**Synthesis of multivitamin-loaded heat stable liposomes from milk fat globule membrane phospholipids by using a supercritical-CO2 based system**

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**Abstract**

Inspired by the heat stability of milk, where fat globules are coated by the milk fat globule membrane (MFGM), heat stable liposomes loaded with multivitamins were successfully synthesized from MFGM phospholipid concentrate. The MFGM phospholipids were first isolated from buttermilk powder, an undervalued dairy byproduct, by means of sequential pure SC-$CO_2$ and ethanol-modified SC-$CO_2$ extraction. The final extract was composed of 75% phospholipids, the highest MFGM phospholipid purity reported so far from buttermilk powder. Extracted MFGM phospholipids concentrate was utilized in liposome synthesis by the rapid expansion of supercritical solution using a venturi-based system (Vent-RESS) for vacuum driven cargo loading. Liposome synthesis was also conducted using sunflower phosphatidylcholine (SFPC) for comparison. To test the performance of the liposomes, vitamins E and C were used as model hydrophobic and hydrophilic bioactives, respectively. MFGM phospholipids mostly produced unilamellar vesicular type liposomes with an average diameter of 533 nm and ζ-potential of -57 mV. The encapsulation efficiency (EE) of vitamins E and C in MFGM liposomes were 77 and 65%, respectively. Even after heating at 90 °C for 30 minutes, MFGM liposomes retained structural integrity as shown in their confocal micrographs, structural characterizations, and EE measurements. In contrast, SFPC liposomes disintegrated at temperatures above 60 °C. Thus,

MFGM liposomes have the potential to protect the nutritional and functional properties of bioactive compounds during extended exposure to thermal treatment. This study proposes a green method to extract dairy phospholipids and fabricate liposomes for the delivery of bioactive compounds with application in the food, pharmaceutical, and cosmetic industries with a great potential for scale-up.

## 1. Introduction

Liposomes are artificial spherical vesicles consisting of one or more phospholipid bilayers enclosing an aqueous core.[1] The aliphatic chains of the phospholipids promote internal hydrophobic interaction while the polar headgroups interact with the internal and external aqueous phases. The unique amphiphilic nature of liposomal systems allow them to entrap both hydrophobic and hydrophilic compounds, enabling the encapsulation of a diverse range of bioactives/drugs.[2,3] Depending on the arrangement of phospholipid bilayer, liposomes can be classified into one of the three categories: (1) unilamellar vesicles (ULV), (2) multilamellar vesicles (MLV), and (3) multivesicular vesicles (MVV).[4] Liposomes can easily be customized for specific applications through modification of their coating material. For example: using different kinds of phospholipids, incorporating additional polymers,[5,6] carbohydrates,[7] surfactants,[8] or other amphiphilic molecules will modify their surface chemistry.[9] These modifications can be tuned to protect encapsulated bioactive molecules from physical and enzymatic degradation. Liposomal systems are also regarded for their biodegradability, biocompatibility, site-specificity, minimal-toxicity, and non-immunogenicity. Due to these advantages, liposomal systems have found extensive applications in the food, pharmaceutical, and cosmetic industries as effective vehicles for bioactive delivery.[10-12]

Existing techniques for liposome synthesis include thin film hydration (TFH), reverse phase evaporation, solvent injection, emulsion method, and detergent removal method.[13] The major drawback of these techniques is the use of toxic organic solvents to dissolve the lipid phase, resulting in negative impacts to the environment in addition to adding additional processing steps to remove solvent from the final product.[14] To avoid these drawbacks, supercritical fluid (SCF) based methods have emerged as effective alternatives for liposome synthesis. SCFs are non-condensable fluids that are highly dense at temperatures and pressures exceeding their critical point. Among SCFs, carbon dioxide ($CO_2$) has attracted significant attention for liposome synthesis because it has a low critical pressure (7.38 MPa) and temperature (31.1 °C), is non-toxic, non-flammable, and is recognized as a safe additive for food and pharmaceutical manufacturing. Several recent review publications have highlighted the potential of synthesizing liposomes by using SC-$CO_2$.[14-16]

Traditionally, variations of phosphatidylcholine (PC) have been used for making liposomes. Due to their unsaturated fatty acid chains, PC has a low phase transition temperature ($T_m$). As such, liposomes made from PC will disintegrate upon heat treatment as they undergo a gel-to-liquid phase transformation. Therefore, liposomes made from phospholipids with a higher phase transition temperature are desirable because they would be able to undergo higher temperature treatment without having any structural disintegration. Several research works have been conducted in an effort to produce liposomes with improved heat stability. However, most of these methods involve the use of lysolipids or other synthetic temperature-sensitive polymers, which are often not cost effective and face challenges to get approval as food additives.[17,18] Thus, there exists a great opportunity for a technology which improves the heat stability of liposomes.[19,20]

Our group has previously reported the development of a novel and sustainable process for synthesizing liposomes which involves the rapid expansion of supercritical solution using a venturi system (Vent-RESS) for concomitant vacuum driven cargo loading.[21-23] In these publications, we have reported the synthesis of liposomes from soy lecithin with varying phospholipid content. Improving on this system to produce heat-stable liposomes, we intended to use milk fat globule membrane (MFGM) phospholipids as a coating material. The MFGM is a unique structure of phospholipids and proteins surrounding the milk fat globule. It contains a wide variety of phospholipids such as sphingomyelin (SM), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidylcholine (PC). The thermotropic nature of MFGM phospholipids allow for the emulsion stability of milk fat globules even at high temperatures.[24-25] Thus, from the bioinspiration of milk fat globules, we hypothesized that a liposomal system coated with MFGM phospholipids could be heat stable.

In addition to their attractive physical properties, MFGM phospholipids possess several health benefits such as antiproliferative activity against cancer cells,[26] improved outgrowths of cortical neurons,[27] and betterment of the gut, metabolic health, and immunity.[28-30] Nevertheless, the availability of food-grade concentrates of MFGM phospholipids remain limited. One good source of MFGM phospholipids is buttermilk, an undervalued byproduct of the butter-making process.[31] Due to its low cost and relative abundance, buttermilk has recently received attention as a source for MFGM phospholipids. For example, methods have been developed to (i) recover MFGM phospholipids from buttermilk powder (spray died buttermilk) by solvent extraction[32] or (ii) concentrate them by extracting nonpolar lipids from the material.[33] Both of these methods have drawbacks, however. The first approach employs toxic organic solvents like chloroform, methanol, and petroleum ether which are not acceptable for use in the food industry. Furthermore, the first

approach extracts both polar and nonpolar lipids resulting in phospholipid extracts with low purity. The second approach separates nonpolar lipids but leaves MFGM phospholipids contaminated with proteins and carbohydrates in the buttermilk powder. To illustrate, Spence et al.[34] microfiltered buttermilk, spray dried, and extracted the resulting powder with SC-$CO_2$, but was only able to achieve a phospholipid concentration of less than 10% – too low for many food applications. However, using solvent modifiers, SC-$CO_2$ technology can be used to obtain much higher purity MFGM phospholipid concentrates.

SC-$CO_2$ is a good solvent for nonpolar compounds like oils, fats, and waxes, but is less effective with polar compounds (i.e. phospholipids). To increase the solubility of polar solutes, a polar co-solvent (such as ethanol) can be incorporated. Therefore, to best isolate the MFGM phospholipids in buttermilk powder, we propose to first extract the nonpolar lipids using pure SC-$CO_2$, then isolate the phospholipids using ethanol-modified SC-$CO_2$.[35] In this study, the second lipid fraction of high-purity MFGM phospholipids was used for liposome synthesis. Vitamins E and C were co-encapsulated in the liposomes as model hydrophobic and hydrophilic bioactive molecules. For comparison, liposomes were also produced using sunflower phosphatidylcholine (SFPC). The heat stability of synthesized liposomes was quantified by analyzing the structure, size, surface charge, and encapsulation efficiency of bioactives.

