Encapsulation of ascorbic acid in liposomes prepared with milk fat globule membrane-derived phospholipids

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ORIGINAL PAPER

Encapsulation of ascorbic acid in liposomes prepared with milk fat globule membrane-derived phospholipids

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Abstract The production of nanoliposomes is considered to be an effective technology for the encapsulation and controlled release of nutraceuticals and bioactive compounds, as well as for enhancing their stability and bioavailability. Although liposomes are generally prepared with phospholipids from soy or egg, in recent years, there has been a growing interest in the health benefits and functional properties of milk fat globule membrane-derived phospholipids, as these ingredients have become commercially available. The objective of this work was to characterize nanoliposomes prepared with milk phospholipids using microfluidization, and to evaluate their encapsulation behavior using ascorbic acid as a model biomolecule. Liposomes prepared by microfluidization with milk phospholipids showed an apparent diameter of about 100 nm. The incorporation efficiencies for ascorbic acid increased as the concentration of phospholipid-rich powder increased from 5 % to 10 % (*w/w*). However, from 10 % to 16 % (*w/w*), the incorporation efficiency reached a plateau value of 26 %. There appeared to be a change in liposome structure from unilamellar to multivesicular/multilamellar vesicles with high levels of phospholipids (16 %) and a high number of passes through the microfluidizer. Storage studies showed less stable dispersions when stored at pH 3 compared with pH 7. After 7 weeks at 4 °C, liposomes stored at pH 3 and pH 7 retained 67 % of the ascorbic acid originally encapsulated, while only 30 % remained when the dispersions were stored at 25 °C. The present work demonstrated that, using microfluidization, it was possible to obtain small, unilamellar milk phospholipids liposomes stable at neutral pH protecting ascorbic acid for at least 7 weeks.

脂肪球膜磷脂脂质体包埋抗坏血酸

摘要：制备纳米脂质体可以有效地对营养成分和生物活性物质包埋和控制释放，并且还可以提高这些物质的稳定性和生物利用率。普遍采用的脂质体有大豆磷

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脂和鸡蛋磷脂。近年来,随着人们对乳脂肪球膜磷脂营养性和功能性的认识,乳脂肪球膜磷脂的商业价值也不断提高。本文采用微射流技术制备纳米级乳磷脂脂质体,以抗坏血酸作为生物分子模型评价乳磷脂的包埋性质。微射流技术制备的乳磷脂脂质体的表观直径约为100 nm。对抗坏血酸的包埋效率随着磷脂浓度从5 %(w/w)到10 %(w/w)增加而增加,当浓度从10 %(w/w)增加到16 %(w/w)时,包埋效率达到26 %时趋向于平衡,原因是在高磷脂浓度(16 %)下大量的物质通过微射流设备后,使得脂质体结构从单层向多层囊泡或多囊泡结构转变。脂质体在pH3和pH7下贮藏后,前者的分散稳定性低于后者。在4 °C(pH3和pH7)贮藏7周后,经脂质体包埋的抗坏血酸的保留率为67 %,而在25 °C时,抗坏血酸的保留率仅为30 %。研究结果表明,采用微射流技术制备的单层乳磷脂脂质体包埋抗坏血酸的稳定可以保持7周(中性pH值)。

Keywords Milk fat globule membrane · Phospholipids · Liposomes · Encapsulation · Ascorbic acid

关键词 乳脂肪球膜 · 磷脂 · 脂质体 · 包埋 · 抗坏血酸

1 Introduction

Liposomes are lipid vesicles containing an aqueous core and a bilayer membrane composed of amphiphilic molecules (mostly phospholipids). Because of the lipid bilayer, liposomes can segregate hydrophilic compounds within their aqueous core, protecting them from unwanted degradation or interactions with other components in the outer phase. Because the consumption of phospholipids themselves have been associated with several health benefits (Imaizumi et al. 1992; Vesper et al. 1999; Van Hooijdonk et al. 2000; Ward et al. 2006; Hellhammer et al. 2010), there has been an increased interest in employing liposomes for controlled delivery of functional components in food products (Larivière et al. 1991, Thompson et al. 2009). The potential for the use of liposomes in the food industry ranges from protecting sensitive ingredients, to increasing the efficacy of food additives or confining undesirable flavors (New 1990; Taylor et al. 2005). However, most research has been reported on liposomes prepared with soy- or egg-derived phospholipids (New 1990; Lasic 1993). The phospholipids from milk can be extracted from a number of by-products such as buttermilk or butter serum (Corredig et al. 2003; Rombaut et al. 2006). Only recently, a new phospholipid extract from the milk fat globule membrane (MFGM) has become commercially available, and milk sources for these phospholipids have been suggested as a viable alternative to the more traditional soy or egg sources (Thompson and Singh 2006; Thompson et al. 2006).

