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Storage Stability and Physical Characteristics of Tea-Polyphenol-Bearing Nanoliposomes Prepared with Milk Fat Globule Membrane Phospholipids

İbrahim Gülseren* and Milena Corredig

Department of Food Science, University of Guelph, Guelph, Ontario, Canada N1G2W1

ABSTRACT: The objective of this work was to better understand the functional properties of milk phospholipids when used as ingredients to prepare liposomes. Liposomal dispersions (10%) were prepared using high-pressure homogenization, and their physical properties as well as their ability to encapsulate tea polyphenols were investigated. The extent of encapsulation, measured by HPLC, increased with tea polyphenol concentration up to about 4 mg·mL^{-1}. At polyphenol concentrations ≥ 6 mg·mL^{-1}, the liposome dispersions were no longer stable. The influence of pH (3−7), storage temperature (room temperature or refrigeration), and addition of sugars (0−15%) were studied for liposomes containing 4 mg·mL^{-1} polyphenols. The liposomal dispersions were also stable in the presence of peptides. The storage stability of the systems prepared with milk phospholipids was compared to that of liposomes made with soy phospholipids. Soy liposomes were smaller in size than milk phospholipid liposomes, the encapsulation efficiency was higher, and the extent of release of tea polyphenols during storage was lower for milk phospholipid liposomes compared to soy liposomes. The results suggest that milk phospholipids could be employed to prepare tea-polyphenol-bearing liposomes and that the tea catechins may be incorporated in the milk phospholipid bilayer more efficiently than in the case of a soy phospholipid bilayer.

KEYWORDS: *milk phospholipid nanoliposomes, soy phospholipid nanoliposomes, tea polyphenols, encapsulation, storage stability, ultrafiltration*

INTRODUCTION

Due to the potential health-promoting effects of bioactive compounds, there has been an increased interest in developing delivery systems that would allow for the inclusion of these compounds in food products at concentrations sufficient to be biologically relevant. For example, improved uptake of bioactive compounds in the daily diet for prevention or slowing down of carcinogenesis could lead to a lower cancer burden for both individuals and societies.[1] A particularly large effort has been dedicated to the design of delivery vectors using food-grade ingredients. Similar requirements stand for the reagents and processes employed in the manufacture of such vectors. Therefore, stable delivery systems that can endure the stresses of food processing environments such as changes in pH and temperature, the presence of osmotic stresses, and interactions with a wide variety of food molecules might be necessary. Liposomes are lipid vesicles that are made out of bilayer-forming lipids, the majority of these compounds being phospholipids. These layers (one or more) are arranged concentrically around a central aqueous phase.[2] Compared to other colloidal delivery systems, liposomes have certain advantages: they are easily biodegraded and weakly immunogenic and possess limited intrinsic toxicity.[3] Furthermore, liposomes enable controlled delivery of both hydrophilic and hydrophobic compounds, protect sensitive ingredients against processing or storage-induced degradation, and increase the efficacy of active compounds.[4]

The methods of liposome preparation have been extensively reviewed.[2,5] Well-designed liposomes with a narrow particle size distribution, high encapsulation efficiency, long-term stability, and optimized release properties could successfully protect bioactive compounds from degradation in food processing.[5] Liposomes have been commonly used as both model systems and delivery vehicles in biomedical and pharmaceutical research. Their utilization in ripening of cheese has previously been investigated.[6] In addition, they were utilized in the encapsulation of food antimicrobials,[7] phytosterols,[8] and purified polyphenols[9] or polyphenol extracts.[10] Soy or egg is usually the preferred source for phospholipids. Recently it has been demonstrated that milk-derived phospholipids can be employed to manufacture liposomes.[11] Liposomes prepared with milk phospholipids can encapsulate both hydrophilic and hydrophobic (i.e., β-carotene) compounds.[4,12] Since this methodology utilizes high-pressure homogenization, it has the capabilities of being scaled-up in industrial settings.

As previously mentioned,[6] liposomes can be destabilized upon mixing with biological liquids such as milk and blood plasma. Some biological compounds may alter the bilayer permeability barrier by inducing changes in bilayer structure. Leakage, for example, can be induced by changes in the lateral packing of the bilayer. Osmosis, partial dehydration, or the absorption of amphiphilic compounds (detergents, bile salts, peptides, proteins, sugars, and amino acids) can cause instabilities.[13] For example, bile salts[14] and small-molecule surfactants such as Triton-X-100[15] have been shown to cause liposomal destabilization. Even traces of surfactants can lead to liposomal leakage.[16] In the case of casein addition, although no

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interactions on the surface of the phosphatidylcholine vesicle were detected, casein molecules were incorporated into the hydrophobic core of the bilayer.[17] It has been recently found that the presence of β-casein induces the clustering of liquid ordered microdomains of milk phospholipid monolayers.[18]

Tea polyphenols have antioxidant properties and are effective at decreasing cancer cells growth. They have been shown to block the formation of carcinogenic compounds, suppress the activation of carcinogens, and trap genotoxic compounds. [19] The major polyphenol of tea (i.e., epigallocatechin-3-gallate, EGCG) has demonstrated antioxidant activity, antibacterial effect, anticarcinogenesis, antihypercholesterolemia, improvement of hyperglycemia, cutaneous photoprotection from UV radiation).[20] However, tea catechins are easily oxidized under ambient conditions and light exposure, and the useful life of aqueous solutions of tea polyphenols has been shown to be about 18 h. [21]

Liposomes containing tea polyphenols have been recently tested as delivery systems on colon cancer cells (HT-29).[22] Both free and encapsulated tea polyphenols caused a reduction in viability of HT-29, showing that small unilamellar liposomes prepared with milk phospholipids can deliver bioactive material to the intestinal cells and do not affect the uptake of polyphenol compounds. The work demonstrated that, although liposome-encapsulated polyphenols were slightly less efficient compared to free polyphenols in reducing the viability of human adenocarcinoma HT-29 cells, they will protect polyphenols from degradation and offer an extended period of stability and delivery of a high concentration of polyphenols over time. In addition to their anticancer properties, various components of milk fat globule membrane, including milk phospholipids and sphingolipids, are known to demonstrate anticancer, anti-cholesteremic, anti-infection, or antiadhesion properties[23−25] and therefore are functional food ingredients that can further encapsulate health-promoting food ingredients.

