Original Article

Applied Food Biotechnology

APPLIED FOOD BIOTECHNOLOGY, 2015, 2(2): 7-14
Journal homepage: www.journals.sbmu.ac.ir/afb

pISSN: 2345-5357

Survey Study of Lipid Effect on Nisin Nanoliposome Formation and Application in Pasteurized Milk as a Food Model

*Say-yed Hesameddin Tafreshi[1], Saeed Mirdamadi[2]**

[1]Quality Assurance Department, Research and Production Complex, Pasteur Institute of Iran, Tehran, Iran
[2]Biotechnology Department, Iranian Research Organization for Science and Technology, Tehran, Iran

Abstract

The use of bacteriocins, mainly nisin, is one of the most significant preservation technologies in the food industry. Nisin encapsulation can improve stability and homogenous distribution in food matrices. In this study, liposomes of four various lipids (lipoid S 100, lipoid S PC-3, lipoid S PC, and lipoid PC (DPPC)) were prepared by dehydration-rehydration method, and compared for entrapment efficiency, and lipid with the highest entrapment efficiency (DPPC) was characterized. The inhibitory effects of encapsulated (DPPC nanoliposomes) and free nisin on the spoilage of pasteurized milk were also studied. All experiments were performed in triplicate. Entrapment efficiency ranged from 14% (lipoid S 100) to 49% (DPPC). DPPC nanoliposomes were large unilamellar vesicles (LUV), and had an asymmetric oval shape (elliptical) with a mean diameter of 136 nm. It was revealed that pasteurized milk spoilage was delayed by both free and encapsulated nisin, but free nisin (with 38 days) was significantly more efficient in comparison with encapsulated nisin (14 days).

Article Info

Article history:
Received 24 Jan 2015
Revised 2 Feb 2015
Accepted 13 Feb 2015

Keywords:
Encapsulation,
Liposome characterization,
Milk spoilage,
Nisin.

Correspondence to:
Saeed Mirdamadi
Tel: 0098 (21) 56275510
Fax: 0098 (21) 56276636
Emails: Mirdamadi@irost.org

1. Introduction

At present, consumers' awareness about chemical preservatives hazards, demand for minimally processed foods, and effect of food pathogens on human health, has led food manufacturers to use advanced preservation technologies in order to increase the shelf life of food products, and inhibit the growth of pathogenic bacteria. A technology intended for this purpose is bio-preservation with bacteriocins, specifically nisin.

Nisin, a well-recognized bacteriocin, is produced by *Lactococcuslactis*, and has broad spectrum of inhibitory effects against Gram-positive bacteria such as *Listeria monocytogenes* and *Clostridium botulinum*.

It is generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) [1-2] and approved by the World Health Organization (WHO) [3] and the European Union (EU) [4].

Despite the many advantages of free nisin, there are also several limitations in respect of its application in food industries, such as adherence to fats and proteins, which decreases its accessibility to microorganisms' cells, inhibits sensitive starter cultures, and heterogeneous distribution in food matrices [5-7]. Nevertheless, attempts to use liposomes in food products to encounter the disadvantages of free nisin have increased [8]. For example, there have recently been statements on nisin nano-encapsulation in niosomes to prevent any undesired conditions [9]. Encapsulation of nisin can improve its stability, activity and homogenous distribution in food matrices [6, 10]. Other nisin encapsulation advantages include long term preservation (slow release), reducing the risk of resistant strains and lower inhibition effect on starter cultures [11-13].

In this study, four various lipids were screened and compared in respect of entrapment efficiency, and subsequently lipid with the highest entrapment

efficiency was compared with free nisin for inhibitory effects on pasteurized milk spoilage. Moreover, pure nisin has been used by other researchers in some nisin encapsulation studies [5-6, 10, 14-17], however in the present study, we examined commercial nisin for readily use in industrial applications.

2. Materials and Methods

2.1. Bacterial Strains and Media

Micrococcus luteus PTCC 1169, as the indicator strain for nisin bioassay, was obtained from the Persian Type Culture Collection (PTCC). All stock cultures were maintained at -80°C in skim milk (Merck, Germany) and 20% glycerol (Merck, Germany). Working culture of the bacterial strain was maintained on BHI agar (HiMedia, India) at 4°C.

2.2. Liposome Preparation

Four various lipids, phosphatidyl choline from fat free soybean lecithin (S 100), hydrogenated phosphatidyl choline from soybean lecithin (S PC-3), phosphatidyl choline from soybean lecithin (S PC) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), (all purchased from Lipoid, Germany) were examined and compared for entrapment efficiency**.**