Since the MFGM derives from the mammary cell, its composition is similar to that of any biological membrane, consisting of phospholipids, glycoproteins, neutral lipids, enzymes, and other minor components (McPherson and Kitchen 1983; Ward et al. 2006). In particular, milk phospholipids contain a high ratio of sphingolipids and glycosphingolipids to other phospholipids. Although there are no required dietary levels for sphingolipids, it has been reported that consumption of sphingolipids has significant health benefits, such as gut maturation (Oshida et al. 2003), and anti-carcinogenic properties (Dillehay et al. 1994; Schmelz et al. 1996). In addition, it has

been shown that consumption of dietary sphingolipids can reduce serum low-density lipoprotein-cholesterol in rats and increase serum high-density lipoproteins (Imaizumi et al. 1992; Schmitt 2009). In addition to sphingolipids, phosphatidylserine has also raised significant interest, because of its links to improved cognitive performance, stress release, and decreased age-induced memory loss (Burling and Graverholt 2008; Helhammer et al. 2010).

To prepare liposomes for food applications, it is necessary to employ techniques that will meet regulatory requirements and do not involve the utilization of solvents. In particular, liposomes prepared by high-pressure homogenization are of interest for the food industry, as their small size and substantial surface area provide unique functionalities to these colloidal particles. Indeed, structures in the nanometer scale have great potential, because of the large surface area available for interactions with target molecules and tissues. In addition, the small size of nanoliposomes makes them less susceptible to physical destabilization.

In the case of food applications, therefore, liposomes prepared with milk phospholipids may provide a number of advantages over the more traditional soy and egg sources. Comparing the amount of literature on the characterization of soy- or egg-derived phospholipid liposomes, and their encapsulation behavior for drug delivery (Lasic 1993; New 1994; Lasch et al. 2003; Zhang et al. 2005; Takeuchi et al. 2005), the information on the encapsulation properties of liposomes prepared with milk phospholipids, and their potential for application in food systems are still scarce (Thompson et al. 2006, 2009). As fractions of milk phospholipids derived from the MFGM become more readily available, there will be an increased demand for these ingredients for the preparation of liposomal nanoparticles, and studies on the properties of these nanoparticles in relevant food matrices will become increasingly critical.

The objective of this work was to evaluate the stability and encapsulation behavior of small unilamellar liposomes prepared with milk phospholipids using microfluidization. Ascorbic acid (vitamin C) was used as a model hydrophilic molecule. Ascorbic acid is added extensively to food products for two different purposes: as a vitamin supplement and as an antioxidant (Kirby et al. 1991). Ascorbic acid is very labile and can degrade rapidly, if not protected. The present study examined the ability of milk phospholipid liposomes to efficiently encapsulate and protect ascorbic acid from degradation.

2 Materials and methods

2.1 Materials

The milk phospholipids, derived from MFGM (NZMP Phospholipid Concentrate 700, stored vacuum-packed at –18 °C) were donated by Fonterra (Fonterra Co-operative Group, Palmerston North, New Zealand). The composition of the phospholipid powder and the fatty acids composition, based on manufacturer's specification, are shown in Tables 1 and 2. In addition, Table 1 reports mineral composition data, as measured by atomic absorption spectroscopy at Laboratory Services (University of Guelph). The powder was rich in phospholipids, with 85 % total lipids, as

Table 1 Composition of milk phospholipid-rich powder based on manufacturer's specification

Parameter	Mean result
Total lipids (%)	85
Phosphatidyl serine (%)	3
Phosphatidyl choline (%)	31
Phosphatidyl ethanolamine (%)	8.7
Sphingomyelin (%)	16.5
Moisture (% *w/w*)	1.74
Lactose monohydrate (% *w/w*)	6.6
Ash (%)	8.3
Minerals (mg/g)	
Calcium	0.28
Magnesium	0.055
Phosphorus	27
Potassium	17
Sodium	7.1

shown in Table 1. Ascorbic acid with 99.6 % purity was obtained from Fisher Scientific (Ottawa, ON, Canada). All other chemicals were of analytical grade and obtained from Fisher Scientific or Sigma-Aldrich Canada Ltd (Oakville, ON, Canada).

