LWT - Food Science and Technology 62 (2015) 341–349

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

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

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ARTICLE INFO

Article history:
Received 9 October 2012
Received in revised form
11 March 2013
Accepted 19 December 2014
Available online 31 December 2014

Keywords:
Antimicrobial peptide
Nisin
Lecithin
Liposome
Microfluidization
Listeria monocytogenes

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.

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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http://dx.doi.org/10.1016/j.lwt.2014.12.046
0023-6438/

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

using a flow of cold water flowing (4 °C) as cooling jacket. To recover the liposomes containing nisin, emulsions were passed through Sephadex G-50 (fractionation range for protein 1500–30,000 M.W.) column and nisin-encapsulating liposomes were eluted by size exclusion chromatography using a centrifuge (Eppendorf, Hamburg, Germany).

2.4. Particle size characterization

The mean diameter and particle size distribution of liposomes were determined using dynamic light scattering (DLS) technique employing a Zetasizer Nano-ZS (Malvern instruments, UK). The apparatus is equipped with a 4 mW He/Ne laser emitting 633 nm, measurement cell, photo multiplier and correlator. Prior to size measurement, the samples were diluted (1:400) with ultra-pure water. The samples were taken in vertical cylindrical cuvettes (10 mm diameter). The scattering intensity was measured at a scattering angle of 173° relative to the source using an avalanche of photodiode detector, at 25 °C. Results are presented as an average diameter of the liposome suspension (z-average mean) with the polydispersity index (PDI). This index ranges from 0.0 for an entirely monodisperse system up to 1.0 for a polydisperse particle dispersion (Colas et al., 2007). The particle size distribution was characterized using PDI, which is a measure of the width of the size distribution. Droplets sizes were obtained from the correlation function calculated by the dispersion technology software (DTS) using various algorithms. All measurements were carried out at 25 °C, with a medium refractive index of 1.335. The measurements were performed in five replicates.

2.5. Electrophoretic mobility

The effect of lecithin type and nisin on the electrophoretic mobility of liposomes was studied by means of a Zetasizer Nano-ZS apparatus (Malvern Co. Ltd., Worcestershire, UK). Samples were prepared as explained above in size measurement.

2.6. Encapsulation efficiency (EE)

The concentration of entrapped nisin in the liposomes was determined using the bicinchoninic acid (BCA) assay. In this assay, the BCA reagent (Sigma Chemical, Lyon, France) was added to liposome sample containing nisin. After incubation at 37 °C for 30 min, absorbance at 562 nm was measured using a UV–Visible spectrophotometer (Ultrospec 4000, Amersham Pharmacia Biotech, UK). During incubation, a small amount of nisin was released from liposomes but this amount is negligible compared to the 24 h required for measurable liposome poration. Bovine serum albumin containing 1 mg ml^{-1} protein was diluted and used as the protein standard. From this colorimetric measurement the unencapsulated nisin concentration was measured. Knowing the overall nisin concentration incorporated in the liposomal solution, encapsulated nisin concentration was calculated as the difference between overall and unencapsulated nisin. The EE was calculated using the following equation: EE% = Encapsulated nisin concentration/Overall nisin concentration.

2.7. Transmission electron microscopy (TEM)

Liposome samples encapsulating nisin were diluted 10-folds with ultra-pure water to dilute the concentration of the vesicles. Equal volumes of the diluted sample and a 2 g/100 ml ammonium molybdate solution were combined and left for 3 min at room temperature. A drop of this solution was placed on a copper mesh for 5 min before the excess liquid was drawn off using filter papers. The mesh was examined using a Transmission Electron Microscope (Philips CM-20, Paris, France) at an operating voltage of 200 KV.