## 2. Materials and Methods

### 2.1. Materials

Vitamin E (α-tocopherol, 95.5%), protamine sulfate, Nile Red, cholesterol (92.5%), and dimethyl sulfoxide (DMSO) (99.9%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Carbon dioxide ($CO_2$) (99.99%) was purchased from Airgas (Ithaca, NY, USA). Calcein and Tris(hydroxylmethyl) aminomethane (TRIS) were purchased from Acros Organics (Morris, NJ, USA) and Bio-Rad (Hercules, CA, USA), respectively. Vitamin C (L-ascorbic acid, 99%) was purchased from TCI America (Portland, OR, USA). Phosphatidylcholine (PC) (Sunlipion® 90, 99%), extracted from non-genetically modified sunflower lecithin was donated by Perimondo (Florida, NY, USA). Dry buttermilk powder was purchased from Land O'Lakes, Inc. (Arden Hills, MN, USA).

### 2.2. Extraction of MFGM phospholipids using ethanol-modified SC-$CO_2$

Sequential extraction of the nonpolar ($1^{st}$ fraction) and polar ($2^{nd}$ fraction) lipids from buttermilk powder was carried out using a laboratory scale SC-$CO_2$ extraction system equipped with a cosolvent pump (SFT-250, Supercritical Fluid Technologies, Inc., Newark, DE, USA). The details of the extraction system are depicted in Fig. 1 (a). First, buttermilk powder (30 g) was blended with nonporous glass beads (30 g) to enhance mass transfer properties. This mixture was then loaded into a high-pressure vessel (100 mL) with glass wool layers at both ends of the vessel. Next, the system was flushed with $CO_2$ under ambient conditions to purge air from the vessel. Afterward, the system was heated to 60 °C and pressurized to 40 MPa with $CO_2$. After 20 minutes of static extraction, the $CO_2$ flow rate was adjusted to 1 L/minute (measured at ambient conditions) using a micro-metering valve. The micro-metering valve was heated to 70 °C throughout the extractions to prevent freezing due to the Joule Thompson effect. The $1^{st}$ fraction (rich in nonpolar

lipids) was continuously collected for 3 h using pure SC-$CO_2$. Next, system was repressured to 30 MPa at the same temperature (60 °C) and ethanol was introduced at a concentration of 15% (w/w). After 20 minutes of static extraction with ethanol, the $CO_2$ flow rate was set to 1 L/min (measured at ambient conditions). The extracted lipid and ethanol mixture were continuously collected for 4 h in a sample vial kept in an ice bath. Subsequently, ethanol was removed from the extracts in a vacuum oven at 40 °C. This 2$^{nd}$ fraction (rich in polar lipids) was later used for liposome synthesis. All the samples were stored under nitrogen at -20 °C until further use. The total lipid yield was calculated using the following equation:

$$\text{Total lipid yield (\%)} = \frac{\text{weight of the solvent free extract}}{\text{weight of buttermilk powder used for extraction}} \times 100 \quad [1]$$

Furthermore, the total lipid content of buttermilk powder was determined using Folch extraction method [36] where chloroform: methanol (2:1, v/v) mixture was used to extract both polar and nonpolar lipids. Briefly, methanol was first mixed with buttermilk powder. Then, chloroform was added to the mixture to extract lipids. Later, the lipid extract was filtered through a Whatman #42 filter paper, and the solvent was removed using a rotary evaporator (Rotavapor-R, Büchi Labortechnik AG, Flawil, Switzerland). The total lipid content of buttermilk powder was determined from the weight of this solvent-free extract.

### 2.3. Liposome synthesis with a SC-$CO_2$ assisted system

Liposome synthesis was performed with two types of phospholipids: (i) SFPC and (ii) MFGM phospholipids. To determine the efficacy of bioactive encapsulation in the synthesized liposomes, vitamins E and C were used as model hydrophobic and hydrophilic bioactives, respectively. The lipophilic cargo was prepared by mixing the phospholipids, cholesterol, and vitamin E at a weight ratio of 5:1:1 at 45 °C until homogeneous mixture was obtained. The mixture was then solidified at 4 °C for loading convenience. Cholesterol was added to increase the rigidity

and the strength of the phospholipid bilayer by restricting the movement of the long alkyl chains, thus increasing liposome stability and preventing structural disintegration.[37] The aqueous cargo was prepared as a solution of 0.125 M vitamin C in a 0.02 M TRIS buffer solution (pH = 7.4).

The liposomes were prepared using the SC-$CO_2$ assisted Vent-RESS system described in our previous publications.[22,23] Fig. 1 (b) depicts a simple schematic representation of the Vent-RESS system. This apparatus consists of three main parts: a high-pressure pump (HPP), a stainless-steel mixing vessel equipped with a stirrer, and a 1.5 mm (internal diameter) expansion nozzle located inside an eductor. For liposome synthesis, the mixing vessel was loaded with the lipophilic cargo, and the vessel was pressurized to 17.2 MPa and heated to 45 °C. The SC-$CO_2$ mixture was then stirred continuously for 1 h to equilibrate. A solenoid valve was used to release pressure for a predetermined time interval. At this stage, the phospholipid-rich SC-$CO_2$ expanded toward the expansion nozzle. To avoid precipitation of phospholipids before they reached the nozzle, SC-$CO_2$ was maintained at a temperature of 45 °C. The aqueous cargo was introduced to the expansion nozzle by a tube with an internal diameter of 1.3 mm mounted at a 45° angle to the SC-$CO_2$ flow.

Upon pressure release by the solenoid valve, the phospholipid-laden SC-$CO_2$ rapidly expanded, generating high velocities through the expansion nozzle. Consequently, a vacuum was formed at the throat of the eductor (i.e. *vena contracta*) due to the Bernoulli effect, enabling suction of the aqueous cargo. The aqueous stream enters the educator and collides with the $CO_2$ stream, fragmenting into submicron droplets. At this stage the $CO_2$ loses its supercritical properties, and nucleation of the dissolved phospholipids begins. To attain stability, phospholipid molecules coalesce around miniscule water droplets, self-assembling into bilayer liposomes (Fig 1(c)). During this process, the eductor-nozzle assembly was heated to 80 °C to prevent phospholipid condensation as a result of cooling by the Joule-Thompson effect and $CO_2$ expansion. Heating the

eductor-nozzle assembly also helps to minimize the interfacial tension between the aqueous and phospholipid phases.[38] The resulting liposomes were collected in 10 mL of TRIS buffer solution (pH = 7.4).

**2.4. Phospholipid analysis**

Identification and quantification of phospholipids in the MFGM extract and liposomes were performed using a nuclear magnetic resonance (NMR) spectrometer (Bruker Avance III HD 500 NMR spectrometer) following the method of MacKenzie et al.[39] In short, a detergent solution containing 10% (w/w) sodium cholate and 1% (w/w) EDTA was prepared in a 20% (v/v) deuterium oxide aqueous solution. The pH of the detergent solution was then adjusted to 7.1 using a 1 M NaOH solution. An aliquot of each sample (~30 mg) was dispersed in 750 µL of the detergent solution, and 50 µL of $K_2HPO_4$ solution (6 mg/mL) was added as an internal standard for the quantification of phospholipids. Then, the samples were sonicated at 60 °C for 10 minutes with occasional mixing by vortex. Proton-decoupled $^{31}P$ NMR spectra were collected at 202.3 MHz with 128 scans using a 2.0 sec recycle delay and 81.5 kHz spectral width. TopSpin 3.5 and MestRenova 14.1 were used to record and analyze the spectra, respectively. The phospholipids were quantified by relating the area of each signal peak to the area of the internal standard of known molar concentration.