Lipid stocks dissolved in chloroform (Merck, Germany) and 3 mL of lipid solutions (10 mM) were poured in a round bottom flask and dried with a vacuum rotary evaporator (Laborota 4003, Germany) at 25°C and 65 rpm in order to constitute lipid film. To remove any solvent residual, the lipid film was next dried under vacuum for 16 h. Hydration of lipid film (for vesicle preparation) was accomplished by adding 4 mL of 0.1 x phosphate-buffered saline (10 x of this buffer contains 17 mM KH_2PO_4, 50mM Na_2HPO_4, 1.5 M NaCl, pH=7.4, all purchased from Merck, Germany) consisting of 1000 IU/mL nisin (Sigma, USA) and 10 mM EDTA (Merck, Germany). The process was carried at 45°C and 65 rpm during 3 h. Downsizing of vesicles was further performed by sonication for 20 s at 24 kHz (Hielscher, UP 200H, Germany). Also, two additional sonication cycles were executed at 45 s interval. In order to unify the downsized vesicles, the vesicles were extruded through a 0.1 µm (100 nm) Nuclepore® polycarbonate filter (Whatman, UK) 25 times. The obtained liposomes were centrifuged at 3500 rpm for 7 min at room temperature, washed twice with 0.1 x phosphate-buffered saline and then re-centrifuged. The resultant supernatants were combined and used to determine the amount of unencapsulated nisin. Finally, the liposomes were re-suspended in 2 mL of 0.1 x phosphate-buffered saline [8, 15, 18].

2.3. Determination of Entrapment Efficiency

In order to calculate the amount of entrapped nisin in liposomes, 200 µL of triton X-100 (Merck, Germany) 2% and 4 µL of 0.5 M HCl (Merck, Germany) were respectively combined with 200 µL liposomes suspensions and kept at 100°C in water bath for 5 min and maintained at -20°C temperature until nisin bioactivity measurement by bioassay method was achieved [6].

The entrapment efficiency percent (% EE) was calculated according to equation (1):

$$\text{Equation (1) } \%EE = 100 \times \frac{\text{Encapsulated nisin (IU)}}{\text{Encapsulated nisin (IU)} + \text{Unencapsulated nisin (IU)}}$$

2.4. Nisin Bioassay

Nisin was determined by agar diffusion method [19]. Temperature was next adjusted on sterilized BHI medium (HiMedia, India) containing 0.75% agar (HiMedia, India) and 1% Tween 20 (Sigma, USA) at 40°C, and inoculated with 1% of a 24-h culture of *M. luteus* (OD_{600nm}=1.7) to produce approximately 10^8 colony forming units (cfu) of the indicator strain per mL of the agar medium. Sterile Petri dishes (100×15 mm) were filled with 25 mL of the inoculated bioassay medium; after solidification, four wells were bored on each Petri dish by means of a sterilized stainless steel borer. Then, from every sample 50 µL, was placed in each well in triplicate, and the fourth well was filled with blank. The Petri dishes were incubated at 4°C for nisin diffusion for 16 h, and incubation was repeated at 37°C for another 24 h. The dishes were then examined for diameter of inhibition zones using a digital caliper (CB-50001S, Medford Tools and Supply, USA) to the nearest 0.01 mm and the average of the three measurements was calculated. In order to construct standard curve, a stock solution of nisin (1000 IU/mL) was prepared by dissolving 0.025 g of commercial nisin (10^6 IU/g, Sigma, USA) in 25 mL of sterile 0.02 M HCl as diluents solution. Standard nisin solutions in the range of 500, 400, 300, 200, 100, 50, 25, 10, 5 and 0 IU/mL were prepared by using sterile 0.02 M HCl solution. The standard curve was computed using $\log_{10}$ nisin concentration vs. average zone diameters of growth inhibition.

2.5. Liposome Characterization

After the selection of appropriate lipid for liposome preparation (lipid with the highest entrapment efficiency), liposome characterization was performed.

2.5.1. Particle Size Analysis

The mean diameter and size distribution of approved liposome were analyzed by dynamic light scattering technique (DLS) using a Zetasizer Nano ZS (Malvern instruments, UK) with 4 mW helium/neon laser at 633 nm; the samples were measured at a detection angle of 173°. Liposomal size distribution

was analyzed with DTS Nano software version 4.20, supplied by manufacturer (Malvern instruments, UK). All measurements were performed at 25°C.

2.5.2. Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) was used to determine the shape and size of the liposomes. The liposome samples for electron microscopy examination were prepared as previously described [6, 12, 17, 20], and the grids were examined by a transmission electron microscope (Philips CM120, The Netherlands) at 100 kV equipped with the EMMENU4 software by the manufacturer. By the use of TEM images, additional size analysis became feasible by employing the Aquinto a4i Docu software (Excel Technologies Inc., USA).

2.5.3. Shelf Life of Liposomes

In order to obtain the shelf life of the prepared liposome and to evaluate the effect of temperature on its stability, the prepared liposome was kept at room temperature (25±1°C) and refrigerator (4±1°C) in 0.1 x phosphate-buffered saline for thermal stability analysis. Sampling was performed every three days and encapsulated nisin was measured and compared with its initial amount at zero time. The shelf life of liposome described here as the length of time in which the encapsulated nisin remained at an acceptable level (95% of initial encapsulated nisin concentration) during storage period.