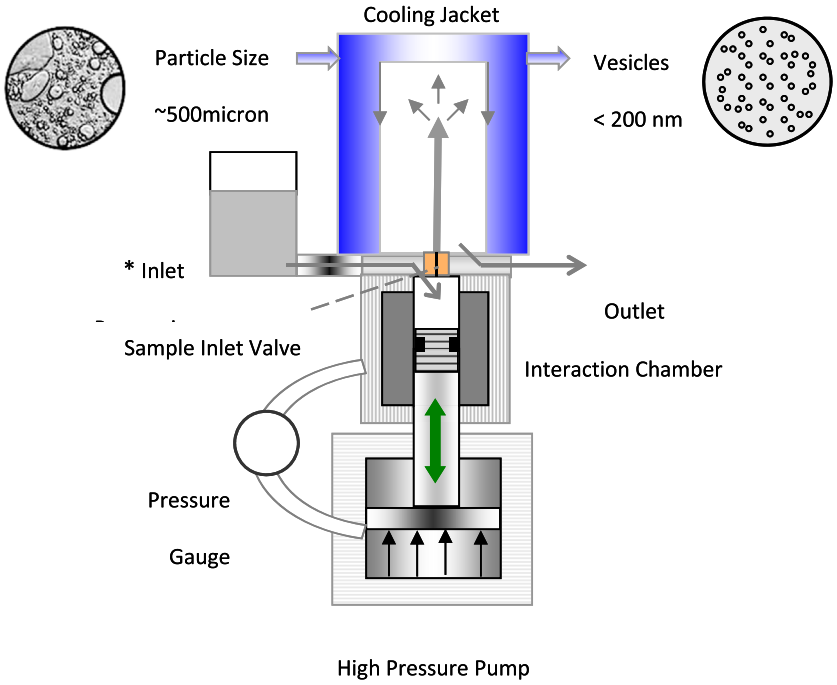

Fig. 1. Schematic diagram of continuous cell disruption system. Cell disruptors and homogenizers are both positive displacement pumps each differs in the way that they create pressure on the sample and transfer it from pressurized chamber to another chamber which is at lower pressure. Homogenizers pressurize the sample in a chamber, which is then released, into a chamber of lower pressure through a homogenizing valve. Cell disruptors use a hydraulic force to accelerate the sample to high pressure and forcing them through a miniature orifice to hit on a disruption head, which is at a lower pressure.

2.8. Microbiological analysis

The minimal inhibitory concentration (MIC) of nisin was determined by the critical dilution method in 96-well plates (Nunc, Roskilde, Denmark). The target strain *Listeria monocytogenes* CIP 82110 (Collection de l'Institut Pasteur, Paris, France) was prepared in Trytone Soytone Broth (Biokar, Paris, France) supplemented with 6 g L^{-1} Yeast Extract (TSB-YE) to a final $OD_{660\ nm}$ of 0.2. Equal volume of inoculated doubled concentrated medium and nisin dilutions was added to the 96 well-plate. The plates were shacked and the $OD_{660\ nm}$ was determined with a Titertek Multiscan MCC/340 (Huntsville, AL). The plates were incubated for 24 h at 37 °C. Afterwards, the corresponding MIC dilutions of nisin, liposome encapsulated (SL 5 g/100 ml) nisin, and liposome encapsulated plus unencapsulated nisin were incubated with the target strain and growth kinetics were recorded.

2.9. Statistical analyses

Statistical analyses were carried out by using the software KyPlot version 2.0 (Koichi Yoshioka, Department of Biochemistry and Biophysics, Graduate school of Allied health Sciences, Tokyo, Japan). For comparison a parametric multiple Tukey test ($p \leq 0.05$) was performed. All errors and errors bars correspond to the standard deviations.

3. Results and discussion

3.1. Fatty acid profile and lipid composition of encapsulating polymer

The fatty acid profile of different lecithin used in this study was analysed to determine the effect of lipid class and fatty acid chain length on the liposome characteristics. The percentage of total polyunsaturated fatty acids was the highest (60 ± 0.4 g/100 g) in SL (Table 1). SL contains >50 g/100 g omega-6 (linoleic acid C18:2 n-6) and >5 g/100 g omega-3 (linolenic acid C18:3 n-3) which belong to the group of essential fatty acids (EFAs). EFAs are the “good fats” and help to support the cardiovascular, reproductive, immune, and nervous systems (Simopoulos, 2009; Yashodhara et al., 2009). However, the most significant proportions of polyunsaturated fatty acids of C20:5 n-3 and C22:6 n-3 was found only in ML. Highest saturated fatty acids contents (C16 and C18) were found in Proliposomes, which contained 87.4 ± 0.3 g/100 g stearic acid.

Table 1
Fatty acid profile of different lecithins by gas chromatography (area %). Results are grouped as saturated fatty acids (SFA), mono-unsaturated fatty acids (MUFA) and poly-unsaturated fatty acids (PUFA).