The objective of the current study was to determine the physicochemical properties of the liposomes prepared with milk phospholipids and loaded with tea polyphenol and evaluate the effect of storage under different pH conditions, in the presence of surface-active peptides, with varying concentrations of sucrose, and during ultrafiltration. Tea polyphenols studied here are water-soluble/dispersible compounds that may interact with lipid bilayers. It was hypothesized that liposomes prepared with different phospholipids may show different behavior, especially when tested under different environmental conditions.

MATERIALS AND METHODS

Materials. Bovine milk phospholipid concentrate 700 (PC-700) and Ultralec F lecithin were donated by Fonterra USA, Inc. (Rosemont, IL, USA) and ADM (Decatur, IL, USA), respectively. The commercial tea polyphenol sample used here was Teavigo, a gift of DSM Nutritional Products (Ayr, Ontario, Canada). According to the manufacturer, PC-700 contained 85.1% lipids (3% phosphatidylserine, 31.5% phosphatidylcholine, 8.7% phosphatidylethanolamine, and 16.5% sphingomyelin). The moisture content was 1.74%, and PC-700 contained 6.6% lactose and 8.3% ash.[26] Ultralec F lecithin was a commercial deoiled soy lecithin sample mostly composed of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol. More detailed information on the composition of phospholipid samples can be found elsewhere.[27]

Teavigo contains a high concentration of (−)-epigallocatechin-3-gallate (EGCG) (min. 94%). Sodium chloride, imidazole, HPLC-grade water, acetonitrile, methanol, chloroform, and glacial acetic acid were obtained from Fisher Scientific. Sodium azide (0.02% by wt) (catalog no. S227I, Fisher Scientific, Fair Lawn, NJ, USA) was added to all solutions to prevent bacterial growth.

Sample Preparation. Liposomes were prepared as previously described.[8,11] Milk or soy phospholipids (10%) were dispersed in buffer (20 mM imidazole, 50 mM NaCl in Milli-Q water, pH 7) using a magnetic stirrer (1 h). The mixtures were prehomogenized using a shear mixer (Power Gen 125, Fisher Scientific, Mississauga, Ontario, Canada) at full speed (30 000 rpm) for 1 min and homogenized using a one-stage high-pressure homogenizer (Nano DeBee, BEE International, South Easton, MA, USA) at a homogenization pressure of 690 bar for two passes, unless otherwise stated. For the encapsulation of tea polyphenols in liposomal dispersions, tea polyphenols (4 $mg{\cdot}mL^{-1}$) were added to the mixture prior to prehomogenization.

Determination of Apparent Particle Diameter and ζ-Potential Using Dynamic Light Scattering. The apparent size and ζ-potential of the liposomes were determined using a dynamic light scattering (DLS) technique (Zetasizer Nano, Malvern Instruments, Worcestershire, UK). The latex reference samples used for size and ζ-potential measurements were purchased from Duke Scientific, Palo Alto, CA, USA (catalog nos. 3550A and 5009A). The liposomal dispersions were appropriately diluted using prefiltered imidazole buffer for size and ζ-potential measurements. Refractive indices of phospholipids and water were taken as 1.45 and 1.33, respectively.

Separation of Unentrapped Polyphenols. Immediately after homogenization free polyphenols were removed from liposomal dispersions with a desalting column (HiTrap desalting column, product no. 17-408-01, GE Healthcare, Uppsala, Sweden) as previously described.[4,8] The elution buffer that was used in the removal of free polyphenols was identical to the sample preparation buffer.

In selected experiments, the liposomal dispersions were concentrated using a centrifugal ultrafiltration method (5000*g*, Avanti J-E centrifuge, supplied with a JA-25.50 rotor and 10 kDa filters, Beckman Coulter, Inc., Mississauga, ON, Canada). These tests were carried out to determine if separation of the unbound material could be carried out using this method and if the liposomes would withstand the separation/concentration process. Different centrifugation times were applied, 0, 15, 30, 60, 90, 120 min with a constant speed of 5000*g*. The ultrafiltered liposomes were analyzed for their size, surface charge, and encapsulation characteristics as detailed above.

RP-HPLC-Based Quantification of Encapsulated Polyphenols. After separation of the liposomes from the unentrapped polyphenols, the liposome fraction was analyzed for encapsulated polyphenols. Separation of the liposomes (by gel filtration, unless otherwise indicated) was followed by a Bligh−Dyer extraction[28,29] to disrupt the liposomes and collect the encapsulated polyphenols in the aqueous phase. This technique is based on the solubilization of lipids in methanol followed by their transfer to the more lipophilic phase upon the addition of acetone. The extraction procedure was followed by centrifugation to ensure the complete separation of the two phases. The samples were centrifuged at 5000*g* for 30 min (Eppendorf 5415D, Brinkmann Instruments, Westbury, NY, USA). Rapidly afterward, the isolated samples were analyzed using a reversed-phase HPLC technique,[30] with minor modifications. The column used for this analysis was a Nova-Pak C18 column (4 μm, 3.9 × 150 mm, part no. WAT086344, Waters Corporation, Milford, MA, USA). The column was supplied along with a guard column (WAT44380) that had the identical chemistry as the analytical column. A binary mobile phase was utilized for the gradient elution of EGCG, and it consisted of 2% acetic acid in HPLC water (buffer A) and 100% acetonitrile (buffer B). A linear gradient of 1−70% acetonitrile was carried out in the first 20 min of analysis, which was followed by two linear gradients to reach 99% B and initial conditions (1% B), each of which account for 5 min. The detection was carried out at 280 nm. Temperature and flow rate were kept constant throughout the analyses (35 °C and 1 $mL.min^{-1}$, respectively). In all cases, the major ECGC peak was acquired around an acetonitrile concentration of 14%. The measurements were performed in triplicate for every treatment and subsample.

Storage Experiments. Storage stability of liposomal dispersions was investigated at varying pH values (pH 3–7) and temperature (4 or 25 °C) or in the presence of hydrophobic peptides or sucrose (5–15%). Immediately after liposome preparation, the pH values of the liposomal dispersions were altered using concentrated acid or base solutions (0.5 M HCl or NaOH). For sugar inclusion experiments (pH 7, 25 °C), a stock sucrose solution (30%, S5, Fisher Scientific, Fair Lawn, NJ, USA) was prepared, and liposomal dispersions were diluted 1:1 using appropriate volumes of sucrose solution and Milli-Q water.