2.6. Free and Encapsulated Nisin Effects on Pasteurized Milk Spoilage Parameters

The indicators of pasteurized milk spoilage consist of alcohol test, clot on boiling test, decreased pH value and increased total colony count. Free and encapsulated nisin (200 IU/mL) were separately added to pasteurized milk (Faculty of Veterinary Medicine, University of Tehran, 2.5% fat, Tehran, Iran). Moreover, liposomes without nisin (empty liposomes) were added to pasteurized milk considered as blank for encapsulated nisin. The milk samples were then tested every other day during the first week (and every 4 days then after) to obtain pH value measurement, clot on boiling test, alcohol test, nisin bioassay and total colony count until spoilage.

2.6.1. Alcohol Test

The test was performed by combining equal amounts of milk and ethanol (68 %, Merck, Germany) in a small test tube and shaking the mixture. If a precipitate (or curd) was formed, the test was considered positive [21].

2.6.2. Clot on Boiling (COB) Test

A test tube containing milk was placed in a boiling water bath for 5 min. In case of clotting, coagulation or precipitation, the test was considered positive [21].

2.6.3. pH Value

pH values of the milk samples were measured by digital pH meter (MP 220, Mettler Toledo, Switzerland); pH meter was calibrated with standard buffer solutions of pH=4.00 and pH=7.00 (Merck, Germany) [22].

2.6.4. Total Colony Count

Plate count skim milk agar medium (Merck, Germany) was used for the enumeration of microorganisms in milk samples based on Iran national standards, ISIRI No. 5484 [23] and ISIRI No. 2406 [24] procedures (in compliance with ISO 6610 and ISO 8261 respectively). In brief, ten-fold serial dilutions of milk sample were prepared with sterile peptone saline solution (Merck, Germany) and total count was performed by the poured plate count method. The final count was carried out after 72 h of incubation at 30°C.

2.7. Nisin Bioassay of Milk Samples

2.7.1 Milk samples without liposome

pH values of these samples were adjusted to 3.0 using 0.2% Tween 80 (Merck, Germany) in concentrated hydrochloric acid (Merck, Germany) and were heated at 100°C for 5 min in water bath. After cooling at room temperature (25°C), the samples were centrifuged at 12000 × g for 10 min; the supernatants were kept at refrigerator (4°C) for 30 min and subsequently filtered through a 0.22 µm filter (Millipore Corp., USA). The filtrates were maintained at -20°C for later use [10].

2.7.2 Milk samples containing liposome

200 µL of triton X-100 (Merck, Germany) 2% and 4 µL of 0.5 M HCl (Merck, Germany) were combined with 200 µL of the samples and held at 100°C for 5 min in water bath. In continuation, the same procedure was followed as previously described in milk samples without liposome [6].

2.8. Statistical analysis

Parameters analyses were performed on a full factorial design and Microsoft Office Excel, 2003 (version 11.0) (Microsoft Corporation, USA) was used in order to plot nisin standard curve and graphs, and calculate its concentration, mean and SD. All experiments were performed in triplicate.

3. Results and Discussion

Nisin is widely used as a bio-preservative in food industries aiming to increase the shelf life of food products, and to prevent pathogenic bacterial growth. In order to achieve these aims, it is possible to use nisin in free or encapsulated forms. The efficiency of these forms for preservation of food products depends highly on food structure, food composition (such as ingredients and additives, nutrients, buffering capacity of food products) and also their physicochemical characters such as pH, temperature, water activity

(a_w), atmosphere (O_2, CO_2), redox potential, microbial load and salt content. The solubility and charge of nisin, binding and interaction of nisin to food components (adsorption to fats or proteins), protease activity, bacterial membrane changes in response to the environmental factors, and food matrices can additionally influence nisin activity in food products [9, 25-26]. For this reason, in this study, lipids screening, nanoliposome formation, and characterization and evaluation of the inhibitory effects of free and encapsulated nisin on pasteurized milk spoilage with commercial nisin were investigated.

3.1. Liposomes Entrapment Efficiency

The entrapment efficiencies of nisin in the liposomes prepared from various lipids are shown in Fig. 1. Among them, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) was found with the highest entrapment efficiency (49%), followed by S PC (30%), S PC-3 (22%) and S100 (14%) respectively. DPPC was used for liposome preparation. The entrapment efficiency of the tested vesicles varied from 14% to 49%. This variation is caused by the interaction of nisin (and other antibacterial peptides) with lipid membranes. For example, nisin-phosphatidylcholine vesicles interaction can change the lipid membrane structure and induce leakage [6]. Also, electrostatic interactions between nisin (containing positive charge) and cationic lipids and on the contrary, anionic lipids such as DPPC (inclined towards higher entrapment efficiency) have been reported [12, 15]. In the present study, the entrapment efficiency of DPPC nanoliposomes (49%) was in the proximity of nanoliposomes entrapment efficiency composed of DPPC:Dicetyl phosphate:Cholesterol with 7:2:1 molar ratio (54.2%) [12], and (47%) of Prolipo® H (hydrogenated phosphatidylcholine) as stated by other investigators [6, 17].