Fatty acid	Soy lecithin	Marine lecithin	Proliposome
C14:0		5.24 ± 0.46	
C16:0	16.60 ± 0.34	18.95 ± 0.48	12.56 ± 0.33
C18:0	3.82 ± 0.03	4.06 ± 0.05	87.44 ± 0.34
SFA	**20.42**	**28.25**	**100**
C16:1		4.88 ± 0.28	
C18:1(n-9)	19.20 ± 0.17	18.62 ± 1.25	
C18:1(n-7)		2.73 ± 1.03	
C20:1(n-7)		1.27 ± 0.56	
C20:1(n-9)		6.13 ± 0.62	
MUFA	**19.20**	**33.63**	**0**
C18:2(n-6)	54.63 ± 0.29	4.25 ± 0.06	
C18:3(n-3)	5.74 ± 0.11		
C18:4(n-3)		1.80 ± 0.18	
C20:4(n-6)		5.07 ± 0.78	
C20:4(n-3)		1.24 ± 0.03	
C20:5(n-3)		8.31 ± 0.02	
C22:5(n-3)		3.65 ± 0.03	
C22:6(n-3)		13.78 ± 0.20	
PUFA	**60.37**	**38.11**	**0**

The neutral and polar lipid classes of lecithins were separated by thin-layer chromatography (Iatroscan). SL contained 69.6 ± 2.2 g/100 g of phospholipids and 25.4 ± 1.4 g/100 g neutral lipids; Polar lipid fraction of ML was found 41.5 ± 1.5 g/100 g which was significantly (Tukey test, $p \leq 0.001$) lower than SL. The commercial proliposomes contained no traces of neutral lipids (data not shown).

3.2. Liposomes characterization

3.2.1. Choice of conditions for liposome fabrication

To prepare multilamellar vesicles for imposing multiple barriers in active agent release, the liposome size diameter must range >100 nm (Taylor, Davidson, Bruce, & Weiss, 2005). Influence of pressure (140, 170, 200, 250 MPa) and number of passes (3–5) on mean diameter of liposomes at 5 g/100 ml SL concentration was measured (Fig. 2) to optimize average diameter.

Liposome size decreased along with process cycles number. At 200 MPa pressure, five passes resulted in appropriate average size (151 ± 4) with a polydispersity index (PDI) 0.23 ± 0.02. However increasing inlet pressure to maximum limit of apparatus 250 MPa results in unexpected higher average size either due to over disruption of liposome or probable formation of multivesicular vesicles. These results are in coherence with previous study of liposome preparation using milk fat globule membrane phospholipids (Thompson & Singh, 2006). From these results, 200 MPa pressure and 5 numbers of passes were chosen as effective parameters for liposome fabrication.

3.2.2. Encapsulation efficiency (EE)

The protein content entrapped in liposome preparations using three different lecithin concentrations was measured to determine the total amount of nisin entrapped (Fig. 3).

With equivalent concentrations of nisin (1 mg mL^{-1}; 3.8 × 10^4 I.U.) added to liposome formulations, the highest encapsulation of antimicrobials was obtained with 5 g/100 ml SL liposomes with a protein content entrapped 47.4 ± 1 g/100 g which was significantly higher than SL 1 and 10 g/100 g and other liposomes at similar lecithin concentration (Tukey test, $p \leq 0.001$). ML and proliposomes had highest EE% for 5 g/100 ml lecithin ranging 32 ± 3% and 33.6 ± 1% respectively.

Relatively higher EE for SL liposomes may be related to the high pressure disruption method used as compared to traditional heating method for proliposomes. Once the size of the liposomes decreased, it resulted in better dispersibility and higher number of vesicles that could be effective for retention. Decreased antimicrobial concentration in higher neutral-lipids containing liposomes, such as ML liposome, was in agreement with results reported in earlier studies (Laridi et al., 2003). Lower phospholipids ratio in ML could reduce the polypeptide affinity, thus reducing the concentration of antimicrobials that can be incorporated.

In conclusion, protein content measurement indicated that nisin can be encapsulated in stable liposomes using 5 and 10 g/100 ml lecithin concentration, with 5 g/100 g as highly effective. However, EE depended on the lipid composition. Effects of fatty acid chain length on liposome stability had been already tested on lipid models (Maherani, Arab-tehrany, Kheirolomoom, Cleymand, & Linder, 2012). The present study revealed that having other factors constant, SL with shorter fatty acid chain length than ML (Table 1) had an advantage for encapsulating nisin. Determination of exact location and distribution of antimicrobials in liposomes (e.g., inside the liposomal core or incorporated in the vesicle membrane) will require further investigations.