2.2 Liposome preparation

For the encapsulation experiment, the milk phospholipids were dispersed in an aqueous buffer (20 mM imidazole, 50 mM sodium chloride, 0.02 % sodium azide as a bacterostatic agent, and 1 % (*w/w*) ascorbic acid, pH 7.0). The dispersion was mixed thoroughly using a Polytron mixer (Brinkmann Inst. Corp., Mississauga, ON, Canada) at 7,000 rpm for 10 min. The phospholipid dispersion was then cycled through a microfluidizer (model M-110Y, Microfluidics Corporation, Newton, MA, USA) for five passes with an input air pressure of 580 bar (Thompson et al. 2006).

To study the effect of phospholipid concentration on the encapsulation efficiency of ascorbic acid, the dispersions were prepared with phospholipid-rich powder concentrations ranging from 5 % to 16 % (*w/w*). To produce liposomes with similar

Table 2 Fatty acid composition (%) of the milk phospholipid-rich powder

Fatty acid	Amount (%)
Myristic acid (14:0)	5.4
Palmitic acid (16:0)	20.9
Palmitoleic acid (16:1)	1.3
Stearic acid (18:0)	10.5
Oleic acid (18:1)	30.5
Linoleic acid (18:2)	4.3
Linolenic acid (18:3)	1.8
Arachidonic acid (20:4)	0.5

apparent diameters (about 100 nm), the number of passes through the microfluidizer was varied from 3 to 10 (higher concentrations required a greater number of passes to obtain 100 nm diameter vesicles).

2.3 Quantification of encapsulated ascorbic acid

Immediately following liposome preparation and after storage, the free (unencapsulated) ascorbic acid was separated from the liposomes containing the entrapped ascorbic acid using gel permeation chromatography. Aliquots (2.5 mL) of the liposome dispersion were passed through a 10×150 mm Sephadex G-25 gravity-fed PD-10 desalting column (GE Healthcare Bio-Sciences Corp, Piscataway, NJ, USA). Imidazole buffer (20 mM imidazole, 50 mM sodium chloride, 0.02 % sodium azide, pH 7) was used as the mobile phase. The fraction containing liposomes was collected in the first 4 mL, and then the rest of the free ascorbic acid was eluted. Control runs were carried out with empty liposomes as well as with free ascorbic acid, to test the performance of the column. Encapsulation efficiency was determined as the ratio between the amount of ascorbic acid recovered in the liposome fraction and the initial amount of ascorbic acid in the original sample. The changes in encapsulation efficiency with storage were evaluated by measuring the amount of ascorbic acid recovered in the liposome fraction after storage and calculating the ratio (amount after storage/amount initially present in the fresh liposome).

The concentration of ascorbic acid in fresh and stored liposomes was measured spectrophotometrically, based on MTT–formazan colorimetic assay, using the L-Ascorbic Acid Assay Kit (Megazyme International Ltd, Wicklow, Ireland), following manufacturer's instructions. In the first sample aliquot, L-ascorbic acid and all the other reducing compounds are measured. In the second sample aliquot, L-ascorbic acid is selectively removed with ascorbic acid oxidase and all remaining reducing substances measured. The reaction involves the reduction of the tetrazolium salt MTT to a formazan compound which is measured spectrophotometically at 578 nm. The difference between the first and second measurement is the quantity of the L-ascorbic acid. It is important to point out that the test does not account for oxidized ascorbic acid.

2.4 Liposome characterization

The average apparent diameter of the liposomes was determined using dynamic light scattering (Nanosizer, Malvern Instruments Ltd, Worcestershire, UK). The dispersions were diluted in imidazole buffer (20 mM imidazole, 50 mM sodium chloride, 0.02 % sodium azide, pH 7), and a medium viscosity of 1.054 mPa s was used for the calculation of the size from the diffusion coefficient data. The initial liposome particle size distribution was monomodal, and the average apparent diameter was measured over time during storage; any statistical deviation from the initial diameter was considered as primary stages of as colloidal instability.

Cryogenic transmission electron microscopy (Cryo-TEM) was employed to provide information on the microstructure of the liposome particles. The sample (4 μL) was pipetted onto a quantifoil (Quantifoil Micro, Jena, Germany) grid with 2-μm holes. The excess sample was blotted off in a vitrobot (FEI), (Eindhoven, Holland) and immediately plunged into liquid ethane held at liquid nitrogen temperature. The specimen was transferred under liquid nitrogen to a Gatan 626 Cryo holder

(Warrendale, PA, USA) and viewed at −176 °C in the Tecnai G2 F20 TEM (Eindhoven, Holland). Images are recorded with a Gatan 4 K bottom mount CCD camera using the Gatan Digital Micrograph software.