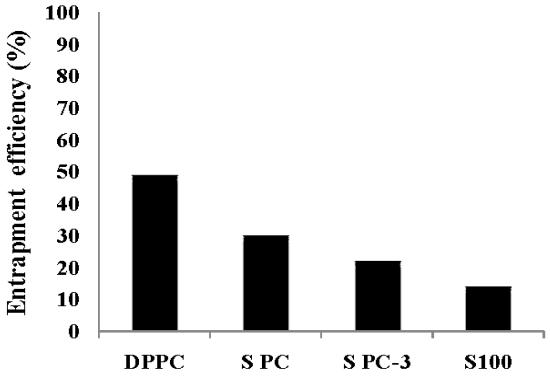


Figure 1. Entrapment efficiencies of the lipids

3.2. Particle Size Analysis

The size distribution of DPPC liposomes is shown in Fig. 2. According to the analysis, the size distribution was narrow and ranged from 125 to 147 nm with a mean diameter of 136 nm (136 ± 11 nm). Based on the intensity distribution data, about 95% of the liposome sizes were between 125 nm and 136 nm and minimum and maximum intensity distributions were 5% and 63% respectively.

As shown in Fig. 2, the mean diameter of DPPC nanoliposomes is 136 nm despite the fact that the nanoliposomes were extruded through 100 nm pore size polycarbonate filters. This finding (that the size of vesicles is larger than the filter pore size via extrusion method) has been reported by other researchers too [27-29].

In various investigations, the size of nisin liposomes ranged from 124 to 2400 nm. For example, in the liposomes containing nisin, the size distribution varied from 140 to 2400 nm with a mean diameter of 740 nm for Prolipo® H, which was prepared by just mixing and without downsizing or homogeneity procedures [6]. However, by application of downsizing and homogenization methods, a range of liposome sizes including 80-120 nm for Prolipo® H were prepared by ultracentrifugation [10], 124 nm phosphatidylcholine liposomes [8], 284±10 nm liposomes consisting of DPPC:Dicetyl phosphate:Cholestrol with 7:2:1 molar ratio [12], 140 nm liposomes prepared from partially purified soy lecithin [30], and 125±6 nm egg phosphatidylcholine and cholesterol (8:1, %w/w) liposomes [31] have been reported, which all are near to our obtained result, 136±11 nm.

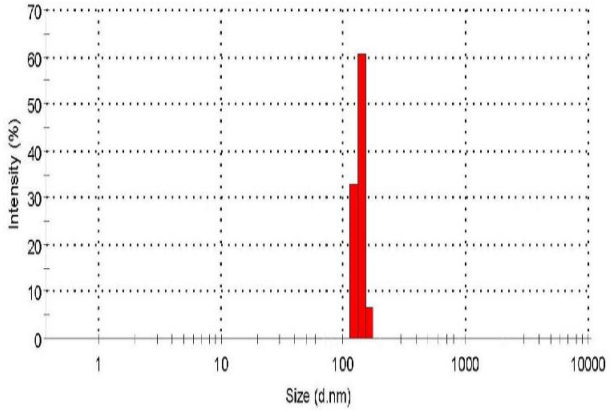
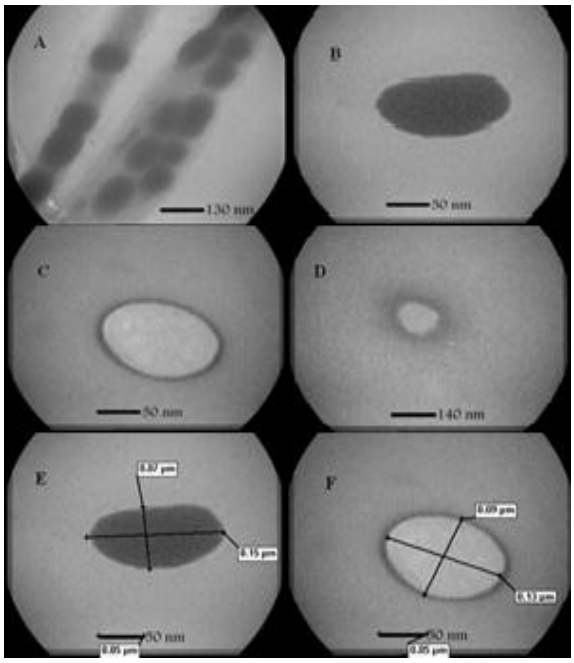


Figure 2. Particle size distribution of DPPC liposomes

3.3. Transmission Electron Microscopy (TEM)

TEM imaging of DPPC liposomes are shown in Fig. 3. TEM images showed that liposomes had asymmetric oval shape (elliptical), and were large unilamellar vesicles with diameters ranging from 120 to 140 nm. Also, on the basis of Aquinto software analysis of the liposome micrographs, DPPC liposomes (viewed as ellipse) had minor axis between 70 nm and 90 nm. Fig. 3 clearly indicates that the shape of nanoliposomes is not spherical (A, B and D), and the liposomal membrane varies in some parts in thickness (C).