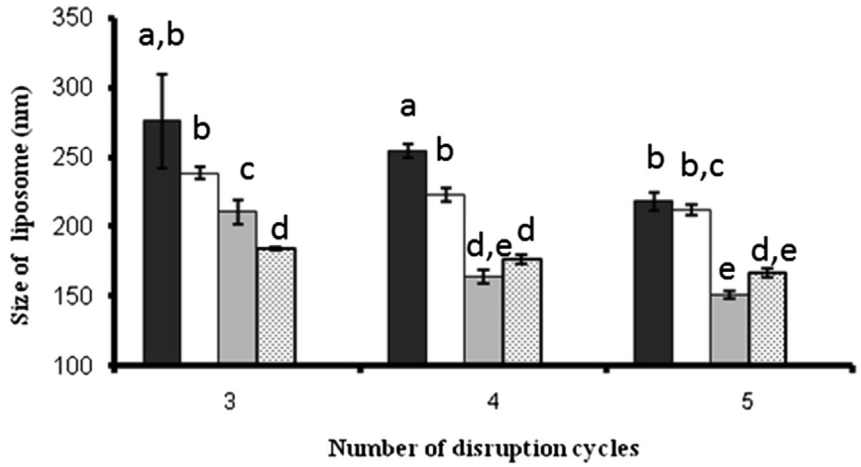

Fig. 2. Mean size of Soy Lecithin (SL) liposomes using 5 percent concentration of SL; effect of pressure (■140 □170; ▨200; ▩250 MPa) and number of disruption cycles (3, 4, 5) at 25 °C. Mean of averaged size from 5 replicates. Same letter means no significant different at 5% confidence level. Measurements were performed in five replicates.

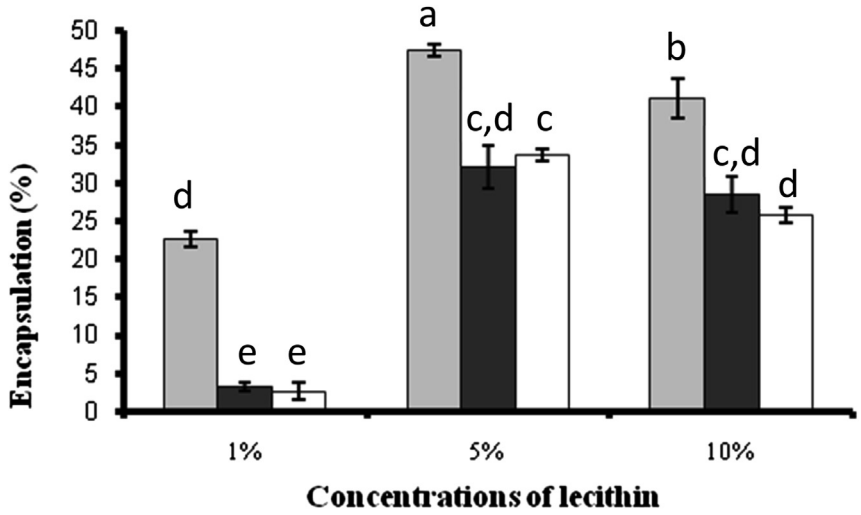

Fig. 3. Effect of lecithin types (Soy Lecithin SL ▨, marine lecithin ML ■, Proliposomes □) at one, five and ten percent lecithin concentrations on encapsulation efficiencies of nisin Z (1 mg mL^{-1}). SL and ML liposomes were obtained at a 200 MPa pressure of continuous cell disruption system with five disruption cycles while heating method was used for proliposomes. Same letter means no significant different at 5% confidence level.