**2.5. Heat treatment**

Both MFGM and SFPC liposomes were subjected to heat treatment at three different temperatures (60, 75, and 90 °C) for 30 minutes by immersing them in a constant temperature water bath. Post heat-treatment, all samples were subjected for morphological characterization by CLSM. Their EE%, diameter, and ζ-potential values were also measured as well for comparative analysis.

### 2.6. Characterization of synthesized liposomes

For all liposomes, characterization was conducted before and after heat treatment. Morphological characterization was carried out using a Zeiss LSM 710 confocal laser scanning microscope (CLSM) equipped with a 63x oil-phase objective lens. Samples were prepared for microscopy following the same protocol as described by Sharifi et al.[22] In short, Nile red (a lipophilic dye) was used to stain the phospholipid bilayer and make the liposomes visible. An aliquot of liposomal dispersion (1 mL) was mixed with 10 μL of Nile red solution (0.2 wt% in ethanol), followed by mild agitation by hand. In the CLSM, the fluorescence emission of Nile Red was recorded at 558-635 nm.

The ζ-potential, diameter, and size distribution of liposomes were determined using a 90 PLUS particle size analyzer (Brookhaven Instruments Corporation, Holtsville, NY, USA) equipped with BI- ζ extension. For measurement, the liposome suspension was diluted 30 times in TRIS buffer to avoid light scattering.

### 2.7. Encapsulation efficiency (EE)

The protamine aggregation method was used to measure the encapsulation efficiency (EE) of vitamin C by the liposomes.[40] Aliquots of liposomal dispersion (0.1 mL) were mixed with an equal volume of protamine sulfate solution (10 mg/mL) to help flocculate the liposomes and were incubated for 5 minutes. The mixture was then diluted with saline solution (0.9% w/v), chilled to 4 °C, and centrifuged for 25 minutes at 2000xg. The supernatant was decanted, leaving behind the concentrated liposomes. For both fractions, the liposomes were then ruptured by the addition of 200 μL of 10% w/v Triton X-100 solution and agitating for 5 minutes by vortex, releasing vitamin C into solution. The concentration of vitamin C was then determined by measuring absorbance at

265 nm using a UV/Vis spectrophotometer (UV1900, Shimadzu Scientific Instruments, Marlborough, MA, USA).

The EE of vitamin E was measured by the method described by Sharifi et al.: 1.5 mL of liposomal dispersion was centrifuged at 4 °C for 20 minutes at 1000xg, and the supernatant was separated from the concentrated liposomes.[23] For both fractions, 0.2 mL DMSO was added to solubilize both the phospholipids and vitamin E liposomes, making homogeneous solutions. The solutions were then diluted to a final volume of 3 mL with additional TRIS buffer and the absorbance at 295 nm was measured using a UV/Vis Spectrophotometer. The EE of vitamin E and C were calculated using equation 2, where VC is the vitamin content:

$$\text{EE}\ (\%) = \frac{\text{VC in centrifuged liposomes}}{\text{VC in centrifuged liposomes} + \text{VC in supernatant}} \times 100 \quad [2]$$

**2.8. Storage stability of MFGM liposomes**

The storage stability of MFGM liposomes was evaluated by their ability to retain encapsulated bioactives after storage as measured by their EE. To this end, the EE of liposomal dispersions in TRIS buffer were measured before and after storage for 4 weeks at 4 °C. The storage stability was reported as a percent of the original encapsulation efficiency.

**2.9. Statistical analysis**

The EE%, diameter, and ζ-potential values of the synthesized liposomes were reported as mean ± standard deviation, and all treatments were performed triplicate for each sample. Statistical analysis was performed in R (Version 3.6.3., R Foundation for Statistical Computing, Vienna, Austria). One-way ANOVA with Tukey's honestly significant difference test with a 95% confidence interval was conducted to determine the statistical significance of differences in means.

## 3. Results and discussion

The isolation and fractionation of MFGM phospholipids from buttermilk powder was carried out utilizing a "green" sequential pure SC-$CO_2$ and ethanol-modified SC-$CO_2$ extraction. The extraction conditions, namely, pressure (30 and 40 MPa), temperature (50 and 60 °C), and ethanol concentration (10, 15, and 20%) were investigated in our previous study.[41] The optimized extraction conditions for the highest phospholipid recovery were implemented in this work. The total lipid content of buttermilk powder was 9% as determined by Folch extraction, where 60% of the total lipids were phospholipids. Similar findings were previously obtained by Gallier et al. [42] and Ubeyitogullari and Rizvi [41]. In this current study, nonpolar lipids were first extracted from buttermilk powder using pure SC-$CO_2$, and the polar lipids (i.e. phospholipids) were concentrated in the 2$^{nd}$ fraction by extraction with ethanol-modified SC-$CO_2$ under previously optimized extraction conditions. The phospholipid contents of the extracts and liposomes were determined using $^{31}$P NMR (Fig. 2). Dihydrosphingomyelin (DHSM), SM, PE, PS, PI, and PC were the major phospholipids in the samples with $^{31}$P NMR signals at δ -0.09 ppm, δ -0.18 ppm, δ -0.23 ppm, δ -0.44 ppm, δ -0.66 ppm, and δ -0.79 ppm, respectively (Fig. 2 (a)). Similar chemical shifts were previously reported by MacKenzie et al.[39] The total lipid yield of the 1$^{st}$ fraction was 2.3 ± 0.2% (w/w), very little of which was phospholipids (0.21 ± 0.03%, w/w) due to nonpolar structure of SC-$CO_2$.[43] On the other hand, our ethanol-modified SC-$CO_2$ extraction (2$^{nd}$ fraction) had a lipid yield of 4.7 ± 0.2% (w/w) and a phospholipid content of 75 ± 2% (w/w) (hereafter this fraction is referred as the MFGM phospholipid concentrate). This procedure produces the highest MFGM phospholipid purity reported so far from buttermilk powder. Overall, 78% of the total lipids and 66% of the MFGM phospholipids present in the buttermilk powder were recovered using this sequential SC-$CO_2$ extraction.

For comparison, Barry et al. attained phospholipid purity of 56% from spray-dried 50 kDa retentate of buttermilk using ethanol-modified SC-$CO_2$ extraction.[35] However, the buttermilk was subjected to enzymatic hydrolysis, ultrafiltration, and spray drying prior to a very long SC-$CO_2$ extraction (13 h), adding processing time.[35] Similarly, a commercial phospholipid concentrate (PC 700) from bovine milk produced by Fonterra Co-operative Group Limited (Rosemont, IL) had a lower phospholipid content (59%).[44] The production steps of PC 700 are a trade secret and cannot be evaluated, but PC 700 contains a fair amount of lactose (6.6%) which limits its potential applications.[42]

The phospholipid composition of the buttermilk powder used in this study was 3.7% DHSM, 32.7% SM, 25.0% PE, 5.1% PS, 4.2% PI, and 29.3% PC as determined by Folch extraction (Fig. 2 (b)), which agrees with previously reported values by MacKenzie et al.[39] and Spence et al.[34] The MFGM phospholipid concentrate had a significantly different phospholipid composition: 6.7% DHSM, 25.8% SM, 18.7% PE, 2.0% PS, 4.3% PI, and 43.7% PC (Fig. 2 (b)). The changes in phospholipid composition can mostly be attributed to the higher solubility of PC in ethanol-modified SC-$CO_2$ as compared to the other phospholipids,[45] resulting in an increased percentage of PC in the MFGM phospholipid concentrate (44% PC) in comparison to the Folch extract (29% PC) (Fig. 2 (b)).

Liposomes were synthesized with the MFGM phospholipid concentrate as well as SFPC for comparison. The ability of these liposomes to encapsulate bioactives was determined using vitamins E and C as model hydrophobic and hydrophilic micronutrients, respectively. Liposome synthesis was carried out by using the Vent-RESS system utilizing SC-$CO_2$ as a solvent as explained in Section 2.3.

