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Physical Stability of Liposomes Prepared from Milk Fat Globule Membrane and Soya Phospholipids

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Previous research has shown that liposomes prepared from a milk fat globule membrane (MFGM) phospholipid fraction had a significantly higher phase transition temperature, thicker membrane, and lower membrane permeability than liposomes prepared from soya phospholipid material. Subsequent investigations into the relative stability of the two liposome dispersions have found that the MFGM phospholipid liposomes are more stable than their soya counterparts in a range of pH conditions, at a variety of storage and processing temperatures, and in the presence of mono- and divalent cations. These results illustrate some potential advantages in the use of MFGM phospholipids for the manufacture of liposomes for use in food systems.

KEYWORDS: Encapsulation; liposome; milk fat globule membrane; MFGM; phospholipid; stability

INTRODUCTION

In recent years, there has been growing interest in the health benefits (*1*−*5*) and functional properties of milk fat globule membrane (MFGM) phospholipids. Research has shown that MFGM material may be successfully used to stabilize emulsions (*6*−*9*) and that vesicle-like structures can be observed in some fractions of cream, cream plasma, and buttermilk (*10*, *11*). Results from our laboratory showed that MFGM-derived fractions may be used to produce bilayer vesicles (or liposomes) in a controlled manner on a large scale (*12*).

Liposomes are phospholipid−bilayer structures with an aqueous core (*13*). They have been used for a wide range of applications in the pharmaceutical and cosmetic industries, including the controlled release of drugs, gene delivery, and as model cells. There are a large number of recent papers and books on various aspects of liposome structure, formation, and characterization (*13*−*21*). These liposomes have traditionally been produced from highly purified phospholipids extracted from soya oil or egg yolk, but the unique composition of the MFGM-derived phospholipid fractions and the lower cost of this raw material may offer some advantages in the manufacture of liposomes for the entrapment of bioactive compounds in food systems.

Subsequent work has shown that the liposomes prepared from the MFGM fraction were significantly different to those produced from soya phospholipids (*22*). The MFGM liposomes had a higher phase transition temperature, thicker membrane, and lower membrane permeability. It is believed that these properties are due to the differences in phospholipid composition between the MFGM and the soya phospholipid fractions.

A liposome dispersion is theoretically at the minimum energy level for the system, with liposomes inherently stable units unless environmental or chemical changes cause a disruption to the system. However, the dynamic nature of the bilayer membrane means that the phospholipids within the membrane are mobile and could interact with other compounds in the environment. The physical instability of liposomes is largely due to aggregation, fusion, or rupture of liposomes and occurs over varying time periods in virtually all liposome dispersions. The ability of the system to maintain its original liposome size distribution is therefore commonly used as an indication of physical stability. Chemical stability may be determined by monitoring the oxidation of the unsaturated fatty acid chains or the hydrolysis of the phospholipids (*23*).

The stabilities of liposome dispersions produced from MFGM phospholipids were compared with those produced from non-hydrogenated soya fractions by measuring changes in average hydrodynamic diameter, peroxide value, and conjugated diene levels of samples under a variety of conditions. These included a range of pH, temperature, and time combinations that may be used for storage and a selection of processing environments commonly encountered in the food industry.

MATERIALS AND METHODS

The MFGM-derived phospholipid fraction (Phospholac 600) was provided by the Fonterra Co-operative Group Ltd. (New Zealand), and a purified soya phospholipid fraction (Sigma product P3644, minimum 30% phosphatidyl choline) was obtained from Sigma-Aldrich (St. Louis, MO). All chemicals used were of analytical grade and obtained from Sigma-Aldrich.

Preparation of Liposomes. The MFGM-derived or soya-derived phospholipid material was used to produce 10% phospholipid dispersions in imidazole buffer (20 mM imidazole, 50 mM sodium chloride, and 0.02% sodium azide in Milli Q water, adjusted to pH 7 with 1 M hydrochloric acid) using a JKA Ultra-Turrax (JKA, Staufen, Germany). The dispersions were cycled through a M-110Y Microfluidizer (Microfluidics International Corp., Newton, MA) with a 75 μm F12Y type interaction chamber five times at ∼1100 bar (17000 psi).