3.2.3. Surface charge properties

Instability of liposomes was attributed to collisions and eventual merging of liposomal membranes of two or more liposomes (Taylor et al., 2005). This process is thermodynamically driven because of system tendency to decrease energetically unfavourable curvature of bilayer membrane in spherical liposomes. Collisions may occur because of random (Brownian) movement of vesicles in solution or because of superimposed convection. Increasing repulsive interactions may reduce collision frequency. Measurement of electrophoretic mobility provided useful information on electrostatic repulsion between liposomes. Liposomes composed of charged polar lipids carrying higher electrical charges with an electrophoretic mobility of −4.32 and −4.10 (μm/s)/(V/cm), at 5 g/100 ml lecithin concentration for SL liposome and proliposomes respectively. They can be expected to be more stable than ML liposomes with significantly lower (Tukey test, $p \leq 0.001$) electrophoretic mobility of −3.77 (μm/s)/(V/cm) (Table 2). Electrophoretic mobility measurements pointed out that introduction of nisin in liposomes significantly affected net surface charge of liposomes compared with nisin-free liposomes. Nisin, as cationic peptide, significantly neutralized liposomes charge (Tukey test, $p \leq 0.001$), reduced the electrostatic repulsion between liposomes but the electrophoretic mobility values were still in agreement with very good liposomes stability. Taylor et al. (2007) observed the same effect when nisin was added in PC:PG (6:4) liposomes but not on PC:PG (8:2) liposomes.

Table 2
Influence of nisin on electrophretic mobilities (0: liposomes without nisin, +: liposomes containing nisin) of soy lecithin liposomes, marine lecithin liposomes and proliposomes.

Lecithin percentage	Nisin content	Liposome type		
		Soy lecithin	Marine lecithin	Proliposome
		Electrophoretic mobility (μm/s)/(V/cm)		
1	0	−4.31 ± 0.16	−3.46 ± 0.07	−3.63 ± 0.07
	+	−3.47 ± 0.13	−2.75 ± 0.10	−2.80 ± 0.21
5	0	−4.32 ± 0.05	−3.77 ± 0.06	−4.10 ± 0.16
	+	−3.41 ± 0.04	−3.32 ± 0.11	−3.20 ± 0.12
10	0	−4.85 ± 0.04	−3.93 ± 0.07	−4.15 ± 0.03
	+	−3.42 ± 0.06	−3.64 ± 0.10	−3.29 ± 0.05

One interest in surface potential reduction concerned the reduction of liposomes/bacteria repulsion and then the more rapid surface action of nisin against bacteria. As bacteria carry a negative electric charge, decrease in negative charge of liposome containing nisin may result in less repulsion between both, thus favouring nisin antimicrobial action.

3.2.4. Physical stability

3.2.4.1. Size measurement. Zeta-sizer results indicated that during the 6-week storage of liposomes containing nisin at 4 °C, there were slight fluctuations in effective diameter (Fig. 4) for 5 and 10 g/100 ml lecithin formulations, which were, however, not statistically significant (Tukey test, $p > 0.05$). Proliposomes average size was nevertheless 2 to 3 times higher than SL and ML due to rapid proliposomes formation limiting the size reduction effect during cell disruption processing. ML and SL liposomes have the same average size for 5 and 10 g/100 ml. Nisin presence generally increased liposome size e.g. SL liposomes 5 g/100 ml had demonstrated 151 ± 4 and 181 ± 5 nm sizes without nisin and with nisin, respectively (Figs. 2 and 4). The increase in liposome size when nisin was encapsulated in SL liposomes could be due to the creation of a more swollen membrane structure. Increase in liposome size upon nisin encapsulation is in agreement with results reported by Were et al. (2003). For 5 and 10 g/100 ml lecithin concentration, the polydispersity index (PDI) of SL liposomes and ML liposomes encapsulating nisin (prepared with constant disruptor method) was <0.3. Proliposomes prepared with heating method had given a PDI value >0.4. The higher PDI values found for 1 g/100 ml lecithin formulations may be due to the fact that liposomes become unstable at lower phospholipids concentration by the pore formation activity of nisin. Proteins might alter the entrapment of phospholipids in the liposomal membrane and promote curvature changes. Liposome size thus was dependent on the lipid composition (fatty acid chain length), nisin and fabrication method.