For a majority of the phospholipids (DHSM, SM, PE, PS, and PI), no significant difference in composition was measured between the initial MFGM phospholipid concentrate and the synthesized liposomes. However, the MFGM liposomes did contain a slightly higher PC content (44 vs. 47%) which can be attributed to its solubility in SC-$CO_2$ and the matrix effect (Fig. 2 (b)).

The CLSM images of liposomes made using MFGM phospholipids are shown in Fig. 3 (a). The lipophilic dye Nile red fluoresced bright red, revealing the phospholipid bilayer. Liposomes synthesized from MFGM phospholipids mostly produced ULV-type morphology; with an average diameter of 532 ± 68 nm and ζ-potential of -57.5 ± 0.3 mV (Fig. 4 ($a_1$) and ($c_1$)). In contrast, SFPC based liposomes produced a mixture of ULV, MLV, and MVV morphologies (Fig. 5 (a)). While the distinct layers of MLV-type liposomes could not be observed in the micrographs, some of the synthesized liposomes had significantly thicker walls than the more common ULVs, indicative of MLV-type lyposomes.[22] Due to their higher proportion of phospholipids, MLVs and MVVs are better equipped to encapsulate fat-soluble compounds whereas ULVs are more suitable to encapsulate water-soluble compounds. The SFPC liposomes had an average diameter of 761 ± 94 nm and ζ-potential of -36.5 ± 1.4 mV (Fig. 4 ($a_2$) and ($c_2$)). For both MFGM and SFPC liposomes, unimodal diameter distribution was observed as shown in Fig. 4 ($b_1$) and ($b_2$), respectively. Both MFGM and SFPC liposomes were able to form stable dispersions since the magnitude of their ζ-potentials were greater than 30 mV for both systems (Fig. 4 ($c_1$) and 4 ($c_2$)).[46] However, the MFGM liposomes had 57% higher surface charge than their SFPC counterparts. One possible explanation for this could be that in SFPC liposomes, negative surface charge is solely contributed by zwitterionic choline headgroups. In contrast, the MFGM liposomes have negatively charged anionic phospholipids (PS and PI) in addition to the zwitterionic phospholipids (PC, SM, DHSM,

and PE).[47,48] This is in line with previous research by Thompson et al., which reported a ζ-potential of -60 mV at pH 7 for MFGM phospholipids.[49]

The CLSM micrographs of MFGM liposomes before and after 30 minutes of heat treatment at three different temperature levels (60, 75, and 90 °C) are shown in Fig. 3 (b-c). No significant change ($p > 0.05$) in liposomal diameter was observed after heating at 60 °C for 30 minutes. However, when heated at 75 and 90 °C, the diameter of MFGM liposomes significantly increased ($p < 0.05$) by factors of 1.6 and 2.0 respectively. This increase in diameter can be attributed to osmotic swelling of the aqueous core enabled by the increased permeability of the phospholipid bilayer at elevated temperatures. Increasing the thermal energy of the system enhances the mobility of phospholipids in the bilayer, resulting in changes in the orientation and packing of phospholipid molecules, ultimately increasing the permeability of the membrane.[50] Since the aqueous core of the liposomes are solutions of vitamin C in TRIS buffer, they have a higher osmotic pressure than the collection buffer, resulting in the migration of water to the core and liposome swelling. Another possible explanation for increase in liposomal diameter could be that at higher temperatures liposome coalesced because inter-particle collisions had enough energy to overcome the electrostatic barrier between liposomes.[51,52] For SFPC liposomes, heat treatment resulted in significant disruption of the liposomal structure. Fig. 5 juxtaposes the liposomes before treatment and the fat droplets and remnant liposomes after heat treatment at 60 °C for 30 (Fig. 5 (b)). Heating at a temperature above 60 °C resulted in the complete disintegration of liposomal structures yielding lipid droplets with irregular shape under CLSM (data not shown). Therefore, for SFPC, only the properties of untreated and 60 °C heat treatment liposomes are reported.

The encapsulation efficiency of vitamins E and C in MFGM liposomes before and after heat-treatment at 60, 75, and 90 °C for 30 minutes are shown in Fig. 6 (a). For untreated MFGM

liposomes, the EE of vitamins E and C were 77 ± 5% and 65 ± 4%, respectively. For vitamin E, the EE held constant ($p > 0.05$) after heating to 60 or 75 °C, but decreased significantly ($p < 0.05$) for the 90 °C treatment (65 ± 4%). For vitamin C, the EE held constant at 60 °C, but decreased significantly after heat treatment at 75 and 90 °C (42 ± 3% and 27 ± 6%, respectively). Several factors could have played a role in the reduced EE at higher temperatures: (i) At higher temperatures, some of the MFGM liposomes could undergo structural disintegration, releasing bioactives. (ii) Due to the osmotic swelling of liposomes at higher temperatures the concentration of encapsulated micronutrients got diluted and subsequently lower EE was observed. Moreover, vitamin C is thermolabile – it could have been oxidized to dehydroascorbic acid then hydrolyzed to 2,3-diketogulonic acid during the heat treatment.[53,54] This effect is expected to increase the EE of vitamin C since degradation of the unencapsulated vitamin C occurs at higher rates than that of vitamin C within the liposomes.[53] However, the leakage and osmotic swelling effects were more dominant than the degradation effect, leading to an increase in the vitamin C content in supernatant (equation 2), and in turn decreasing the EE of vitamin C.

Untreated SFPC liposomes demonstrated an EE of 89 ± 3% for vitamin E and 72 ± 5% for vitamin C. While these initial EE values for SPFC were slightly better than for MFGM, they dropped significantly to 56 ± 9% and 19 ± 7%, respectively ($p < 0.05$) after heat treatment as a result of structural disintegration (Fig. 6 (b)). These EE values for SFPC liposomes agree with those from our previous works.[21,23]

The encapsulation efficiencies of MFGM liposomes reported in this study (77% hydrophobic, 65% hydrophilic) are on par with previously published liposomes. In a 2016 study, Jin et al. compared the encapsulation of curcumin (a hydrophobic bioactive) in liposomes made from MFGM (29% lipids extracted from buffalo milk), to liposomes made from soybean lecithin.

Under optimized conditions, the EEs for curcumin were 74 and 63%, respectively.[55] Jin et al. attributed MFGM's higher EEs to the presence of more suitable phospholipid structures and composition for liposomal entrapment of curcumin. Farhang et al. used a high-pressure homogenization process to synthesize liposomes from a MFGM extract (59.2% phospholipids) and observed a maximum EE of 26% for vitamin C.[56] Thompson et al. used liposomes prepared from MFGM (72-74% phospholipids) to encapsulate β-carotene and potassium chromate as model hydrophobic and hydrophilic compounds, respectively.[57] They measured an EE ca. 45% for β-carotene when ethanol was used to combine β-carotene with phospholipids prior to liposome synthesis by thin-film hydration (TFH). For potassium chromate, a maximum EE ca. 60% was reported. While the EE of the liposomes produced in this study generally compare favorably with that of other published works, a direct comparison would be unrealistic; there are too many differences between methods. There were different sources, compositions, and purities of MFGM phospholipids, a wide variation in operational parameters and techniques, varying use of solvents for dissolution of lipophilic cargo, and several different bioactives used to quantify encapsulation efficiency.

While the measured EE of vitamin E in our MFGM liposomes decreased to 71 ± 3% after storing the dispersion at 4 °C for 4 weeks, the change was not statistically significant ($p > 0.05$). During storage, the retention of vitamin C in MFGM liposomes decreased by 19%, which could have potentially been caused by the leakage of aqueous cargo over time (Fig. 8).