Peptide Preparation. An aqueous dispersion of sodium caseinate (5 mg·mL^{-1}, Alanate 180, Lamoyne, PA, USA) was prepared in 10 mM sodium phosphate buffer (pH 7) by stirring for 1 h and overnight refrigeration (4 °C) to ensure complete hydration. Trypsin was obtained from Sigma Aldrich Corp. (T1426, Oakville, ON, Canada). Tryptic digestion was carried out for 2 h at an enzyme to protein ratio of 1:1000 on a thermomixer (Eppendorf 5436, Brinkmann Instruments, Mississauga, ON, Canada, 37 °C, 1000 rpm). Immediately afterward, the digest was heated to 95 °C and held at this temperature for 5 min. The samples were rapidly cooled on ice, and once room temperature was reached, they were centrifuged for 30 min at 5000*g* to remove insoluble aggregates (Eppendorf 5415D centrifuge). Finally, the samples were filtered through 0.45 μm PVDF syringe filters (Millipore, Millex HV, Billerica, MA, USA) and mixed with liposomal dispersions within 2 h of preparation. The mixtures were analyzed for their size, surface charge, and encapsulation characteristics as described above. Prior to analyses, the mixture was kept stirred for 2 h.

To determine the molecular mass distribution of the prepared peptides, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) was carried out. Aliquots (2 μL) of sample were mixed directly with the matrix solution (2 mg of sinapinic acid in 100 μL of 50:50 acetonitrile/water, with 0.1% TFA) in an analyte to matrix ratio 1:1 (v/v), with 1 μL being spotted on a MALDI plate and allowed to dry at room temperature. In some instances cocrystallization formation was subpar, which required the preparation of a new sample with an analyte to matrix ratio 1:2 (v/v). MALDI-TOF MS analysis was performed using a Reflex III (Bruker, Germany) equipped with a 337 nm nitrogen laser (Mass Spectrometry Facility, University of Guelph). Positive ions were detected in linear mode scanning from 1000 to 20 000 m/z and using ion suppression up to 500 m/z. For all experiments, the ion sources 1 and 2 were held at 20 and 16.35 kV, respectively. Four-point external calibration was performed, using the cytochrome *c* (12 361.1 Da), horse myoglobin (16 952.6 Da), trypsinogen (23 981.9 Da), and BSA (66 431 Da) peaks prepared in acetonitrile/water solution (1 mg sinapinic acid in 100 μL of 50:50 acetonitrile/water, with 0.1% TFA). All MALDI-TOF MS reagents were purchased from Sigma-Aldrich. The mass accuracy with external calibration is estimated to be below 100 ppm.

RESULTS AND DISCUSSION

Physicochemical Characterization of Milk Phospholipid Liposomes. The apparent hydrodynamic size of the liposome dispersion was analyzed as a function of polyphenol concentration (0–5 mg·mL^{-1}) using dynamic light scattering (Figure 1). In all cases, the liposomes showed a mean apparent diameter of about 140 nm. The presence of the EGCG in the liposomes dispersion did not affect their particle size nor their stability up to 5 mg·mL^{-1}. All the loaded liposomes showed a similar particle size to that of empty milk phospholipid liposomes. At concentrations of about 6 mg·mL^{-1}, visual destabilization occurred. On the basis of the molecular weights of EGCG and PC as the most concentrated phospholipid in the sample, the molar ratio of phospholipid to EGCG is approximately 10 (i.e., 10.05) at the destabilization concentration (6 mg·mL^{-1} tea polyphenols). All liposomes showed a negative zeta potential, with a charge of about −20 mV.

The extent of polyphenol encapsulation was determined as a function of initial tea polyphenol concentration in the mixture

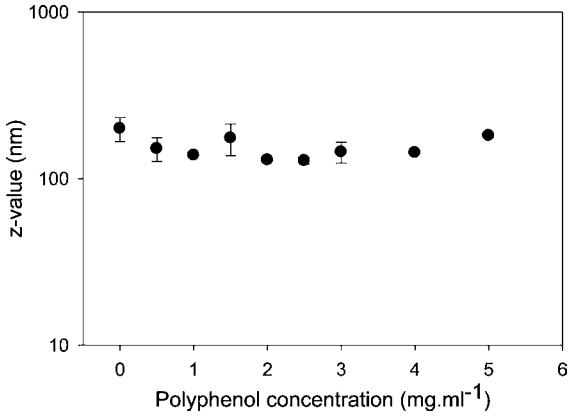

Figure 1. Apparent diameter (*z*-value) of milk phospholipid liposomes as a function of the initial tea polyphenol concentration (0–6 mg·mL^{-1}) determined using dynamic light scattering. Values are the average of three independent experiments, and the error bars indicate the standard deviation.

(Figure 2). Up to a polyphenol concentration of about 4 mg·mL^{-1}, the amount of tea polyphenol recovered in the

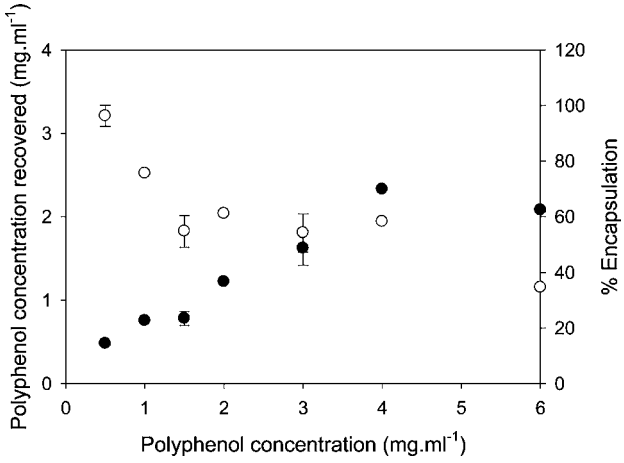

Figure 2. Amount of tea polyphenols recovered in the milk phospholipid liposome fraction (filled symbols) and encapsulation efficiency (empty symbols) as a function of polyphenol concentration (0–6 mg·mL^{-1}) present in the initial suspension. Values are the average of three independent experiments, and the error bars indicate the standard deviation.

liposomal phase increased with the initial polyphenol concentration. At very low concentrations (<1.5 mg·mL^{-1}) almost all the polyphenols were recovered in the liposomal fraction, and the amount of polyphenols encapsulated in the liposomes reached a plateau around 60% at higher concentrations. Beyond 4 mg·mL^{-1}, there seemed to be a decrease in the encapsulation efficiency, and this was related to the decrease in colloidal stability, as at ≥6 mg·mL^{-1}, visible liposomal destabilization took place in much less than 1 h after homogenization.