According to Laridiet al. investigation, nisin can change the morphological characteristics of liposomes, and result in asymmetrical shape and size by effecting the structure and conformation of lipid bilayers in liposomal membrane. These effects may cause variation in liposomes' structure and membrane thickness [6].



The images presented in this study resulting from ultrathin section (A, B), negative stain (C, D) and Aquinto software analysis (E and F) of tested grids. Magnification of the images is ×37600. A and B micrographs revealed the oval shape of the liposomes and unilamellar vesicles are clearly observed in C and D images with two different bars.

Figure 3. Transmission electron micrographs of DPPC liposomes and Aquinto software analysis

3.4. Shelf Life of DPPC Liposomes

The shelf life of DPPC liposomes at 4°C and 25°C is presented in Fig. 4. As clearly shown, the shelf life of DPPC liposomes at 4°C is two folds in comparison with that at 25°C; consequently, the liposomes were preserved at 4°C. The results of thermal stability testing revealed that temperature had a significant role on the liposomes' shelf life and stability, so that the shelf life observed for DPPC nanoliposomes at 4°C was longer than that of stored at 25°C (12 days compared to 6 days). In this research, the appropriate temperature storage of DPPC nanoliposomes was determined as 4°C.

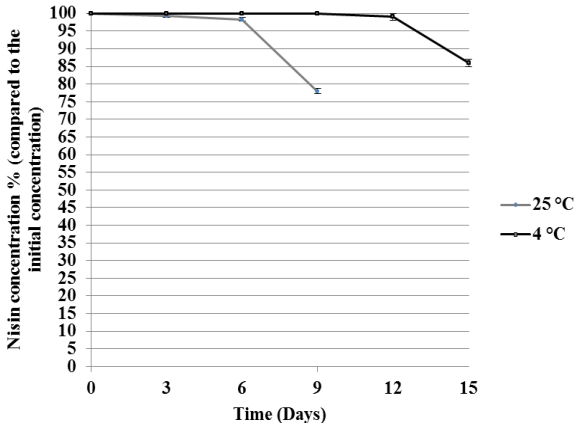


Figure 4. Shelf life of DPPC liposomes at 4°C and 25°C

3.5. Effect of nisin on Milk Spoilage

The effects of free and encapsulated nisin on pasteurized milk spoilage are summarized in Table 1. As indicated, the pasteurized milk was spoiled after 4 days (positive alcohol test). A similar pattern of spoilage was seen for the pasteurized milk containing liposomes without nisin (empty liposomes); therefore, it can be concluded that the empty liposomes had no inhibitory effect on the spoilage of pasteurized milk. Spoilage of pasteurized milk was also delayed by free and encapsulated nisin, but free nisin significantly deferred spoilage time compared to encapsulated nisin (38 days and 14 days, respectively). Alcohol test became positive when the pH value decreased from 6.80 to 6.25±0.05 in all pasteurized milk samples. In addition, a gradual pH value reduction in both nisin containing pasteurized milk samples (free and encapsulated) was quite apparent. It was also found that the pasteurized milk samples failed the COB test, when pH value of the samples ranged from 5.22 to 5.36. A distinguished increase in total colony count was remarked in the pasteurized milk and empty liposome samples (liposomes without nisin) compared to the pasteurized milk samples containing both nisin forms (free and encapsulated). Also, it can be stated that decrease in the nisin activity was intensely higher in milk samples containing encapsulated nisin compared to the free nisin milk samples within the same period of time.

Precipitation of milk protein occurred in both alcohol and COB tests (clear indications of putrid or sour milk), but alcohol test was more sensitive than COB test. This finding was also confirmed by Tassew and Seifu investigation [32]. According to Table 1, it is observed that alcohol test manifested pH milk abnormality in the ranges of 6.2 and 6.3, while in COB test, this proved positive at lower pH (about 5.3).

Table 1. Effects of free and encapsulated nisin on parameters of pasteurized milk spoilage