The structural endurance of a liposome is solely dependent on its phospholipid bilayer. The bilayer is considered to be in a stable, ordered formation when the hydrocarbon chains are fully extended and aligned in parallel. However, heat treatment adds energy to de-align molecules, adding "kinks" to the hydrocarbon chains, reducing packing efficiency and increasing membrane

fluidity. The ordered gel formation transforms into a disordered liquid crystalline state, enabling degradation of the liposomal structure.[18, 58] Therefore, liposomes made of phospholipids with a higher phase transition temperature ($T_m$) would be able to withstand treatment at elevated temperature without having any structural disintegration. The heat stability of MFGM liposomes can be attributed towards the presence of saturated phospholipids with high $T_m$ (i.e. dipalmitoylphosphatidylcholine, palmitoylphosphatidylethanolamine, and SM).[45, 59] The SM family of phospholipids contains several long chain saturated fatty acids (C24:0, C23:0, C22:0, C18:0, and C16:0). These fatty acids lead to the unique biophysical properties of the MFGM which include a high $T_m$ (~34.3 °C) and better interaction with cholesterol and tocopherol, which enable the formation of an ordered domain.[59-61]. Furthermore, the addition of polycyclic amphiphilic molecules like cholesterol which possess a high $T_m$ (~147-149 $^{o}$C) contributes to the heat stability of MFGM liposomes. These molecules promote the formation of a liquid ordered phase, an intermediate state between liquid crystalline and gel phases which doesn't evolve as a function of temperature.[60-62]

In contrast, SFPC liposomes are made up of only one type of phosphatidylcholine (i.e. 1-oleoyl-2-linoleoyl-sn-glycero-3-phosphocholine) which has a $T_m$ below -4 °C due to a higher degree of unsaturation. Even though the PC liposomes made in this study gain structural integrity by the addition of cholesterol and vitamin E, it is not enough to prevent degradation upon heat treatment. Previous research has reported similar results. In 1993, Zuidam et al. studied the effect of autoclaving (121 °C, 15 minutes) on the degradation and leakage from SFPC liposomes encapsulating several model hydrophilic and hydrophobic compounds.[63] They synthesized liposomes from saturated phospholipids (i.e. palmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG)) and cholesterol using thin-film hydration. Autoclaving

these liposomes resulted in a 39 ± 4% degradation of the model lipophilic N-trifluroacetyldoxorubicin-14-valerate. They also observed ca. 20 ± 5% leakage and 26 ± 8% degradation of hydrophilic model compound calcein after autoclaving. Peng el al. developed hybrid liposomes (HBLs) from soybean phospholipids (71% PC and 10% PE) and amphiphilic chitosan by TFH and dynamic high-pressure microfluidization.[64] They encapsulated curcumin as a model hydrophobic bioactive in the synthesized HBLs and measured an EE of 8.08 ± 0.18%. They reported the heat stability of HBLs autoclaved at 121 °C for 20 min in pure water and in PBS buffer as no substantial change was observed in liposomal diameter, but they did not discuss the effect of heat treatment on the retention of curcumin. However, both of these protocols are substantially laborious in addition to the use of toxic organic solvents, which are eliminated in our proposed approach.

## 4. Conclusions

A “green” sequential pure SC-$CO_2$ and ethanol-modified SC-$CO_2$ extraction was used to isolate and fractionate MFGM phospholipids from buttermilk powder. The final extract was composed of 75% phospholipids, the highest MFGM phospholipid purity reported so far from buttermilk powder. Phospholipid compositions were characterized by $^{31}$P NMR spectroscopy. The phospholipid composition of this extract was found to be 5.6% DHSM, 25.8% SM, 18.7% PE, 2.0% PS, 4.3% PI, and 43.7% PC. The highly pure MFGM phospholipid concentrate was used to synthesize liposomes, which had a phospholipid composition of 6.7% DHSM, 25.8% SM, 14.3% PE, 2.4% PS, 3.9% PI, and 47.1% PC. The MFGM-based liposomes demonstrated ULV-morphology with an average diameter of 533 nm and a ζ-potential of -57 mV. In contrast, SFPC-based liposomes gave a mixture of ULV, MLV, and MVV morphologies, and had an average diameter of 761 nm and a ζ-potential of -37 mV. To evaluate the effectiveness of MFGM liposomes for bioactive encapsulation, vitamins E and C were used as model hydrophobic and hydrophilic bioactives; the encapsulation efficiencies were 77 and 65%, respectively. For comparison, SFPC-based liposomes had EEs of 88 and 72% for vitamins E and C. To determine heat stability, both MFGM and SFPC liposomes were heated to 60, 75, and 90 °C for 30 minutes. MFGM liposomes demonstrated enhanced heat stability as established by their CLSM images, structural characterization, and EE. Even after heating at 90 °C for 30 minutes, MFGM liposomes retained 65 and 27% of vitamin E and C, respectively. In contrast, SFPC liposomes disintegrated at the measured temperatures above 60 °C. Our synthesis method for heat-stable, multivitamin-loaded liposomes is a green, sustainable, and novel technology amenable to industrial scale-up. This approach has potential use for effective bioactive delivery in pharmaceutical and food applications.

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**Conflicts of interest**

There are no conflicts to declare.

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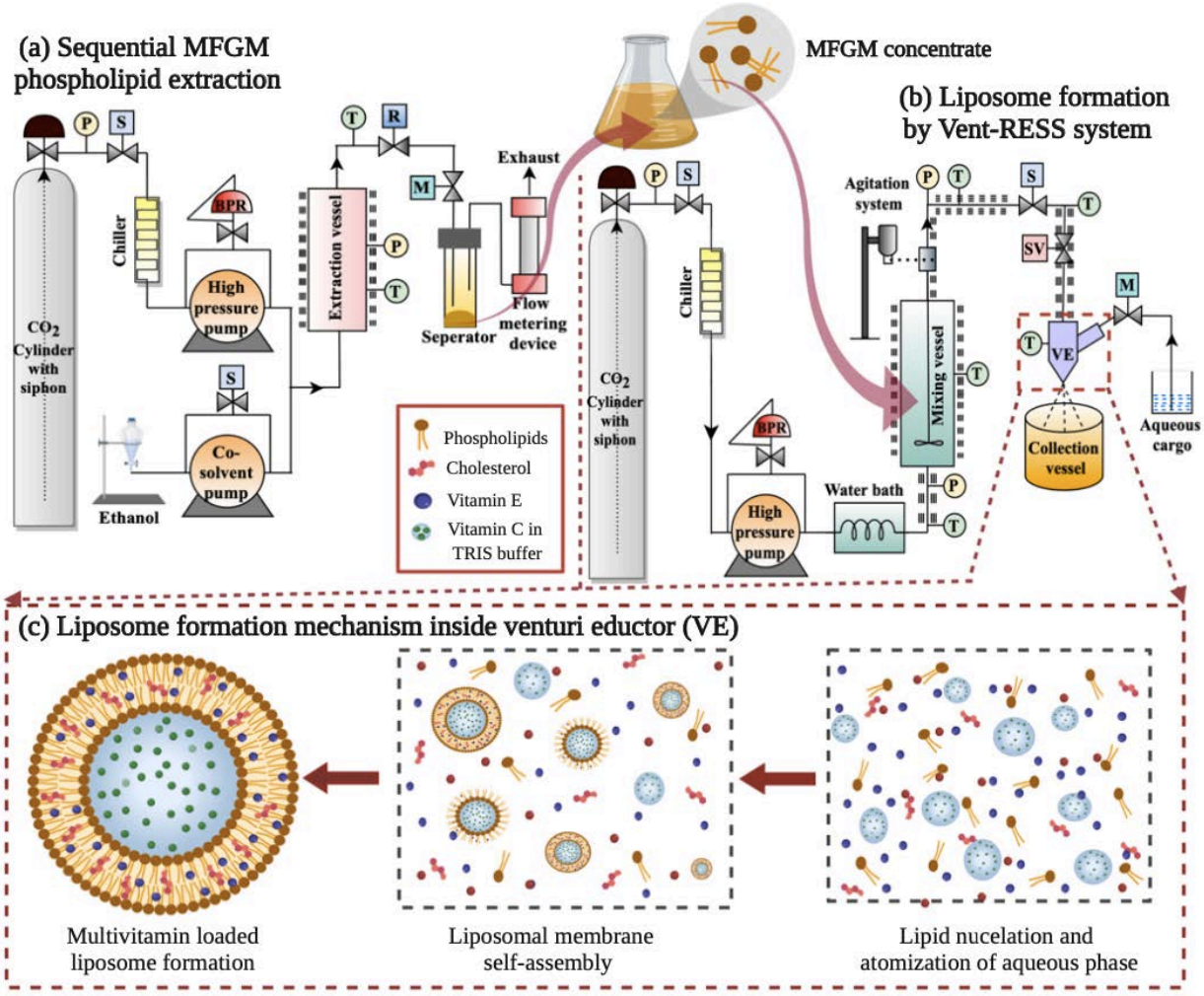