To determine the extent of lipid oxidation of the liposome dispersions, the peroxide value was measured using the IDF standard method (Shantha and Decker 1994), with slight modifications, to remove the ascorbic acid. Aliquots of the whole liposome suspensions (0.3 mL) were first mixed with 2 mL of chloroform–methanol (7:3 *v/v*) on a vortex mixer for 4 s, and then 9 mL of water was added. After mixing the suspension on a vortex mixer for 30 s, the samples were centrifuged at 4,000×*g*, for 3 min (IEC Centra CL2 centrifuge, Fisher Sci.). The extraction with 9 mL of water was repeated three times in total to ensure all ascorbic acid was removed. The determination of the peroxide values was then carried out as described in IDF method 74A:1991 (International Dairy Federation 1991), based on the co-oxidation of Fe(II) to Fe(III) by hydroperoxides and the formation of the red Fe(III)–thiocyanate complex. Absorbance was measured at 500 nm (Shimadzu UV-260, Spectrophotometer, Mandel Scientific, Guelph, ON, Canada). The peroxide values are reported as milliequivalents peroxide per 1,000 g sample.

2.5 Stability of liposome dispersions during storage

The stability of the liposomes stored at 4 °C and 25 °C was evaluated over a 7-week period. Prior to storage, a set of the liposome dispersions were adjusted to pH 3, with HCl 6 N and stored for 7 weeks. In addition, another set of pH 7 liposomes was diluted 1:1 (*v/v*) with a 10 % (*w/v*) dextrose solution and stored for 7 weeks. The liposome dispersions were assessed weekly by measuring changes in average hydrodynamic diameter, peroxide value, and amount of ascorbic acid retained.

2.6 Statistical analysis

A minimum of three replicate determinations were performed for each experimental treatment. Results are reported as mean±standard error of the mean for each treatment. All statistical analyses were conducted by Excel ($P<0.05$), using one- and two-way ANOVA (analysis of variance) where appropriate. Differences between treatments were identified using Tukey's multiple-comparison test ($P<0.05$).

3 Results

3.1 Determination of liposome size and structure

The apparent diameter of the liposomes prepared with varying concentrations of phospholipids (5 %, 8 %, 10 %, and 16 % phospholipid-rich powder) was measured using dynamic light scattering (DLS). All the liposomes dispersions showed a monomodal size distribution (Fig. 1), and the average sizes are summarized in Table 3. A greater number of passes was required to obtain similar size liposomes as the concentration of the phospholipid increased. The encapsulation efficiencies were compared using liposomes with similar average diameters (~100 nm). These

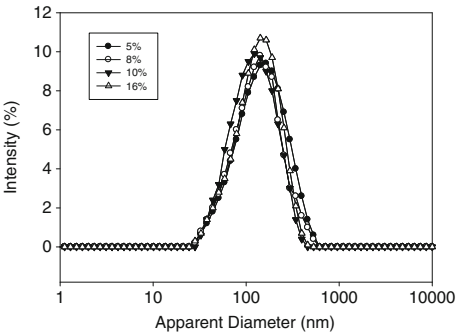

Fig. 1 Size distribution of liposomes prepared with varying concentrations of milk phospholipid-rich powder (5–16 %), as measured using dynamic light scattering. Representative data

samples were prepared by passing the 5 %, 8 %, 10 %, and 16 % phospholipid-rich powder samples through the microfluidizer 3×, 5×, 7×, and 10×, respectively.

The liposomes prepared were about 100 nm diameter and unilamellar. Electron microscopy confirmed the size measured by DLS, although liposomes smaller than 100 nm were visible in the micrographs. The apparent discrepancy between the two techniques is merely due to the nature of the light scattering measurement. Figure 2 shows a representative Cryo-TEM image of the phospholipid liposomes prepared with ascorbic acid. Cryo TEM was applied only for two concentrations (10 % and 16 % *w/w*) of phospholipid-rich powder samples.

3.2 Encapsulation of ascorbic acid

The amount of ascorbic acid encapsulated was measured immediately following liposome preparation. The liposomes were separated from the ascorbic acid present in solution, using column chromatography. After separation of the liposomes, the sizes were measured again using DLS, and the amount of entrapped ascorbic acid was measured as indicated above. The average apparent diameter of the liposomes did not change with the separation and remained about 100 nm, and the distribution was monomodal. The encapsulation efficiencies calculated for the liposomes prepared with varying phospholipid-rich powder concentrations are shown in Fig. 3. There was a clear proportionality of the amount of ascorbic acid entrapped and the volume fraction of the liposomes, up to 10 % phospholipid-rich powder concentration. After that, the encapsulation efficiency reached a plateau. The reason for this plateau can be found

Table 3 Average apparent diameter (nanometers) as a function of phospholipid-rich powder concentration as well as the number of passes through the microfluidizer