3.2.4.2. Transmission electron microscopy (TEM). The microscopical approach is commonly used to characterize the structure/morphology/geometry of nanocarriers. Electron microscopy techniques have been widely used to measure the size and interaction in the form of aggregation or fusion (Khosravi-Darani, Pardakhty, Honarpisheh, Rao, & Mozafari, 2007). To prove the stability during storage period and method ability to efficiently produce liposomes (and not other lipidic structures such as micelles and emulsions) TEM studies were performed. TEM experiments confirmed the presence of predominantly spherical and multilayer structures. Fig. 5b and f shows clearly a disruption of several lecithin layers and prove the multiple layer structure of the liposomes. SL liposomes (5 g/100 ml lecithin and 1 mg mL^{-1} nisin) kept at 4 °C were still intact after 6 weeks according to physical stability study by zeta-sizer, however TEM experiments revealed changes in their morphology and occurrence of fusion phenomenon after 20 weeks of storage (Fig. 5b). These modifications could be due to pore formation and release of nisin. On the other hand proliposomes were unstable during storage period while presence of glycerin and alcohol in their formulation exposed them as nano-gel structure (Fig. 5e and f). The pore formation phenomenon was clearly observed in case of ML liposomes where pore structure is semi-attached with liposome (Fig. 5d). These are very promising results referring to controlled release of nisin either through pore formation or modification in membrane structure but further research will be required to measure the diffusion kinetics of active agents from inside out. Therefore, this method is fully able of producing nanoliposomes with different compositions (Khosravi-Darani et al., 2007).

3.3. Antimicrobial activity

As a primary test for antilisterial activity, free nisin (non-encapsulated) revealed a MIC value of 7.8 mg L^{-1} against *Listeria monocytogenes* CIP 82110. Afterwards, different solutions including control (without nisin addition), non-encapsulated free nisin solution (MIC and ½ MIC), encapsulated nisin (with SL 5%), and blend of free and encapsulated nisin (1:1) at corresponding MIC value were tested for antimicrobial activity against *L. monocytogenes* CIP 82110. As expected, the control sample without nisin had not shown antimicrobial action against *L. monocytogenes* CIP 82110 (Fig. 6). Active solution with nisin (MIC value) inhibited listeria development up to 24 h but afterwards there was gradual and steady growth of bacteria due to transitory effect of nisin already observed in many studies (Delves-Broughton, Blackburn, Evans, & Hugenholtz, 1996). However, the reduction of nisin concentration to 3.9 mg L^{-1} (1/2 MIC) could only control the increase in bacterial population up to 15 h and then steady growth of *L. monocytogenes* CIP 82110 was observed. The SL liposomes containing nisin had demonstrated lesser antimicrobial activity as bacterial growth started before 10 h of incubation possibly due to insufficient nisin bioavailability. Nevertheless the bacterial growth was reduced by half (peak values) as compared to control. This reduction in

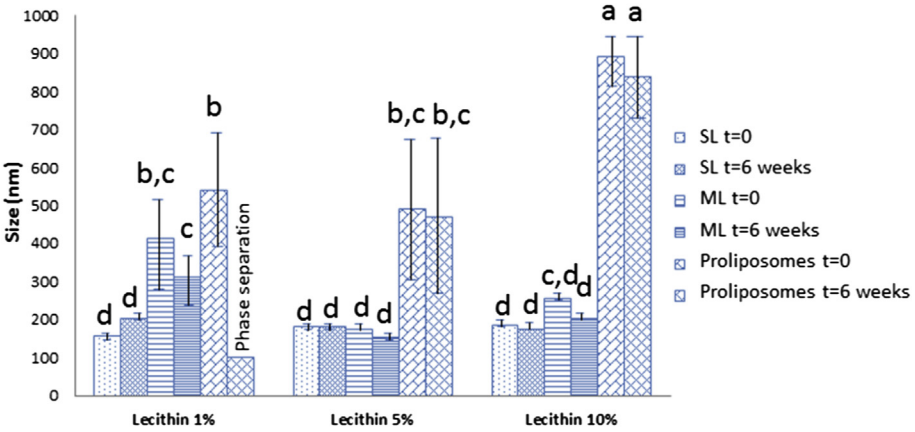

Fig. 4. Mean diameter values of liposomes encapsulating nisin (1 mg mL^{-1}) during a 6-week storage at 4 °C. Mean size for different concentrations of lecithins (1, 5, 10 g/100 ml) for soy lecithin SL liposomes, marine lecithin ML liposomes and proliposomes at day 0 and after 6 week of storage. Same letter means no significant different at 5% confidence level. The key explaining the pattern in bars or symbols/ lines used in graphs should not appear on or next to the figure.