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Measurement of Stability of Liposome Dispersions. *Hydrodynamic Diameter.* The average hydrodynamic diameter of the liposome dispersions was determined using photon correlation spectrometry. The liposome dispersions were diluted to the required turbidity (<250 kilocounts/s) with imidazole buffer, and samples were analyzed in triplicate at 25 °C using a Zetasizer 4 (Malvern Instruments Ltd., Worcestershire, United Kingdom). The sampling time was 99 s, with a scattering angle of 90°, a liposome refractive index of 1.45 (*24*, *25*), a medium viscosity of 1.054 cP, and a refractive index of 1.34 for the aqueous phase.

Transmission Electron Microscopy (TEM). A negative-staining TEM technique was used to provide visual confirmation of the structure of the liposome dispersions. The liposome dispersions were diluted to ~1% phospholipid with distilled water and then mixed with an equal volume of a 2% ammonium molybdate solution and left for 3 min. A drop of this solution was then placed onto the surface of a copper mesh disk for 5 min, and the excess liquid was drawn off using filter paper. The mesh was examined using a Philips 201C Transmission Electron Microscope (Eindhoven, Netherlands).

Determination of ζ-Potential. The ζ-potential of liposome dispersions at a range of pH values was measured using the Zetasizer 4 (Malvern Instruments Ltd.) with an AZ104 cell. Liposome dispersions were prepared in 0.1 M NaCl, and the pH was adjusted using 1 M HCl and 1 M NaOH. Five measurements of 25 s duration at 100 mV were used to measure the ζ-potential at the stationary layer 14.63% of the capillary diameter in from the wall.

Lipid Oxidation of Liposome Dispersions. The peroxide value was determined using a technique based on ISO 3960:2001 (International Organization for Standardization). Briefly, 1 mL of the liposome dispersion was dissolved in 6 mL of acetic acid:chloroform (3:2). The sample was mixed for 1 min with 0.5 mL of saturated potassium iodide solution, followed by a further addition of 6 mL of distilled water. The solution was titrated against 0.1 M sodium thiosulfate solution, with 0.5 mL of a 1.0% starch solution added once the yellow color had almost disappeared. Titration then continued until no blue color remained. The peroxide value was calculated as milliequivalents peroxide/1000 g sample.

The levels of conjugated dienes and trienes formed during oxidation were measured using a method based on IUPAC Method no. 2.505 (*26*) and Lethuaut et al. (*27*). A 25 μL aliquot of the liposome dispersion was dissolved in 10 mL of isopropanol, mixed for 4 s, and centrifuged for 5 min at 2500*g* (CentraMP4R centrifuge, International Equipment Company, Needham Heights, MA). The absorbance was read against a blank containing 10 mL of isopropanol and 25 μL of Milli-Q water at 232 (linoleic hydroperoxides and conjugated dienes) and 268 nm (conjugated trienes and secondary products).

Stability of Liposome Dispersions during Storage. To examine the stability of the liposomes during storage, pH-adjusted samples were held at 5, 20, 30, and 35 °C. Liposome dispersions were assessed weekly for changes in average hydrodynamic diameter, peroxide value, and level of conjugated dienes.

Stability of Liposome Dispersions during Heat Treatments. A selection of heat treatments was chosen to represent common temperature/time combinations used during the processing of foods. These included extended time at elevated temperatures (55 °C for 15 h), pasteurization (75 °C for 2 min), high-temperature treatment (90 °C for 2 min), and ultrahigh temperature (UHT) treatment (141 °C for 15 s). For the UHT treatment, the samples were heated to 141 °C and held for 15 s using a mini UHT plant (Alfa Laval, Sweden), with a flow rate of 1 L/min. The other samples were all heated to the desired temperature using an insulated, stirred water bath. After the specified heat treatment, all samples were rapidly cooled and then stored at 5 °C. The average hydrodynamic diameter of the liposome dispersions was determined 1 day after the heat treatment and again 7 days later.

Effect of Ionic Concentration on the Stability of Liposome Dispersions. Samples of the liposome dispersions were mixed 1:1 with aqueous solutions containing NaCl or $CaCl_2$ (0.01−4 M) and left for 1 h before determining the average hydrodynamic diameter.