At a concentration of 4 mg·mL^{-1}, the percent encapsulation was roughly 58%. These results are in agreement with the literature. It has been previously reported[31] that nanoliposomes prepared with appropriate amounts of soy lecithin and cholesterol show an entrapment efficiency of 61.5% for tea polyphenols from green tea. In this case, the methodology used in the preparation of liposomes was a reversed-phase

evaporation technique. The extent of total tea polyphenol leakage was around 0.4% and 2.9% at refrigeration and room temperature, respectively, after 15 days.[31] While high encapsulation efficiencies have been reported for liposomes prepared by reversed-phase evaporation, high values of entrapment for hydrophilic compounds have not been reported for liposomes prepared with high-pressure homogenization. For example, values of encapsulation efficiency of about 30% have been measured for ascorbic acid in milk phospholipid liposomes prepared by high-pressure homogenization or microfluidization.[4,8] Ascorbic acid is highly soluble in water and can be expected to remain in the aqueous core of the liposomes. However, this may not be the case for EGCG. It has been previously demonstrated that EGCG is incorporated in lipid bilayers and may alter membrane fluidity. Indeed, leakage of intramembranous materials and aggregation of liposomes were observed.[32,33] It was demonstrated that at low concentrations EGCG and ECG prevent leakage of calcein from liposomes, possibly due to their gallic acid ester contents, which is responsible for their bilayer affinity, while at high concentrations, the polyphenols may disrupt the membrane structure.[33] Therefore, it is possible to assume that EGCG was incorporated in the bilayer during the process of homogenization, and at elevated polyphenol concentrations destabilization occurred. The present results also are in full agreement with recent studies using a more heterogeneous polyphenol extract (i.e., grape seed extract) in liposomal encapsulation.[10] However, while in the case of the grape seed extract there was an increase in the apparent size of the soy liposomes, in the present work, the incorporation of EGCG did not significantly increase the mean particle diameter. The discrepancy may be caused by the type of polyphenols or by the source of phospholipids.

To further test the behavior of tea-polyphenol-loaded liposomes, dispersions containing 4 mg·mL^{-1} of EGCG were analyzed, as this amount corresponded to high encapsulation efficiency with no detrimental effects on particle size and colloidal stability. The utilization of polyphenol (or other bioactives)-bearing liposomes in food products would require them to resist a variety of stressors including changes in medium pH, temperature, particle concentration (i.e., dilution or concentration), the presence of surface-active molecules, and osmotic stresses. In this study, we made an effort to analyze the influence of some of these factors.

Separation of Free EGCG Using Ultrafiltration. Ultrafiltration can be regarded as an appropriate technique for the recovery and utilization of the nonencapsulated and valuable bioactive molecules.[34] In particular, this technique could be employed as an efficient method to separate liposomes from their unencapsulated contents.[35] This method was evaluated as an alternative means to separate unencapsulated EGCG from liposomes. Figure 3 illustrates the effect of ultrafiltration duration (up to 2 h) on the apparent size and zeta potential of the liposomes, as well as their encapsulation efficiency. At a constant centrifugation rate (5000*g*), although there were no changes in particle size, some reduction in the magnitude of ζ-potential was measured (Figure 3A). However, there were no changes in the stability of the liposomal dispersion, and there was a slight significant change in the encapsulation efficiency (Figure 3B) after long centrifugation times ($p < 0.05$). These results indicated that ultrafiltration could be employed in the concentration of milk phospholipid liposomes and the recovery of nonencapsulated bioactive molecules. However, it was concluded that at long ultrafiltration times some deposition of EGCG may occur on the membrane filter, causing a slight overestimation of the encapsulation efficiency.

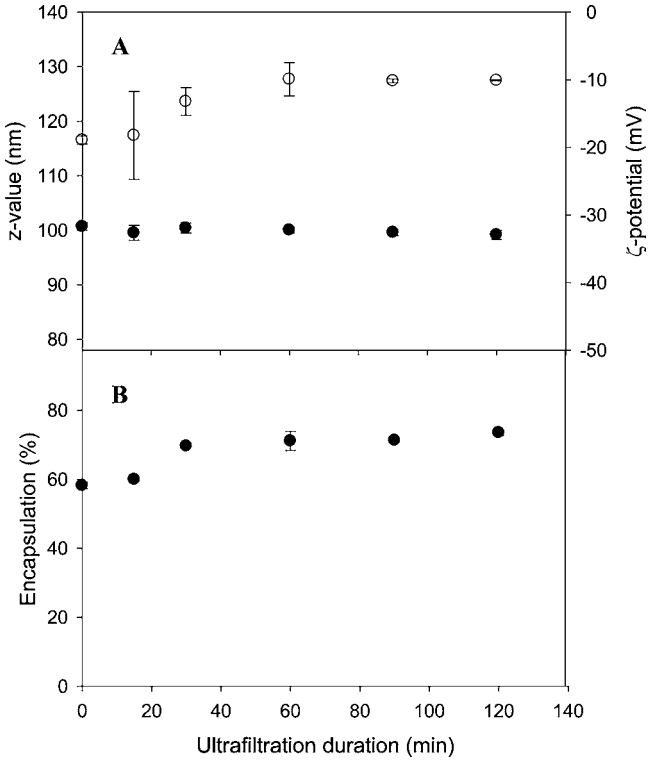

Figure 3. Effect of ultrafiltration time on the mean particle size (*z*-value) (filled symbols) and ζ-potential (empty symbols) (A) and encapsulation efficiency (B) of milk phospholipid liposomes. Values are the average of three independent experiments, and the error bars indicate the standard deviation.