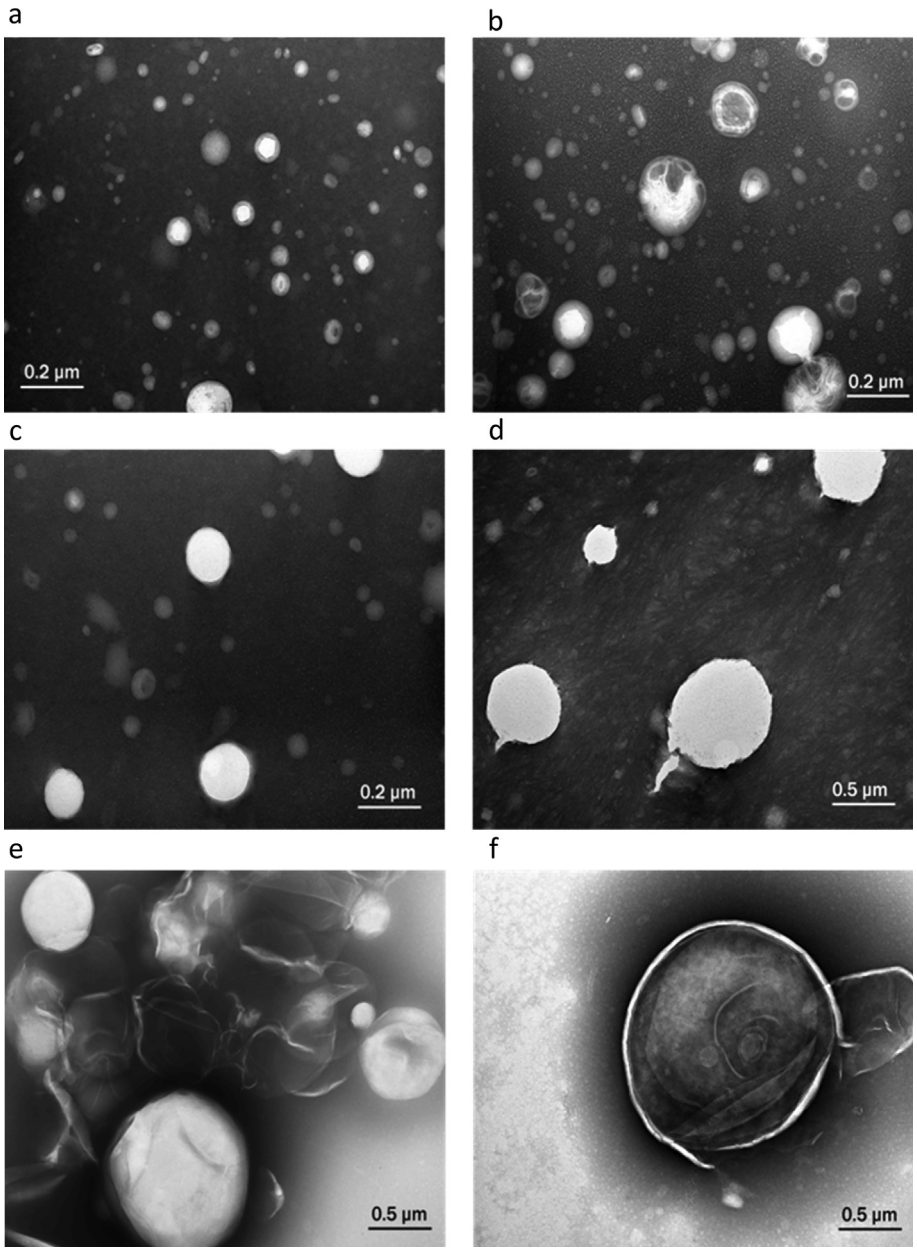

Fig. 5. Structure and stability study of different liposomes at 4 °C for 20 weeks. Soy lecithin SL liposomes, marine lecithin ML liposomes and proliposomes at 0 day (a, c, e) and after 20 weeks (b, d, f).