Phospholipid-rich powder concentration (*w/w*)	One pass	Three passes	Five passes	Seven passes	Ten passes
5 %	109 ±0.5^{a}	92±0.4^{b}	88±0.3^{c}		
8 %	115±0.5^{a}	106±0.8^{b}	97±0.4^{c}		
10 %	124 ±0.5^{a}	119±0.5^{b}	114±0.6^{c}	104±0.7^{d}	
16 %	130 ±0.2^{a}	124±0.6^{b}	120±0.2^{c}	117±0.9^{d}	105±0.8^{e}

Within a row, different letters indicate statistically significant differences ($P<0.05$)

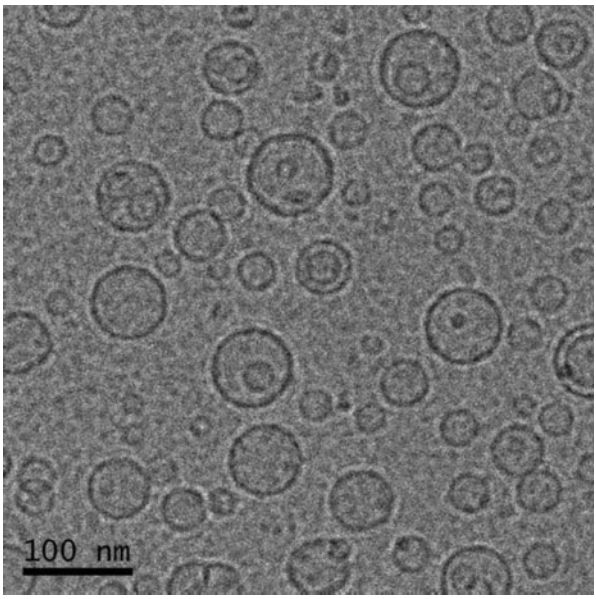

Fig. 2 Cryo-TEM micrograph of nanoliposomes containing ascorbic acid prepared with milk-derived phospholipids (10 % *w/w* powder) in 20 mM imidazole 50 mM NaCl buffer, using seven passes through the microfluidizer. Bar size is 100 nm

in the different structure of the liposomes prepared with 16 % milk phospholipid-rich powder. To determine if there were differences in the structure of the liposomes prepared with 16 % milk phospholipid-rich powder, compared with those prepared at a lower concentration (see Fig. 2), Cryo-TEM was also performed on the liposomes prepared with 16 % phospholipid (Fig. 4). These liposomes were passed ten times through the microfluidizer, to obtain a comparable size to those prepared with a lower concentration of phospholipids (see Table 3). The electron microscopy analysis suggested that, at the high concentration of phospholipids, the liposomes obtained had different structural features, with more multi-vesicles and multilamellar liposomes compared with the suspensions prepared with a lower amount of phospholipids.

All experiments on the stability of encapsulated liposomes were then conducted on unilamellar, small liposomal dispersions prepared with 10 % milk phospholipid-rich powder. These liposomes showed an encapsulation efficiency of 26 % in the fresh samples (see Fig. 3).

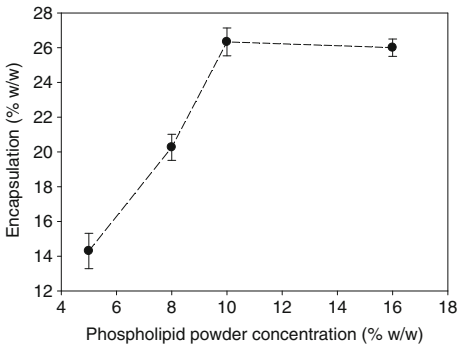

Fig. 3 Encapsulation (percent of total) of ascorbic acid in liposomes as a function of the milk phospholipid-rich powder concentration. Values are the average of three independent experiments with standard error