Encapsulation Behavior and Storage Stability of Liposomes Prepared with Milk or Soy Phospholipids. To better understand the encapsulation behavior of liposomes loaded with EGCG, dispersions prepared with milk phospholipid liposomes were compared to those prepared with soy-derived phospholipids. The storage stability and release of EGCG of the loaded liposomes prepared with milk phospholipids was tested and compared to that of liposomes prepared under similar conditions, but containing soy phospholipids, as the latter have been commonly used in liposome preparation in the literature. In all cases, the liposomes were prepared containing 4 mg·mL^{-1} of EGCG.

Effect of Storage pH at Room Temperature. Immediately after preparation, aliquots were taken and the pH was adjusted to 3, 5, or 7. Mean particle size and surface charge characteristics were analyzed for 14 days during storage at room temperature. In agreement with previous results,[12,22] soy liposomes had a lower mean size than milk phospholipid liposomes (Table 1). Size and ζ-potential values were comparable between empty and tea-polyphenol-bearing liposomes;[22] hence only loaded liposomes are compared in Table 1. For milk phospholipid liposomes, the apparent mean diameter did not show a significant change when stored at pH 5 and 7. An increase in size with storage was observed for these dispersions at pH 3, possibly due to the decreased surface charge at this pH. Indeed, in the case of milk phospholipid liposomes, the surface charge decreased to about −5 mV at low pH, from a value of about −20 mV at pH 7. In the case of soy

Table 1. Apparent Mean Diameter (D) and ζ-Potential (ZP) of Milk or Soy Phospholipid Liposomes As Affected by pH during Storage at Room Temperature (22 °C)[a]

	day 0		day 7		day 14	
storage conditions	D (nm)	ZP (mV)	D (nm)	ZP (mV)	D (nm)	ZP (mV)
milk, pH 3	129 ± 10	−4 ± 2	159 ± 1	−5 ± 1	151 ± 1	−7 ± 1
milk, pH 5	107 ± 1	−12 ± 1	121 ± 1	−12 ± 1	118 ± 1	−13 ± 1
milk, pH 7	106 ± 2	−19 ± 1	168 ± 4	−19 ± 1	138 ± 2	−21 ± 1
soy, pH 3	ND	ND	ND	ND	ND	ND
soy, pH 5	124 ± 1	−34 ± 1	139 ± 1	−33 ± 4	137 ± 3	−32 ± 1
soy, pH 7	90 ± 1	−30 ± 2	90 ± 1	−33 ± 3	93 ± 1	−33 ± 1

[a]The data are the average of three independent experiments, with standard deviations. The physical properties of the visibly unstable samples were not determined (ND).

phospholipid liposomes, the average diameter of the dispersion was about 90 nm at pH 7, with a higher value of surface charge (approximately −30 mV) compared to milk phospholipid liposomes. While the dispersions were stable during storage at pH 5 and 7, at pH 3 the soy liposomes showed rapid destabilization immediately following the pH adjustment step.

It is well established that structure and fluidity of lipid bilayers can be controlled by environmental pH.[36] The fluidity is found to be greater when the molecules forming the bilayers are charged than when they are uncharged, due to the increased intermolecular separation caused by electrostatic repulsion.[37] In most cases, liposomes have nonzero surface electric potentials, even though the liposomes are composed of neutral lipids. However, even at low ζ-potential values (i.e., close to 0), liposomes might remain stable.[38] The present results clearly identified differences in the stability at low pH between EGCG-bearing liposomes prepared with milk- or soy-derived phospholipids. As detailed above, previous investigators have demonstrated that tea polyphenols have the capacity to disrupt phospholipid bilayers.[31,32] The bilayer integrity at acidic pH for milk liposomes may be more pronounced than that for soy liposomes since the mean size of soy liposomes was previously shown to increase with decreasing pH.[39] This, in turn, could explain the instability of tea-polyphenol-bearing soy liposomes at pH 3. The milk phospholipid liposomes carry a lower extent of surface charge, which could imply a reduced molecular separation at the bilayer at any pH value. As acidification takes place, molecular rearrangements in the bilayer could alter the encapsulation characteristics of the liposomes. Very little is known about this behavior, and further studies are necessary to better understand the differences in the integrity of different phospholipid bilayers, especially when loaded with other molecules.

Figure 4 illustrates the changes in encapsulation efficiency for milk phospholipids and soy phospholipids, as a function of storage time and pH. In general, the encapsulation efficiency decreased with storage time; however, while in the case of milk phospholipids there was a significant decrease after 1 day of storage at all pH values, in the case of soy phospholipids, the loss of encapsulated EGCG was only after prolonged storage. Immediately after preparation, milk phospholipid liposomes showed higher encapsulation efficiencies than soy liposomes, at all pH values. After 2 days milk phospholipids showed a decrease, which reached a plateau at about 45%, 40%, and 25% for pH 7, 5, and 3, respectively (Figure 4A). With acidification, the extent of encapsulation decreased for milk phospholipid liposomes, whereas the differences between soy liposomes at pH 5 and 7 were slight. Encapsulation efficiencies were lower than for milk phospholipid liposomes in the case of soy

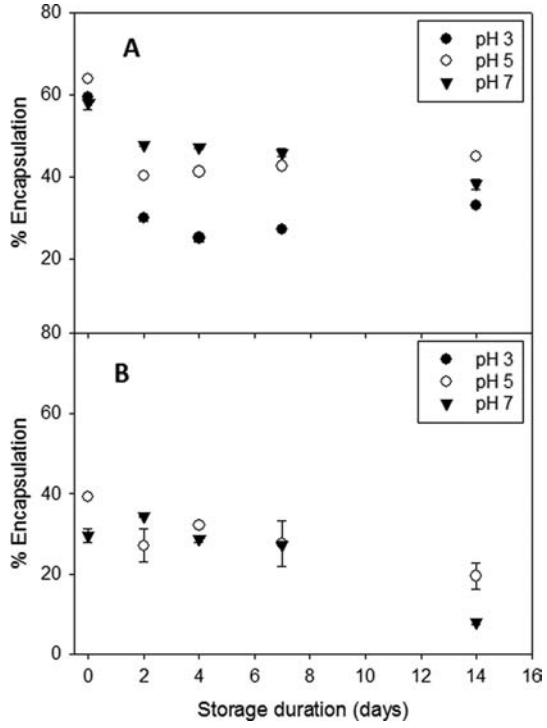

Figure 4. Effect of pH during storage at room temperature (22 °C) on encapsulation efficiency of tea polyphenols for milk phospholipid liposomes (A) and soy phospholipid liposomes (B). Values are the average of three independent experiments, and the error bars indicate the standard deviation.