pathogen growth was due to slow release of nisin from liposome and probably as a result of active liposomes interactions with bacteria. However, blend of free and encapsulated nisin (1:1) at MIC had indicated better control of pathogen as compared to 100% encapsulated nisin or free nisin. This effect is possibly due to the fact that free nisin controlled the initial bacterial growth burst and eventually fresh nisin release from liposome and liposome interactions with *L. monocytogenes* CIP 82110 could have improved the antimicrobial potential. In other study, commercial nisin loaded liposomes, prepared from partially purified soybean phosphatidylcholine, was less efficient in controlling *Listeria monocytogenes* ATCC 7644 growth, when compared to free nisin, in both BHI and skin milk at 30 °C (Malheiros, Daroit, da Silviera, et al., 2010). This system is more effective when used at refrigeration temperature and effective to control *L. monocytogenes* in milk. Thus liposome composition is important to control *Listeria* populations because interactions of liposome with target cells can occur by adsorption onto the cell surface, fusion with the cell membrane, release of drug by micropinocytosis, or due to a specific endocytosis (Torchilin, 2005). Nonetheless, in future more extensive microbiological study against a spectrum of pathogens using model food system for longer storage time will be required to exhibit actual potential of these active liposomal solutions encapsulating nisin for improving food shelf-life.

4. Conclusion

Active liposome production with present method utilized was without organic solvent, rapid, efficient and industrially applicable. Liposomes prepared from soy lecithin SL 5 g/100 ml provided best

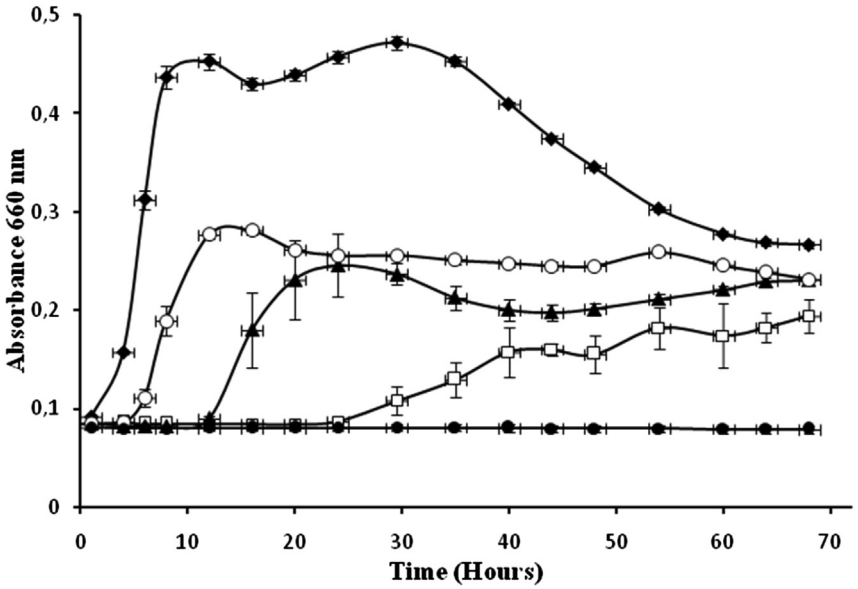

Fig. 6. Growth kinetics of *Listeria monocytogenes* CIP 82110 incubated at 37 °C with unencapsulated (free), encapsulated (SL liposome prepared with soy lecithin 5 g/100 ml, 200 MPa pressure, 5 cycles), and blend of free and encapsulated (1:1) nisin at corresponding minimal inhibitory concentration (7.8 mg L^{-1}). The treatments include; (◆) control, (□) unencapsulated/free nisin at MIC, (▲) unencapsulated nisin at ½ MIC, (○) encapsulated nisin, and (●) blend of nisin (free/unencapsulated and encapsulated nisin) at MIC. Three replicates were made for each growth kinetic.

EE (47%) and high physical stability. All ingredients used in the present study are non-toxic (even nutritive as PUFA), GRAS (nisin) with bio-additive notion. The microscopical study confirmed successful formation of liposomes as well as the fusion and pore formation phenomenon in large liposomes that indicated slow release of lantibiotic nisin. The initial microplate antimicrobial assay revealed that blend of free and encapsulated nisin (1:1) had indicated better control of *L. monocytogenes* CIP 82110 as compared to 100% encapsulated nisin or free nisin. In near future, the diffusion rates of active agent from nanoparticles should be measured for predicting the bioavailability of antimicrobials or food biopreservation. Owing to this study, it would be possible to add the active ingredients in lower concentrations to pharmaceutical/food systems while improving their bioavailability through controlled release from liposome. Food application will nevertheless require additional microbiological experiments on real foods to check the encapsulated nisin efficiency on actual systems.

Acknowledgement

The authors acknowledge the technical assistance of Céline Caillet at Laboratoire Environnement et Minéralurgie (LEM).

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