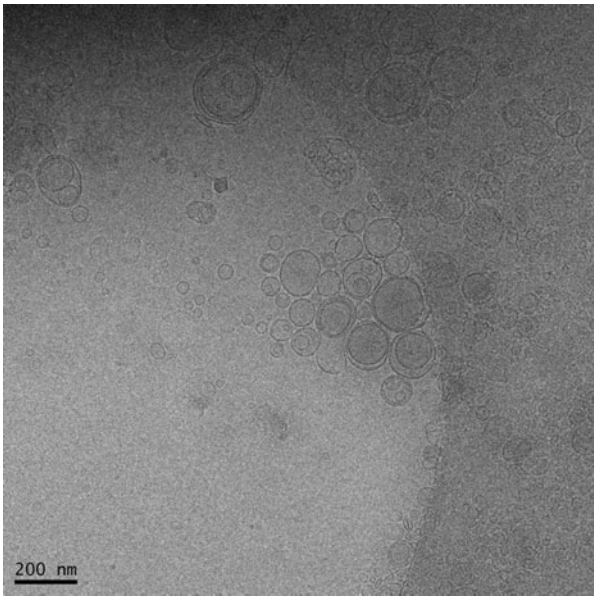

Fig. 4 Cryo-TEM micrograph of nanoliposomes containing ascorbic acid, prepared with milk phospholipid-rich powder (16 % *w/w*) in 20 mM imidazole 50 mM NaCl buffer, using ten passes through the microfluidizer. Bar size is 200 nm

3.3 Effect of environmental conditions (pH, temperature, and sugar concentration) on storage stability of the liposomes

The changes in the apparent size of the liposomes were monitored during storage at two temperatures (4 °C and 25 °C), two pH values (pH 3 and pH 7) and in the presence of 5 % sugar. Figure 5 illustrates the effect of pH, temperature, and storage time on the average diameter of the liposome dispersions prepared with milk phospholipids containing ascorbic acid. All dispersions showed stable particle size over storage time, apart from those stored at pH 3. In this case, the vesicles showed an increase in the apparent diameter, caused by the appearance of a small population of larger sizes. In this case, the change in size has to be taken simply as an indication of

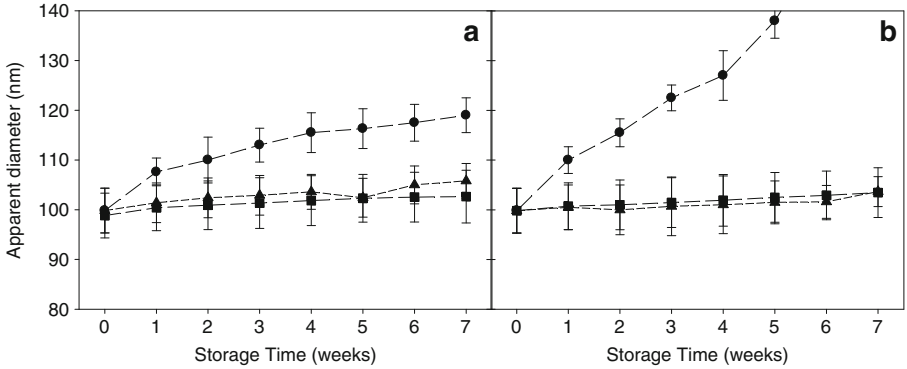

Fig. 5 Changes in apparent diameter of liposomes with entrapped ascorbic acid during storage at **a** 4 °C and **b** 25 °C for 7 weeks. Vesicles were stored at pH 3 (*circles*), pH 7 (*squares*), and at pH 7 in the presence of 5 % dextrose (*triangles*). Values are the average of three independent experiments, and bars represent the mean error

fusion of the vesicles, as measurements of size would no longer be accurate because of physical instability.

The amount of ascorbic acid recovered over time under different storage conditions was also measured for 7 weeks, and the results are summarized in Fig. 6. The encapsulation resulted in an increase of the stability of the ascorbic acid. In control experiments with free ascorbic acid, after 2 days there 57±4 % and 36±5 % ascorbic acid remained in vials stored at 4 °C or room temperature, respectively. The same samples showed 28±3 % and 4.3±1 % residual ascorbic acid when stored for 1 week at 4 °C or room temperature, respectively. The 4.5±1 % ascorbic acid was recovered in the unencapsulated samples stored for 2 weeks at 4 °C. In all cases, there was a decrease in the amount of ascorbic acid recovered within the liposomal core, and a faster degradation was noted when liposomes were stored at 25 °C compared with 4 °C. In addition, in spite of the lower physical stability of the liposomes stored at pH 3 compared with pH 7, the loss of ascorbic acid seemed to be similar. On the other hand, there was a steeper decrease in the amount of ascorbic acid recovered in the liposomes when these liposomes were stored in the presence of dextrose. These results suggested that the liposomes were affected by osmotic pressure changes.

To better identify the effect of storage on oxidative degradation of the liposome suspension, the peroxide value was measured during storage, as shown in Fig. 7. At 4 °C, there were very little peroxide values up to 3 weeks of storage, under all conditions, and there was an increase in the last 4 weeks (Fig. 7a). On the other hand, when stored at a higher temperature (25 °C, Fig. 7b), the peroxide values increased after 1 week of storage for samples stored at pH 3, while samples stored at pH 7 showed a lower extent of oxidation.