liposomes. In this case (Figure 4B), there was a slower release rate, as after 14 days encapsulation efficiencies were <20% for pH 5 and 7. These results were in full agreement with previous work, which demonstrated a higher stability of milk phospholipid liposomes compared to soy counterparts.[10] Milk liposomes had thicker membranes and lower membrane permeability and improved stability in a wide pH range.[40] The disruptive effect of tea polyphenols being more pronounced on the membrane of soy liposomes is coherent with these earlier findings. Previously, in order to reduce the leakiness of soy liposomes, usage of a layer-by-layer (LBL) deposition technique[10] and addition of cholesterol[2,21] were utilized. In phospholipid monolayers, the fluid phase coexists with a noncrystalline gel phase.[41] The lateral order of lipid bilayers is affected by the presence of cholesterol[42] or sphingolipids.[43] Although lipid composition is the primary factor in the formation of membrane structures, proteins may

Table 2. Apparent Mean Diameter (D) and ζ-Potential (ZP) of Milk or Soy Phospholipid Liposomes As Affected by pH during Storage at 4 °C[a]

	day 0		day 7		day 14	
storage conditions	D (nm)	ZP (mV)	D (nm)	ZP (mV)	D (nm)	ZP (mV)
milk, pH 3	129 ± 10	−4 ± 2	157 ± 3	−6 ± 1	141 ± 1	−8 ± 1
milk, pH 5	107 ± 1	−12 ± 1	124 ± 1	−12 ± 1	120 ± 1	−13 ± 1
milk, pH 7	106 ± 2	−13 ± 1	ND	ND	ND	ND
soy, pH 3	ND	ND	ND	ND	ND	ND
soy, pH 5	124 ± 1	−34 ± 1	122 ± 3	−30 ± 1	125 ± 1	−33 ± 1
soy, pH 7	89 ± 1	−36 ± 2	91 ± 1	−32 ± 1	91 ± 2	−33 ± 1

[a]The data are the average of three independent experiments, with standard deviations. The physical properties of the visibly unstable samples were not determined (ND).

influence the size and function of the liquid-ordered microdomains.[44]

The smaller size and lower encapsulation efficiency of soy phospholipid liposomes compared to milk phospholipid liposomes may suggest a different partition of EGCG between the core and the bilayer membrane between soy and milk liposomes. Values of encapsulation efficiency of about 30% are reported for hydrophilic components present in the core of nanoliposomes,[4] and milk phospholipid liposomes show higher encapsulation efficiencies in spite of the lower surface area available for entrapment compared to soy liposomes.

Effect of Storage pH at 4 °C. Liposomes that underwent the gel phase transition consisting of lipids with a high transition temperature were previously shown to be more resistant to disruption compared to fluid liposomes.[45] Regardless of the extent of saturation, the fluidity and curvature of hydrophobic tails increase with temperature.[46] Therefore, lower membrane fluidities can be expected under refrigeration conditions compared to room-temperature storage, possibly resulting in a lower extent of polyphenol release over time. However, especially around the phase transition temperatures, the release is known to be maximal due to the coexistence of two phases and the defects on boundaries between these domains. [47]

The milk phospholipid samples have a sphingomyelin (SM) content of about 22%.[10] Waninge et al. (2003)[48] demonstrated that SM content is an important factor in determination of gel state to liquid crystalline transition temperature of recombined milk membrane lipids. At concentrations of approximately 24% SM, phase transitions take place between 5 and 20 °C. Therefore, it could be anticipated that the milk phospholipid liposomes undergo liquid crystalline to crystalline (gel) phase transition during cooling from room temperature to refrigeration temperature (4 °C).

Table 2 summarizes the average size and ζ-potential of liposome dispersions after refrigerated storage at pH 3, 5, and 7, for soy and milk phospholipid liposomes. As shown in Table 2, overall, size and surface charge characteristics were quite similar to the samples that were kept at room temperature (Table 2). This finding implies that the overall structure of the majority of nanoliposomes was altered minimally with the storage temperature. However, milk liposomes refrigerated at pH 7 visibly destabilized due to the phase transition. Milk liposomes were stable at pH 5, while at pH 3, as already shown in Table 1, the dispersions showed some aggregation over time, which could be in part related to the reduction in the magnitude of ζ-potential. As previously indicated, at pH 3, soy liposomes were not stable and, consequently, were not included in refrigeration experiments. Liposomes stored at pH 5 and 7 were stable at refrigeration temperatures that can once again be related to the magnitude of ζ-potential.

Figure 5 illustrates the variation in the encapsulation efficiency for the liposome dispersions stable at refrigerated

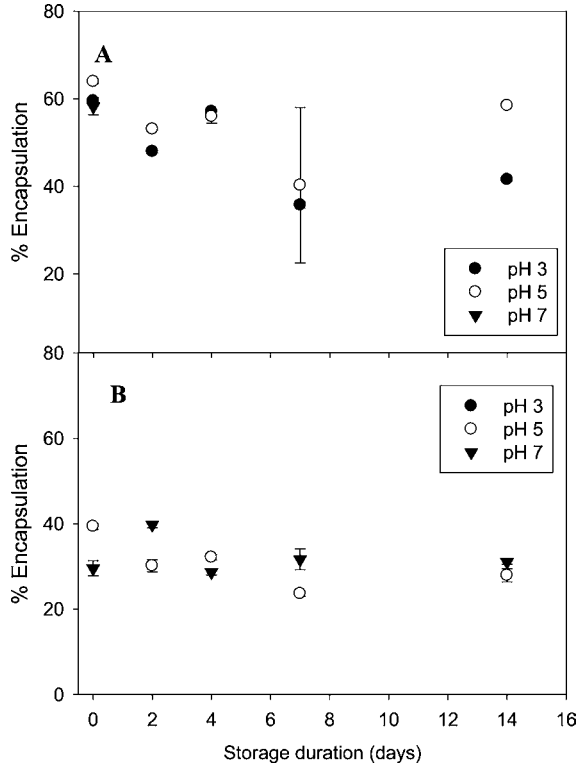

Figure 5. Effect of pH during storage at 4 °C on encapsulation efficiency of tea polyphenols for milk phospholipid liposomes (A) and soy phospholipid liposomes (B). Values are the average of three independent experiments, and the error bars indicate the standard deviation.

temperatures. Milk phospholipid liposomes stored at pH 5 and 3 showed higher encapsulation efficiencies compared to those stored at room temperature (Figure 4). Stability was improved also for soy phospholipid liposomes, which did not show a significant decrease in encapsulation efficiency when stored at pH 7 and showed a slight decrease after 1 day in the case of storage at pH 5. It was concluded that milk phospholipids demonstrated better ability to retain EGCG compared to the soy counterpart.