**Fig. 1.** Schematic representations of (a) the MFGM phospholipid extraction unit, (b) the Vent-RESS system, and (c) the liposome formation mechanism inside the venturi eductor. Abbreviations: T: temperature sensor, P: pressure sensor, SV: solenoid valve, M: metering valve, S: safety valve, HPP: high-pressure pump, BPR: back-pressure regulator; VE: venturi eductor.

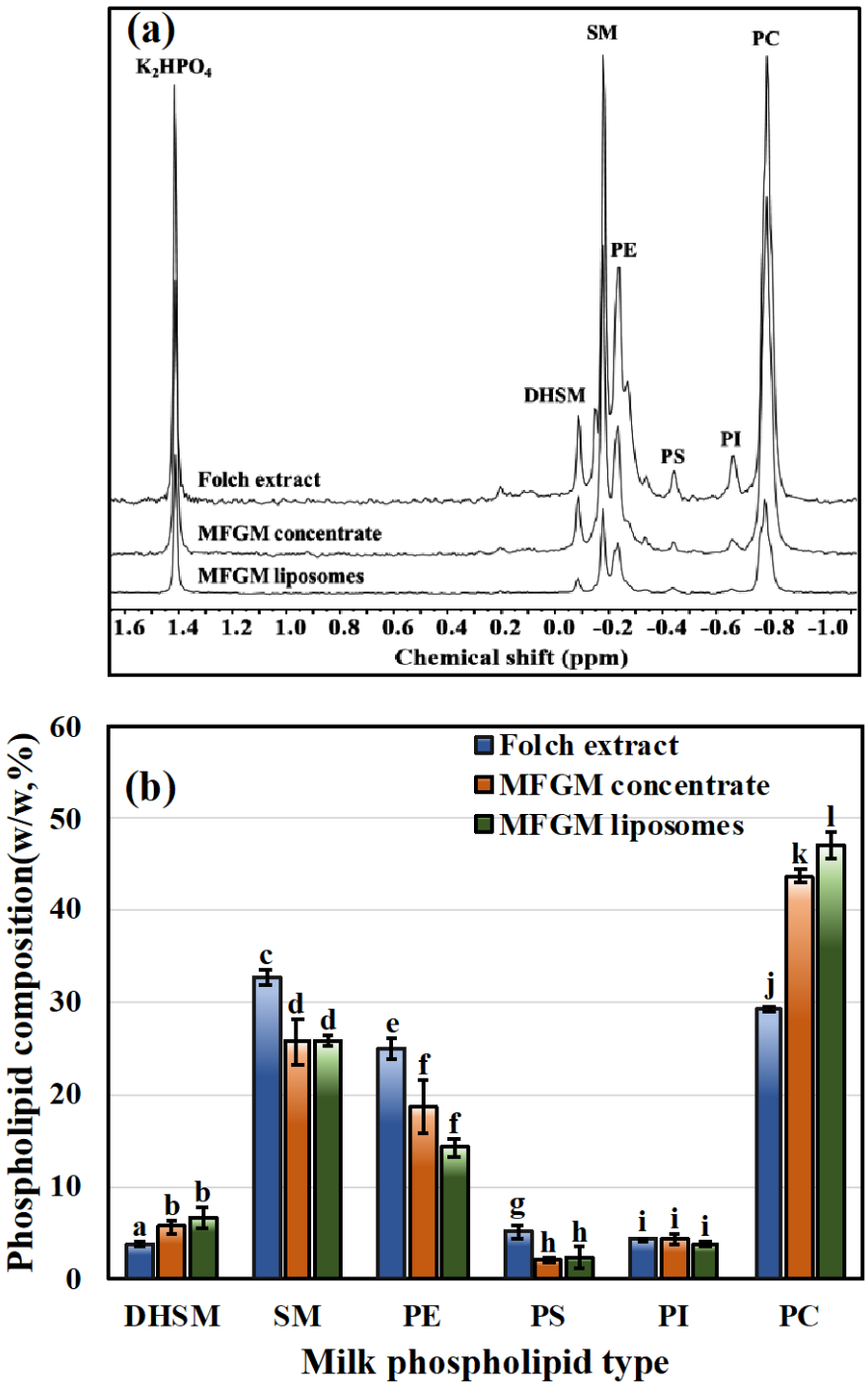


**Fig. 2 (a)** $^{31}$P NMR spectra and **(b)** phospholipid composition of the Folch extract, the MFGM phospholipid concentrate, and the MFGM liposomes. Lower-case labels indicate statistically significant differences ($p < 0.05$). DHSM = dihydrosphingomyelin, SM = sphingomyelin, PE = phosphatidylethanolamine, PS = phosphatidylserine, PI = phosphatidylinositol, PC = phosphatidylcholine.