4 Discussion

Increasing the number of passes through the microfluidizer showed a statistically significant decrease in the size of the phospholipid liposomes ($P<0.05$), as previously reported (Thompson et al. 2006; Thompson and Singh 2006). The liposomes formed in the presence of ascorbic acid looked identical to empty liposomes, in terms of size

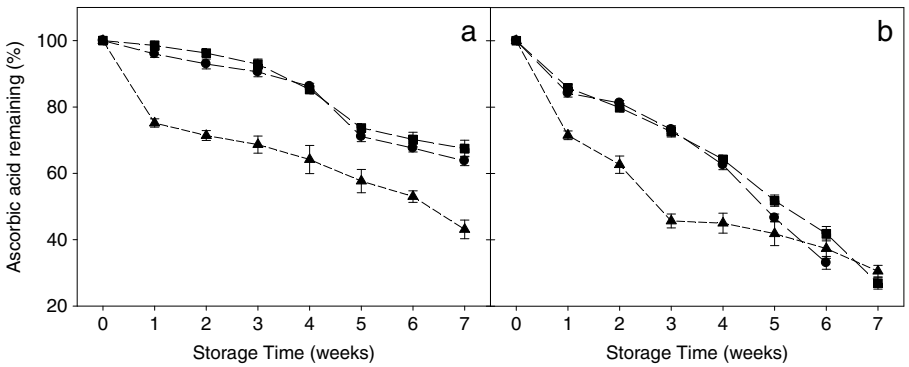

Fig. 6 Amount of ascorbic acid remaining in liposomes as a function of time during storage at **a** 4 °C and **b** 25 °C for 7 weeks. Vesicles stored at pH 3 (*circles*), pH 7 (*squares*), and in the presence of 5 % dextrose (*triangles*). Values are the average of three independent experiments, and bars represent the mean error

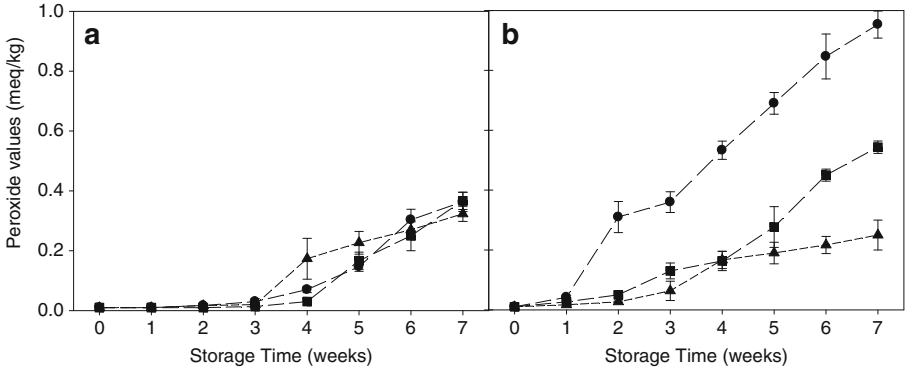

Fig. 7 Peroxide values measured in liposome dispersions containing ascorbic acid as a function of time during storage at **a** 4 °C and **b** 25 °C for 7 weeks. Vesicles stored at pH 3 (*circles*), pH 7 (*squares*) and at pH 7 in the presence of 5 % dextrose (*triangles*). Values are the average of three independent experiments, and *bars* represent the mean error

and lamellarity (Fig. 2), when compared with those shown in previous literature. In the range between 5 % and 10 % phospholipid-rich powder, increasing the phospholipids concentration increased the ascorbic acid entrapment; however, at higher concentrations, there was no further increase in the encapsulation efficiency (Fig. 3). These results are inconsistent with the expectation that the encapsulation efficiency is directly proportional to the concentration of phospholipid in the dispersed phase (Thompson et al. 2009). It was therefore hypothesized that, in the case of small phospholipid vesicles prepared with microfluidization, there is a limit to the volume fraction of colloidal particles that can be formed during the collisions in the microfluidizer; and at the higher concentrations, small unilamellar vesicles can no longer be obtained. Hence, the excess phospholipids are utilized to create multilayered membranes.

This hypothesis was confirmed by analyzing Cryo-TEM images. Electron microscopy observations of liposomes prepared with 16 % phospholipid-rich powder (*w/w*), after ten passes through the microfluidizer revealed that these specimens showed a higher number of vesicles with an altered overall structure and with sizes around 200 nm (Fig. 4). Although some unilamellar vesicles were still present (Fig. 4), there was a greater percentage of liposomes with multi-vesicular and multilamellar structures compared with the images shown in Fig. 2. In addition, there were quite a few liposomes>100 nm, which was the average apparent diameter, as measured by DLS (Table 3). The distribution of the liposomes was still monomodal when measured by DLS. The change from small unilamellar to more large unilamellar and multilamellar liposomes observed with 16 % milk phospholipid-rich powder has never been reported in the literature and indicated a feature unique to microfluidized liposomes, whereby above a critical phospholipids concentration the vesicles formed no longer unilamellar. This explained the plateau values for encapsulation efficiency reached at high phospholipids concentrations (Fig. 3).