Storage Stability of Liposomes in the Presence of Sucrose. In this section, the influence of sucrose on liposome

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Table 3. Apparent Mean Diameter (D) and ζ-Potential (ZP) of Milk or Soy Phospholipid Liposomes As Affected by Sucrose Concentration (5−15%) during Storage at Room Temperature and pH 7[a]

	day 0		day 7		day 14	
storage conditions	D (nm)	ZP (mV)	D (nm)	ZP (mV)	D (nm)	ZP (mV)
milk, 5% sucrose	104 ± 2	−19 ± 1	116 ± 1	−18 ± 1	119 ± 1	−21 ± 1
milk, 10% sucrose	110 ± 14	−19 ± 1	119 ± 1	−19 ± 1	121 ± 2	−19 ± 1
milk, 15% sucrose	104 ± 2	−18 ± 1	114 ± 3	−19 ± 1	116 ± 1	−21 ± 1
soy, 5% sucrose	89 ± 1	−35 ± 2	87 ± 1	−29 ± 2	87 ± 1	−32 ± 3
soy, 10% sucrose	87 ± 1	−36 ±	86 ± 1	−36 ± 1	85 ± 1	−36 ± 1
soy, 15% sucrose	87 ± 1	−33 ± 3	86 ± 1	−31 ± 2	85 ± 1	−34 ± 1

[a]The data are the average of three independent experiments, with standard deviations.

stability was investigated. Sucrose concentrations between 5% and 15% were added to liposomes, and stability was tested during storage at room temperature (22 °C). These concentrations were used as comparable to those present in common soft drinks and juices. Due to mixing with sucrose solutions, the volume fraction of liposomes in the mixture was reduced to 5%. The action of sucrose in bilayers in an excess of water is only colligative; that is, extrusion of water through the membranes may occur by osmosis.[49]

There are two possible consequences to the interactions between membranes and sugars. The interaction of membranes and small sugars might induce an attraction, building up sugar at the interface at low sugar concentrations, or at high concentrations, a compression of the lipids may take place. [50] There were no changes in particle size or surface charge after the addition of sucrose at varying levels (5−15%) for both sets of nanoliposomes (Table 3). It may be possible that, although some water extrusion occurred, part of the water extruded from the vesicles may be the hydration water around the polar head groups of the phospholipids, [51] with a zero net change in the mean size of the liposomes.

The presence of sucrose caused a release of EGCG from all liposomes, regardless of their phospholipid composition; however the amount of sucrose added did not seem to affect the behavior. In the case of milk phospholipid liposomes there was a slow decrease in the encapsulation efficiency (Figure 6A), whereas soy liposomes seemed to leak the majority of their polyphenol content shortly after sucrose addition (Figure 6B). This difference in the release of EGCG may be related to the differences in the membranes' fluidities.

Interactions of Milk Phospholipid Liposomes with Tryptic Peptides of Sodium Caseinate. The stability of the liposomes containing EGCG was tested in the presence of tryptic peptides prepared with sodium caseinate digestion. It has been previously reported that casein molecules can be incorporated in the hydrophobic core of liposomal bilayers.[17]

As mentioned above, milk phospholipid liposomes were stable at acidic conditions. This could imply their utilization as intestinal delivery systems for encapsulated polyphenols. Consequently, the influence of tryptic peptides on the stability of the liposomes was investigated as a model system of interaction between peptides and liposome vesicles. To better control the source of peptides, casein hydrolysates were prepared using trypsin. To evaluate the size distribution of the peptides prepared, the samples after trypsinolysis were analyzed by MALDI-TOF (Figure 7). In the extracts used in this work, the major peaks observed were <6500 Da. The two most abundant peptides showed a mass of 2864 and 6316 Da. These findings were comparable with previous literature, [52] although it is important to point out that in the present work

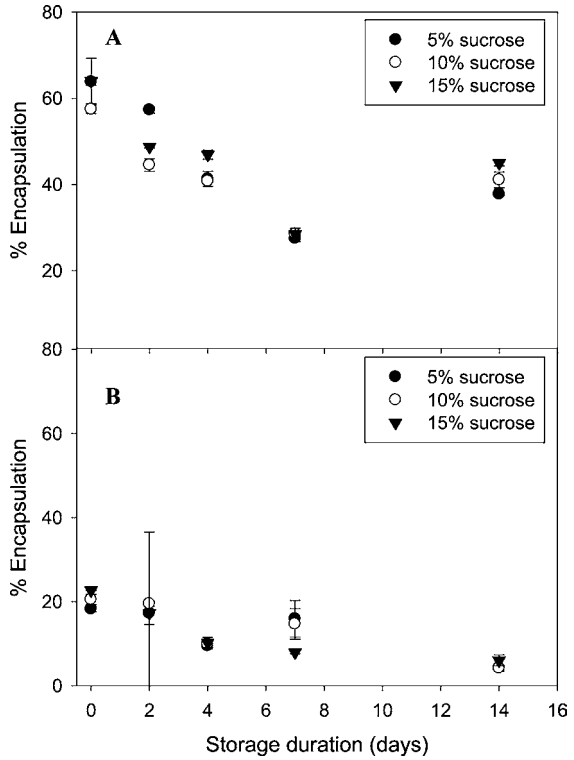

Figure 6. Effect of sucrose concentration during storage at room temperature (22 °C) and pH 7 on encapsulation efficiency of tea polyphenols for milk phospholipid liposomes (A) and soy phospholipid liposomes (B). Values are the average of three independent experiments, and the error bars indicate the standard deviation.

sodium caseinate and not micellar casein was used as a substrate.