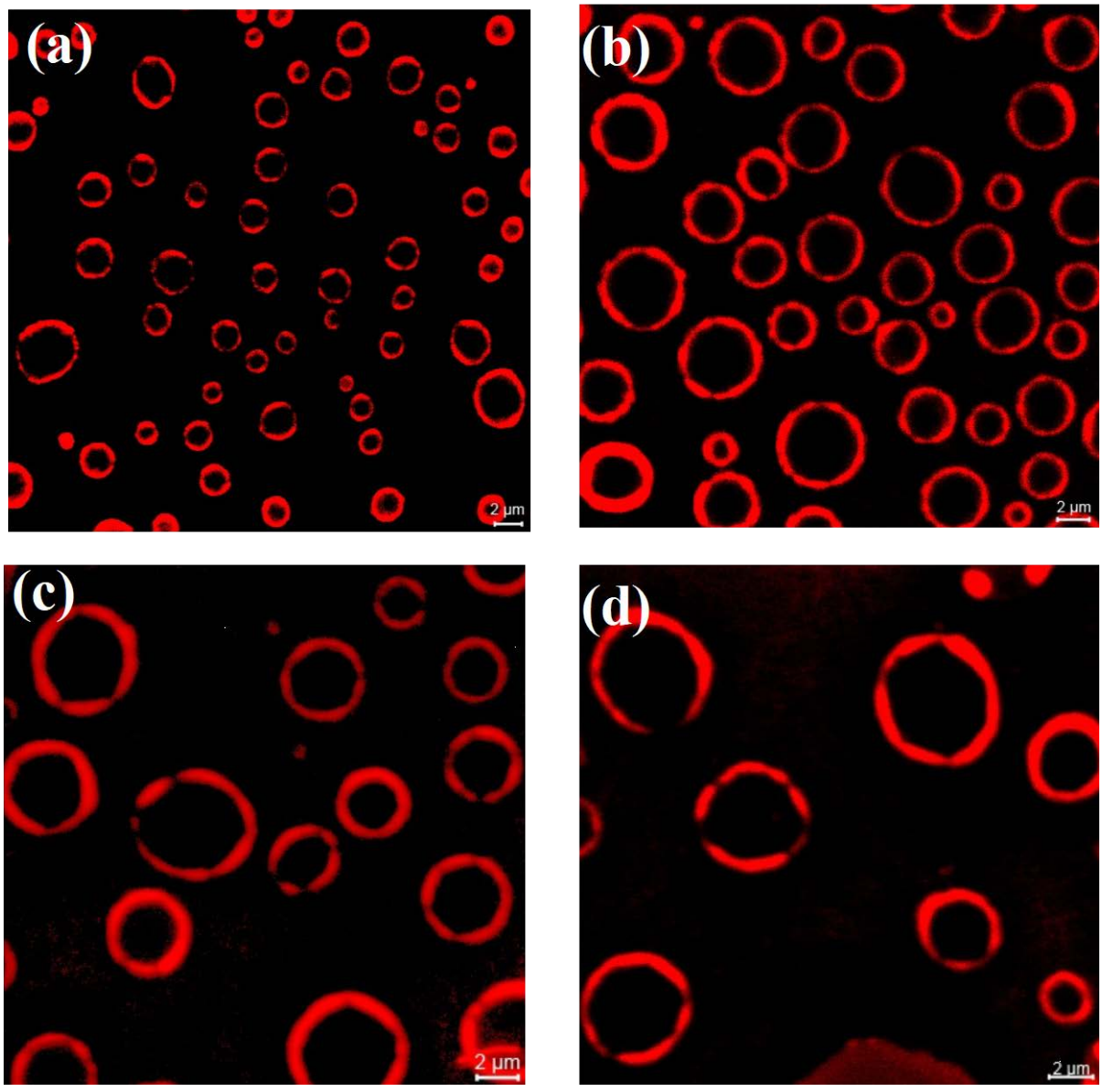


**Fig. 3** CLSM images of MFGM liposomes **(a)** before and after heat-treatment at **(b)** 60, **(c)** 75, and **(d)** 90 °C for 30 minutes.

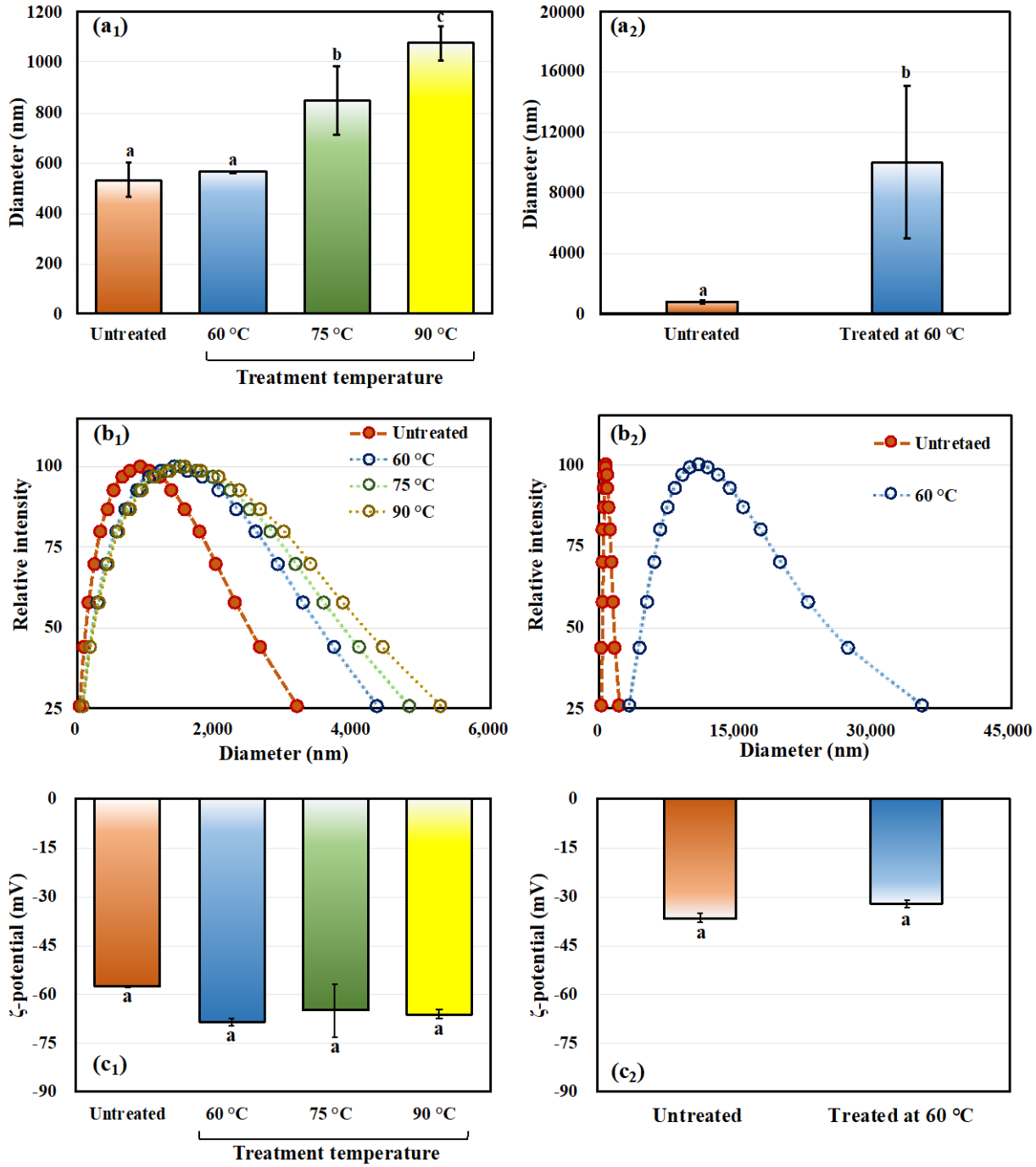


**Fig. 4 ($a_1$)** Average diameter, **($b_1$)** diameter distribution, and **($c_1$)** ζ-potential of MFGM liposomes; and **($a_2$)** average diameter, **($b_2$)** diameter distribution, and **($c_2$)** ζ-potential of SFPC liposomes, before and after heat-treatment for 30 minutes. Alphabetical labels indicate statistically significant differences between treatments ($p < 0.05$).