The maximum encapsulation efficiencies obtained in the present work were 26 % and significantly lower than those reported by Kirby et al. (1991). The source of the discrepancy derives from the method of preparing the liposomes. In the study by Kirby et al. (1991), a maximum of 50 % encapsulation was reported for liposomes

containing egg phosphatidylcholine together with cholesterol and α-tocopherol. However, in this case, large multilamellar liposomes were formed using a dehydration/rehydration method. On the other hand, the encapsulation efficiencies reported in the present work are in agreement with those shown in previous research using microfluidization to prepare milk derived phospholipid liposomes (Thompson et al. 2009).

The storage stability of small unilamellar liposomes prepared with milk phospholipids was followed either at 4 °C or 25 °C and under three model conditions, pH 3, pH 7, or in the presence of 5 % dextrose solution. For this part of the study, a suspension prepared with 10 % phospholipid-rich powder was studied, as it yielded a high encapsulation (26 %) and the liposomes were unilamellar in nature. There were no significant differences ($P>0.05$) in the observed average diameter over 7 weeks in all samples stored at pH 7, with or without 5 % sugar. On the other hand, the samples stored at pH 3 clearly showed colloidal destabilization over time. The low pH lowered the extent of the charges of the phospholipid molecules, altering their ability to coalesce upon collision. Temperature also had an effect, as the liposomes stored at 25 °C (Fig. 5b) destabilized faster than those stored at 4 °C (Fig. 5a). Because of the visible phase separation of these samples, the apparent size distribution was no longer accurate, and the aggregation noted in Fig. 5 has to be taken as an indication of colloidal instability.

There was visible phase separation in the liposome suspensions stored at pH 3 and 25 °C, and the physical destabilization was most probably the cause of the high values of peroxides measured under those conditions (Fig. 7b). On the other hand, when looking at the amount of ascorbic acid still recovered in the liposomes remaining, it seemed clear that the liposomal core protected ascorbic acid from a rapid loss. In general, there were lower losses of ascorbic acid (as well as lower peroxide values) during storage at 4 °C compared with 25 °C.

Unencapsulated ascorbic acid, stored in buffer solution, was completely degraded after 1 or 2 weeks of storage at 25 °C or 4 °C, respectively (data not shown). The rapid loss of unencapsulated ascorbic acid was also consistent with earlier reports (Kirby et al. 1991), who showed complete loss of free ascorbic acid after 18 days refrigerated storage and 6 days at room temperature. Figure 6 shows that amongst the three experimental treatments, the highest losses of ascorbic acid were incurred in the presence of 5 % sugar. These results suggest that, in the presence of sugar, the osmotic balance was shifted and ascorbic acid diffused out of the core. The losses once again were higher during storage at 25 °C compared with 4 °C.

While the losses of encapsulated ascorbic acid occurred right from the start, a secondary destabilization occurred later, as indicated by the peroxide values (Fig. 7). While at 4 °C, the rate of peroxide formation increased only after week 3 (Fig. 7a), at 25 °C, the losses were very rapid.

5 Conclusion

As more information becomes available on the health benefits associated with the consumption of MFGM components, the advantages of using milk phospholipids derived from the MFGM in formulation of delivery systems for foods become more apparent. More research is then needed to better understand the differences in

physico-chemical properties and encapsulation behavior between the MFGM and other more established liposome systems, such as those prepared with egg and soy phospholipids. In addition, it is increasingly important to understand if the small unilamellar milk phospholipid vesicles prepared with methods such as microfluidization are a suitable delivery platform. The present work demonstrated that it is possible to protect ascorbic acid by encapsulation in liposomes prepared with milk-derived phospholipids. Using microfluidization, it was possible to obtain small, unilamellar MFGM liposomes, stable at neutral pH for at least 7 weeks. The values of encapsulation efficiency reached a plateau at about 10 % milk phospholipid-rich powder, and at higher concentrations, small multivesicles form. After storage at 4 °C and pH 7, 67 % of the original encapsulated ascorbic acid was still retained after 7 weeks. The liposomes showed lower stability at pH 3 than at pH 7, and they showed an increased permeability when stored in 5 % sugar solutions.

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