Milk samples	Tests	Time (days)											
		0	2	4	6	10	14	18	22	26	30	34	38
Pasteurized milk	Alcohol test	-[1]	-	+[1]	+								
	Clot on boiling test	-	-	-	+								
	pH	6.81[2] ±0.02	6.72 ±0.01	6.30 ±0.01	5.22 ±0.02								
	Total colony count (cfu/mL) × 10^3	18 ±1.00	32 ±1.20	75 ±2.00	250 ±3.00								
Pasteurized milk with empty liposomes (without nisin)	Alcohol test	-	-	+	+								
	Clot on boiling test	-	-	-	+								
	pH	6.80± 0.01	6.69± 0.02	6.25± 0.02	5.33± 0.02								
	Total colony count (cfu/mL) × 10^3	18± 0.900	34± 2.000	78± 3.000	240± 2.000								
Pasteurized milk with liposomes containing nisin (encapsulated nisin)	Alcohol test	-	-	-	-	-	+	+					
	Clot on boiling test	-	-	-	-	-	-	+					
	pH	6.80± 0.02	6.78± 0.02	6.75± 0.01	6.62± 0.01	6.45± 0.01	6.21± 0.01	5.36± 0.02					
	Total colony count (cfu/mL) × 10^3	18± 1.000	9.4± 0.400	7.2± 0.300	5.7± 0.300	32± 0.800	71± 1.000	180± 1.100					
	Nisin activity (IU/mL)	198± 4	135 ±4	120± 3	85 ±3	72 ±2	65 ±4	42 ±4					
Pasteurized milk with free nisin	Alcohol test	-	-	-	-	-	-	-	-	-	-	-	+
	Clot on boiling test[3]	-	-	-	-	-	-	-	-	-	-	-	-
	pH	6.82± 0.02	6.81± 0.02	6.80± 0.02	6.78± 0.01	6.72± 0.02	6.72± 0.01	6.66 ±0.02	6.54 ±0.02	6.53± 0.01	6.52 ±0.01	6.40 ±0.01	6.20 ±0.02
	Total colony count (cfu/mL) × 10^3	18 ± 1.000	0.44 ± 0.010	0.36 ± 0.010	0.22 ± 0.010	0.09 ± 0.050	0.03 ± 0.005	0.027 ± 0.004	0.014 ± 0.003	0.014 ± 0.002	0.03 ± 0.004	0.027 ± 0.003	0.030 ± 0.004
	Nisin activity (IU/mL)	200± 2	199± 2	175± 3	171± 2	170± 3	168± 4	166 ±3	165 ±2	163 ±2	160 ±3	160 ±2	158 ±3

1- In the table, (-) has considered for milk sample passing the test and (**+**) for milk sample failing the test.
2- Data have shown as Mean ± SD.
3- (Data not shown). After 42 days COB test proved positive.

Total colony count increased both in the pasteurized milk and the pasteurized milk with empty liposomes, though in the case of samples containing free and encapsulated nisin, at first, a decrease in total colony count was observed that gradually increased. Total colony count reduction in the free nisin milk samples is considerably more than in the encapsulated nisin milk samples.

Although a vast concentration between 10 IU/mL and 400 IU/mL has been asserted for milk preservation [33], in our experiments, an effective concentration was obtained with 200 IU/mL. In the 38-day pasteurized milk samples, 79% (158 IU/mL) of the free nisin's initial activity had remained intact (21% loss). This result suggests that preservation and inhibition of pasteurized milk spoilage can be achieved by lower nisin concentration. On the contrary, nisin activity in the pasteurized milk samples with encapsulated nisin (nanoliposomes) decreased from 198 IU/mL to 42 IU/mL. This means that 21% of the initial activity of encapsulated nisin remained unchanged. The cause of reduction can be due to the effect of nisin's solubility changes and protease inactivation [25].

Based on this study, free nisin was more effective than encapsulated nisin for preservation and delay of pasteurized milk spoilage which is highly in conformity with the previous studies, as they have reported that free nisin exerted statistically greater inhibition of *Listeria* on milk compared to liposomal nisin [25, 30].

Laridi*et al.* stated that liposome stability in milk was affected by milk fat concentration [6], and according to Kirby *et al.*, liposomes are largely limited to areas between fat globule and casein matrix [34]. Also, some interactions between fat globules and liposome membranes, and subsequently destabilization of liposomes membrane were mentioned by other investigators [35-36]. In addition, the presence of calcium ions in milk is another important factor in lipid membrane destabilization, which eventually leads to large vesicle aggregations [6].

4. Conclusion

Several advantages and disadvantages for free and encapsulated nisin have so far been suggested by different investigators. In this study however, the followings were concluded:

(i) Characterization of encapsulated nisin, especially entrapment efficiency, has significant role in food bio-preservation.

(ii) In addition to foods' structure and composition, their physicochemical properties, nisin's characteristics and other relative factors, nisin efficiency for bio-preservation of food stuffs basically depends on food matrices. Also, in order to obtain efficient bio-preservation with nisin, selection of an appropriate nisin form (free or encapsulated) and stability study of food product final formulation are necessary.

(iii) Effective concentration and correct form (free) of commercially available nisin (and not as a pure form and non-commercial nisin) with the objectives of pasteurized milk spoilage inhibition are additionally accomplished.

Milk spoilage is an overall issue in warm countries (transportation of pasteurized milk over long distances and poor refrigeration conveniences), and application of free nisin for spoilage prevention of pasteurized milk has several advantages; including availability of commercial nisin, lower cost of free nisin in comparison to encapsulated nisin, and longer shelf life.

5. Acknowledgment

We gratefully acknowledge Dr. Farzaneh Aziz Mohseni, head of Persian Type Culture Collection (PTCC) for her true assistance in providing bacterial strain and Mr. Abbas Rahimi from Pasteur Institute of Iran's Nano-biotechnology Department for particle size analysis. We would also like to thank and appreciate Dr. Saeed Sepehriseresht's collaborations in TEM laboratory of Tehran Heart Centre for electron microscopy imaging and also Majid Tavoosi in Iranian Research Organization for Science and Technology (IROST), Advanced Material and Renewable Energies Institute, for his technical assistance with the Aquinto a4i Docu software analysis.