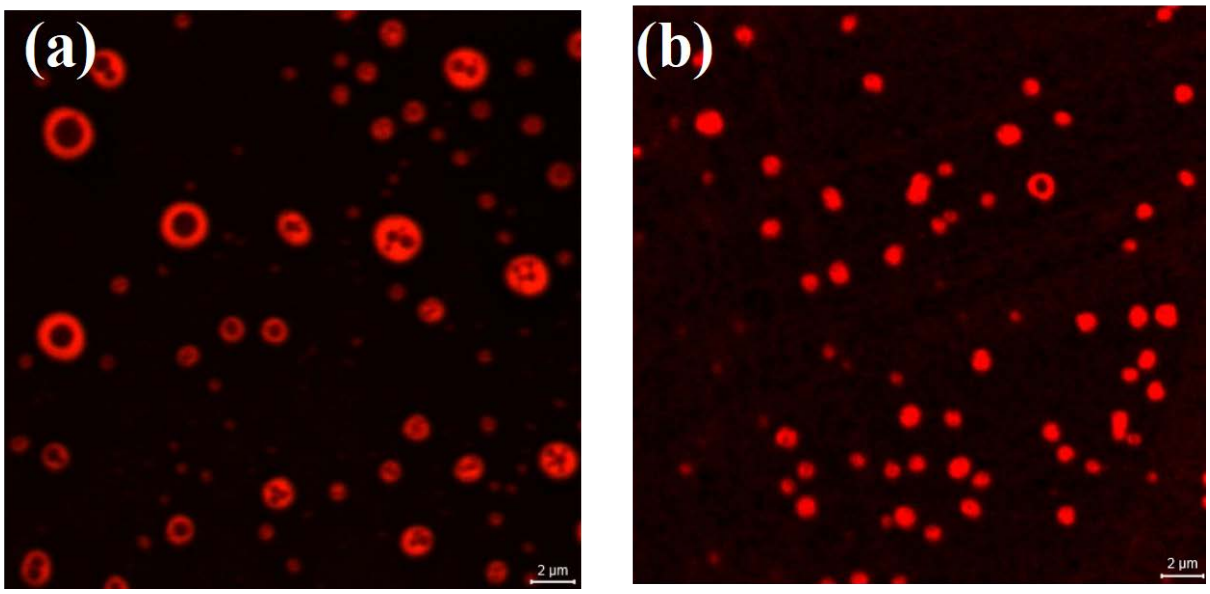


**Fig. 5** CLSM images of SFPC liposomes **(a)** before and **(b)** after heat-treatment at 60 °C for 30 minutes.

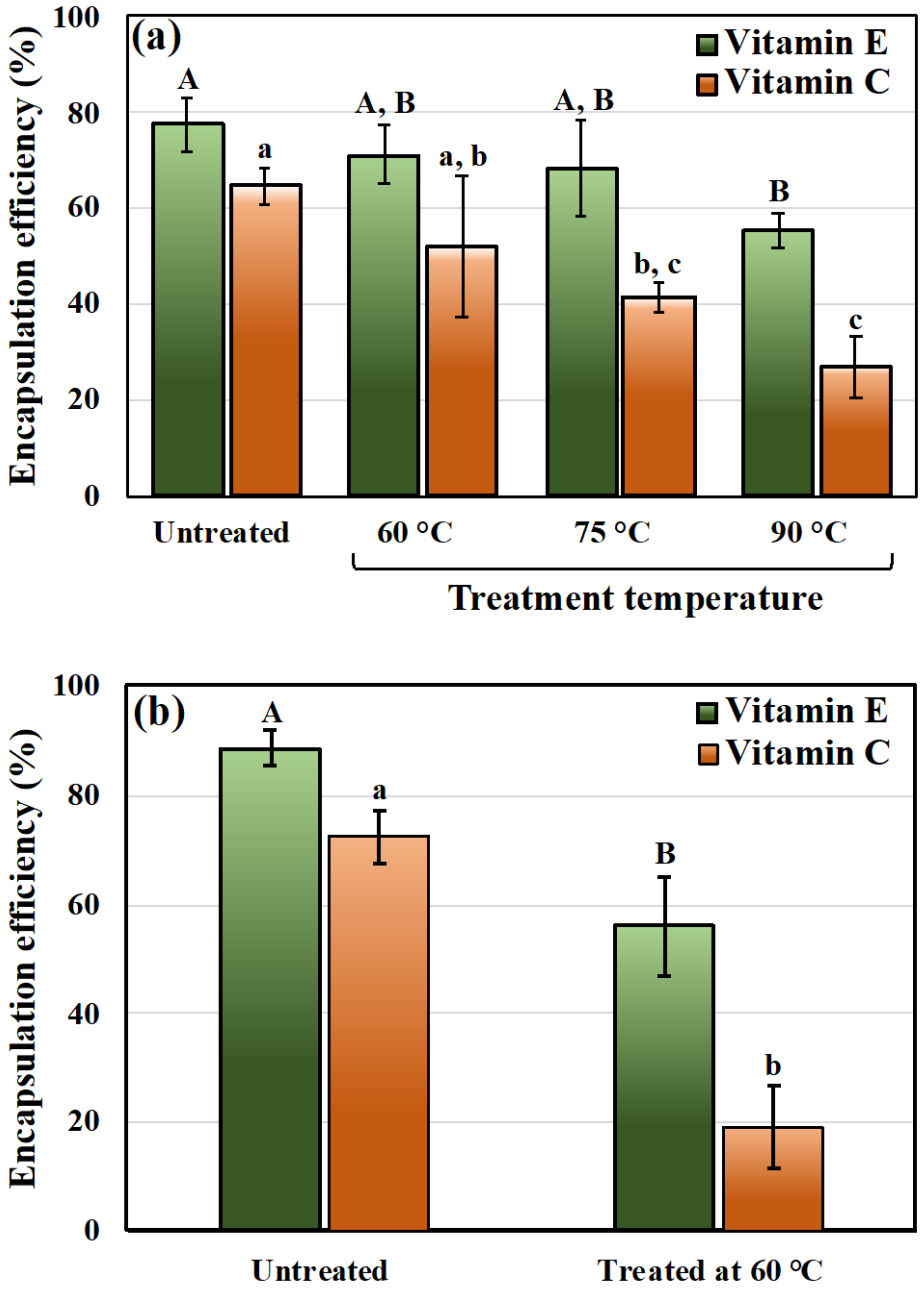


**Fig. 6** The encapsulation efficiencies of vitamins E and C in (a) MFGM liposomes and (b) SFPC liposomes before and after heat treatment for 30 minutes. Upper- and lower-case letters label statistically significant differences in the EEs of Vitamin E and Vitamin C, respectively ($p < 0.05$).

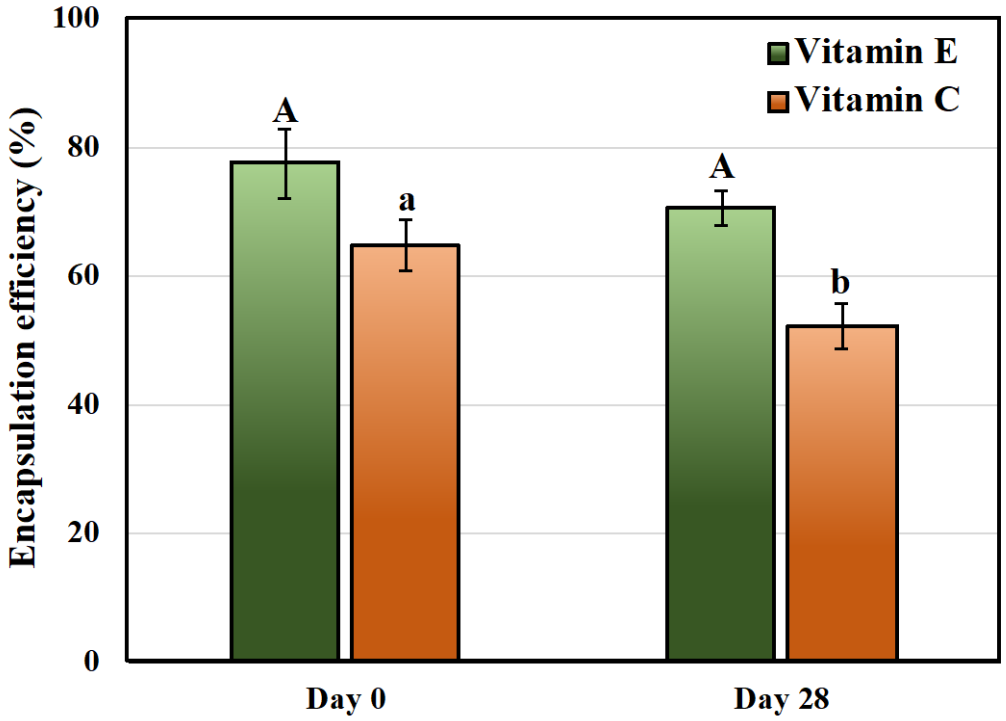


**Fig. 7** The encapsulation efficiencies of vitamins E and C in MFGM liposomes on day 0 and day 28 of storage at 4 °C. Upper- and lower-case letters label statistically significant differences in the EEs of vitamins E and C, respectively ($p < 0.05$).