionized water was boiled and degassed to remove all oxygen traces. One, 5 and 10 g/100 ml of proliposome mixture was mixed with an aqueous solution containing nisin (3.8×10^4 I.U.). The mixture was stirred (50–60 rpm) for 15 min, diluted with deionized water to obtain 1 mg mL^{-1} of nisin Z concentration and re-stirred for 15 min. The entrapment process was carried out above transition temperature i.e. at 65 °C.

In case of constant cell disruptor system technique, the method was optimized using SL with different concentrations (1, 5, 10 g/100 ml), number of cycles (3–5) and pressure (140, 170, 200, 250 MPa). From this data optimum pressure and number of passes were used for preparing liposome-encapsulated-nisin. Soy/marine lecithins and water were mixed for 15 min, by magnetic stirring. Further it was thoroughly mixed by Ultraturax T-25 (Avantek, Strasbourg, France) at 13,500 rpm for 3 min. The phospholipids dispersion was then passed through a constant cell disruptor system (Constant Systems Ltd, Northants, UK) with a vertical interaction chamber for specific number of cycles at given pressure (Fig. 1). The homogenization temperature was kept below 10 °C by

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