6. Declaration of interest

The Authors declare that there is no conflict of interest.

References

1. Liu X, Chung Y, Yang S, Yousef AE. Continuous nisin production in laboratory media and whey permeate by immobilized *Lactococcus lactis*. Process Biochem. 2005; 40: 13-24. DOI: 10.1016/j.procbio.2003.11.032.
2. Deegan LH, Cotter PD, Hill C, Ross P. Bacteriocins: Biological tools for biopreservation and shelf-life extension. Int Dairy J. 2006; 16: 1058-1071. DOI: 10.1016/j.idairyj.2005.10.026.
3. Souza EL, Silva CA, Souza CP. Bacteriocins: Molecules of fundamental impact on the microbial ecology and potential food biopreservatives. Braz Arch Biol Technol. 2005; 48: 559-566. DOI: 10.1590/S1516-89132005000500008.
4. Anton R, Barlow S, Boskou D, Castle L, Crebelli R, Dekant W, Engel K, Forsythe S, Grunow W, Heinonen M, Chr. Larsen J, Leclercq C, Mennes W, Rosaria Milana M, Pratt I, Rietjens I, Svensson K, Tobback P, Toldrá F. Opinion of the scientific panel on food Additives, flavourings, processing aids and materials in contact with food on a request from the commission related to the use of nisin (E234) as a food additive. Eur Food Safety Authority J. 2006; 314, 1-16.
5. Benech RO, Kheadr EE, Lacroix C, Fliss I. Impact of nisin producing culture and liposome-encapsulated nisin on ripening of *Lactobacillus* added-Cheddar cheese. J Dairy Sci. 2003; 86: 1895-1909. DOI: 10.3168/jds.S0022-0302(03)73776-X.
6. Laridi R, Kheadr EE, Benech RO, Vuillemard JC, Lacroix C, Fliss I. Liposome encapsulated nisin Z: Optimization, stability and release during milk fermentation. Int Dairy J. 2003; 13: 325-336. DOI: 10.1016/S0958-6946(02)00194-2.
7. Sobrino-López A and Martín-Belloso O. Use of nisin and other bacteriocins for preservation of dairy products. Int Dairy J. 2008; 18: 329-343. DOI: 10.1016/j.idairyj.2007.11.009.
8. Were LM, Bruce B, Davidson PM, Weiss J. Size, stability and entrapment efficiency of phospholipid nanocapsules containing polypeptide antimicrobials. J Agric Food Chem. 2003; 51: 8073-8079. DOI: 10.1021/jf0348368.
9. Kopermsub P, Mayen V, Warin C. Nanoencapsulation of nisin and ethylenediaminetetraacetic acid in niosomes and their antibacterial activity. J Sci Res. 2012; 4: 457-465. DOI: http://dx.doi.org/10.3329/jsr.v4i2.9407.s.
10. Benech RO, Kheadr EE, Laridi R, Lacroix C, Fliss I. Inhibition of *Listeria innocua* in cheddar cheese by addition of nisin Z in liposomes or by in situ production in mixed culture. Appl Environ Microbiol. 2002; 68: 3683-3690. DOI: 10.1128/AEM.68.8.3683-3690.2002.

11. Ingebrigtsen L. and Brandl M. Determination of the size distribution of liposomes by SEC fractionation, and PCS analysis and enzymatic assay of lipid content. AAPS Pharm Sci Tech. 2002; 3: 1-7 (article 7). DOI: 10.1208/pt030207.

12. Colas JC, Shi W, Rao VSNM, Omri A, Mozafari MR, Singh H. Microscopical investigations of nisin-loaded nanoliposomes prepared by Mozafari method and their bacterial targeting. Micron 2007; 38: 841-847. DOI: 10.1016/j.micron.2007.06.013.

13. Taylor TM, Bruce BD, Weiss J, Davidson PM. Listeria monocytogenes and *Escherichia coli* O157:H7 inhibition in vitro by liposome-encapsulated nisin and ethylenediaminetetraacetic acid. J Food Safety. 2008; 28: 183-197.

14. Winkowski K, Ludescher RD, Montville TJ. Physicochemical characterization of the nisin-membrane interaction with liposomes derived from *Listeria monocytogenes*. Appl Environ Microbiol. 1996; 62: 323-327.

15. Bonev BB, Chan WC, Bycroft BW, Roberts GCK, Watts A. Interaction of the lantibioticnisin with mixed lipid bilayers: A ^{31}P and ^{2}H NMR Study. Biochem. 2000; 39, 11425-11433. DOI: 10.1021/bi0001170.

16. Wiedemann I, Breukink E, Van Kraaij C, Kuipers OP, Bierbaum G, De Kruijff B, Sahl HG. Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. J Biol Chem. 2001; 276: 1772-1779. DOI: 10.1074/jbc.M006770200.