The centrifuged and filtered digests were mixed with liposomal dispersions, and the changes in the physical characteristics were studied. Polyphenol-bearing milk phospholipid liposomes (containing 4 mg·mL^{-1} tea polyphenols) were mixed with the tryptic digest. Figure 8 summarizes the changes in ζ-potential and apparent diameter, as well as in encapsulation efficiency after mixing of liposome dispersion with the hydrolysate on a volume concentration basis. The size of the liposomes decreased significantly with dilution in the peptide solutions, as shown in Figure 8A. However, there were no changes in the zeta potential of the liposomes as a function of dilution in the peptide solution (Figure 8A).

As the light scattering measurements indicate only the overall changes in size and scattering characteristics of liposomes, and these measurements do not *per se* reflect the barrier properties

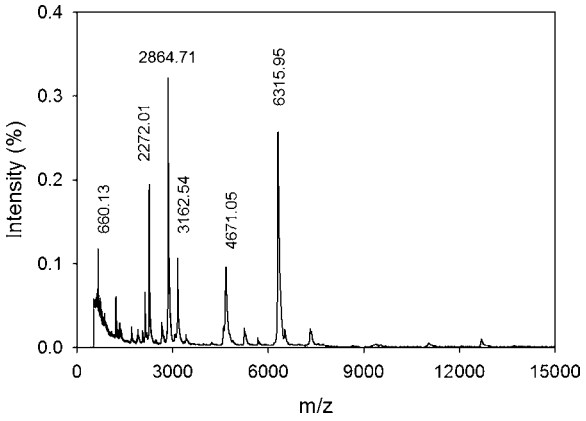

Figure 7. Frequency distribution of tryptic peptide fractions prepared from sodium caseinate (see methods) as analyzed by MALDI-TOF. The distribution is representative of duplicate samples.

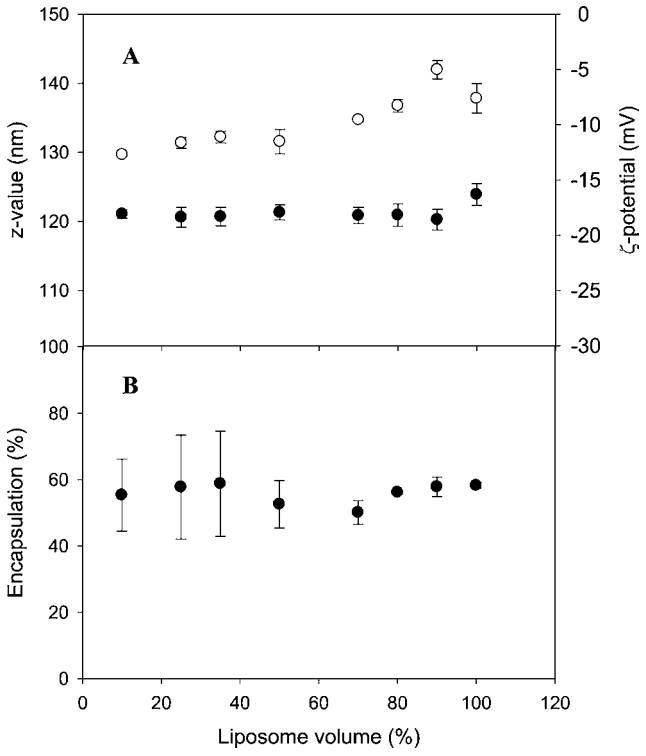

Figure 8. Effect of addition of tryptic peptides to milk phospholipid suspension on apparent diameter (empty symbols) and ζ-potential (filled symbols) (A) and encapsulation efficiency (B) for milk phospholipid liposomes. Values are the average of three independent experiments, and the error bars indicate the standard deviation. The *x* axis represents % of original volume of liposomes (10% phospholipids) diluted in tryptic digest (0.5% protein).

of liposomal membranes,[53] encapsulation efficiency was also measured as a function of dilution in the peptide solution. The encapsulation efficiency was stable (Figure 8B), which points out that the barrier properties were not negatively affected in the presence of tryptic peptides. When similar experiments were repeated after 24 h of storage at room temperature, no significant changes were observed in the physical characteristics of MFGM liposomes (data not shown). The ability of milk phospholipids to be stable in the presence of large amounts of peptides is important to better understand their stability during digestion. While nanoliposomes might be prone to lipolytic action[35] *in vitro* or *in vivo*, they seem to retain their functionality in the presence of low concentrations of surface-active peptides. These findings are coherent with previous observations that *in vitro* digestion with proteases (for example, pepsin) did not significantly affect the stability of MFGM liposomes.[54] It is well known that polyphenols interact with proteins,[55] and in the current investigation, a small extent of interactions may occur. In the peptide experiment, the concentration of free polyphenols was low and the experimental time frame was kept short (i.e., a low extent of polyphenol leakage). Consequently, the polyphenol−peptide interactions were likely to occur only between free polyphenols and peptides. Due to the low concentration of polyphenols available, we assume that these interactions are unlikely to influence the nature of liposome−peptide interactions.

In conclusion, tea-polyphenols-bearing nanoliposomes were prepared from soy and milk phospholipids using a food-grade process, and their encapsulation characteristics were studied under varying storage conditions and during interactions with model food molecules. Although at elevated polyphenol concentrations, liposomal membranes are prone to disruption, under the majority of the conditions studied here, milk nanoliposomes showed an enhanced encapsulation and physical stability behavior when compared with soy liposomes, possibly due to their higher saturated phospholipid content and thicker membrane forming characteristics due to the presence of liquid-ordered domains,[44] which in turn enabled the incorporation of polyphenols within the bilayer. It might be possible to optimize the polyphenol release (rate and duration of release) and liposomal stability characteristics (changes in size and surface charge) through simple physical manipulation in order to generate high-performance polyphenol delivery vehicles that retain functionality in food products and demonstrate maximum biological functionality.

AUTHOR INFORMATION

Corresponding Author

*E-mail: igulsere@uoguelph.ca. Phone: +1 (519) 824-4120, ext 58132. Fax: +1 (519) 824-6631.

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Notes

The authors declare no competing financial interest.

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