17. Benech RO, Kheadr EE, Lacroix C, Fliss I. Antibacterial activities of nisin Z encapsulated in liposomes or produced in situ by mixed culture during Cheddar cheese ripening. Appl Environ Microbiol. 2002; 68: 5607-5619. DOI: 10.1128/AEM.68.11.5607-5619.2002.

18. Gao FH, Abee T, Konings WN. Mechanism of action of the peptide antibiotic nisin in liposomes and cytochrome c oxidase-containing proteoliposomes. Appl Environ Microbiol. 1991; 57: 2164-2170. DOI: 0099-2240/91/082164-07.

19. Pongtharangkul T. and Demirci A. Evaluation of agar diffusion bioassay for nisin quantification. ApplMicrobiolBiotechnol. 2004; 65: 268-272. DOI: 10.1007/s00253-004-1579-5.

20. Vojta A, Scheuring J, Neumaier N, Mirus O, Weinkauf S, Schleiff E. Determination of liposome size: A tool for protein reconstitution. Anal Biochem. 2005; 347: 24-33. DOI: 10.1016/j.ab.2005.09.003.

21. Wasiksiri S, Chethanond U, Pongprayoon S, Srimai S, Nasae B. Quality aspects of raw goat milk in lower southern Thailand. Songklanakarin J Sci Technol. 2010; 32: 109-113.

22. Hassan A, Amjad I, Mahmood S. Microbiological and physicochemical analysis of different UHT milks available in market. Afr J Food Sci. 2009; 3: 100-106.

23. Milk and milk products Enumeration of colony forming units of microorganisms colony count technique at 30°C. 2007. Institute of Standards and Industrial Research of Iran, ISIRI No. 5484, Tehran, Iran. (http://www.isiri.org, Date of Access: February 23, 2012).

24. Milk and milk products-General guidance for the preparation of test samples, initial suspensions and decimal dilutions for microbiolgical examination. 2007. Institute of Standards and Industrial Research of Iran, ISIRI No. 2406, Tehran, Iran. (http://www.isiri.org, Date of Access: February 23, 2012).

25. Schmidt SE, Holub G, Sturino JM, Taylor TM. Suppression of *Listeria monocytogenes* Scott A in fluid milk by free and liposome-entrapped nisin. Probiotics Antimicro Prot. 2009; 1: 152-158. DOI: 10.1007/s12602-009-9022-y.

26. Suganthi V, Selvarajan E, Subathradevi C, Mohanasrinivasan V. Lantibioticnisin: Natural preservative from Lactococcuslactis. Int Res J Pharm. 2012; 3: 13-19.

27. Nayar R, Hope MJ, Cullis PR. Generation of large unilamellar vesicles from long-chain saturated phosphatidylcholines by extrusion technique. BiochimBiophysActa. 1989; 986: 200-206. DOI: 10.1016/0005-2736(89)90468-9.

28. Mac Donald RC, Mac Donald RI, Menco BPM, Takeshita K, Subbarao NK, Hu LR. Small-volume extrusion apparatus for preparation of large unilamellar vesicles. BiochimBiophysActa. 1991; 1061: 297-303. DOI: 10.1016/0005-2736(91)90295-J.

29. Mozafari MR, Reed CJ, Rostron C. Cytotoxicity evaluation of anionic nanoliposomes and nanolipoplexes prepared by the heating method without employing volatile solvents and detergents. Pharmazie 2007; 62: 205-209. DOI: 10.1691/ph.2007.3.6045.

30. Da Silva Malheiros P, Daroit DJ, Da Silveira NP, Brandelli A. Effect of nanovesicle-encapsulated nisin on growth of *Listeria monocytogenes* in milk. Food Microbiol. 2010; 27: 175-178. DOI: 10.1016/j.fm.2009.09.013.

31. Zou Y, Lee HY, Seo YC, Ahn J. Enhanced antimicrobial activity of nisin-loaded liposomal nanoparticles against foodborne pathogens. J Food Sci. 2012; 77: M165-M170. DOI: 10.1111/j.1750-3841.2011.02580.x.

32. Tassew A and Seifu E. Microbial quality of raw cow's milk collected from farmers and dairy cooperatives in Bahir Dar Zuria and Mecha district, Ethiopia. AgricBiol J N Am. 2011; 2: 29-33. DOI: 10.5251/abjna.2011.2.1.29.33.

33. Delves-Broughton J. Nisin as a food preservative. Food Aust. 2005; 57:525-527.

34. Kirby CJ, Brooker BE, Law BA. Accelerated ripening of cheese using liposome-encapsulated enzymes. Int J Food Sci Technol. 1987; 22: 355-375. DOI: 10.1111/j.1365-2621.1987.tb00499.x.

35. Laloy E, Vuillemard JC, Simard RE. Characterization of liposomes and their effect on the properties of Cheddar cheese during ripening. Le lait. 1998; 78: 401-412. DOI: 10.1051/lait:1998439.

36. Kheadr EE, Vuillemard JC, El Deeb SA. Accelerated Cheddar cheese ripening with encapsulated proteinases. Int J Food Sci Technol. 2000; 35: 483-495. DOI: 10.1046/j.1365-2621.2000.00398.x.