In some experiments, 10 mL of the MFGM or soya liposome dispersion was dialyzed against 100 mL of the other liposome dispersion for 24 h at 20 °C, with the external dispersion replaced every 8 h. These dialyzed samples were then mixed 1:1 with the NaCl or $CaCl_2$ solutions as described above.

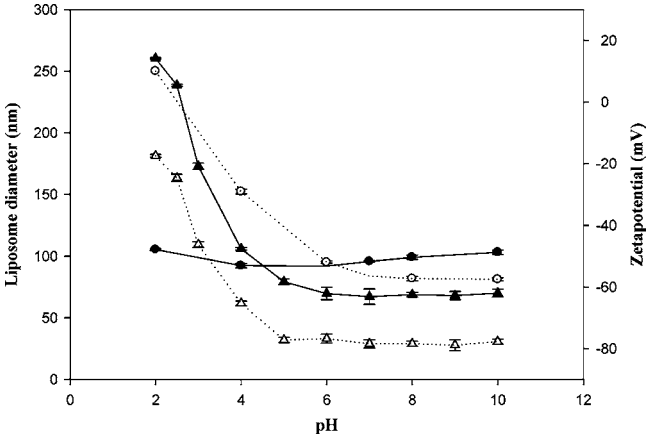

Figure 1. Effect of pH on the average hydrodynamic diameter (MFGM liposome dispersions, ●; soya liposome dispersions, ○) and ζ-potential (MFGM liposome dispersions, ▲; soya liposome dispersions, △). Samples were measured 24 h after pH adjustment. Each point is the mean of three measurements with error bars ± 1 standard deviation.

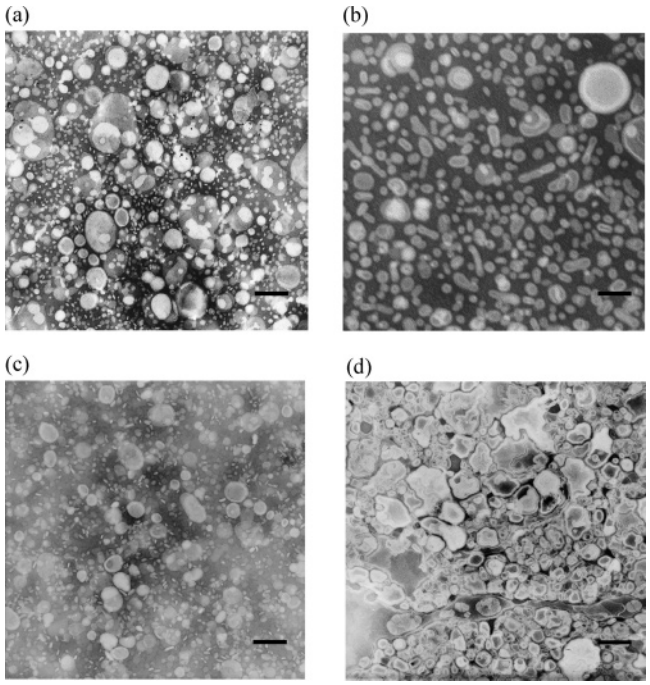

Figure 2. Negative-staining TEM micrographs of MFGM- and soya-derived liposome dispersions. (**a**) MFGM dispersion at pH 7, (**b**) MFGM dispersion at pH 4, (**c**) soya dispersion at pH 7, and (**d**) soya dispersion at pH 4. Bar = 500 nm.

RESULTS AND DISCUSSION

Effect of pH on the Stability of Liposome Dispersions. The changes in liposome diameter caused by pH adjustment are shown in **Figure 1**. There was little change in the average diameter of the MFGM phospholipid liposome dispersion (~95 nm) across the entire pH range, but while the soya phospholipid dispersions had an average diameter of ~80 nm between pH 6 and pH 10, there were rapid increases in average diameter in all samples at pH < 6. This increase in liposome diameter at the low pH values was reflected in a dramatic increase in sample turbidity, with the dispersion at pH 2, forming a solid, pale yellow sediment after 72 h.

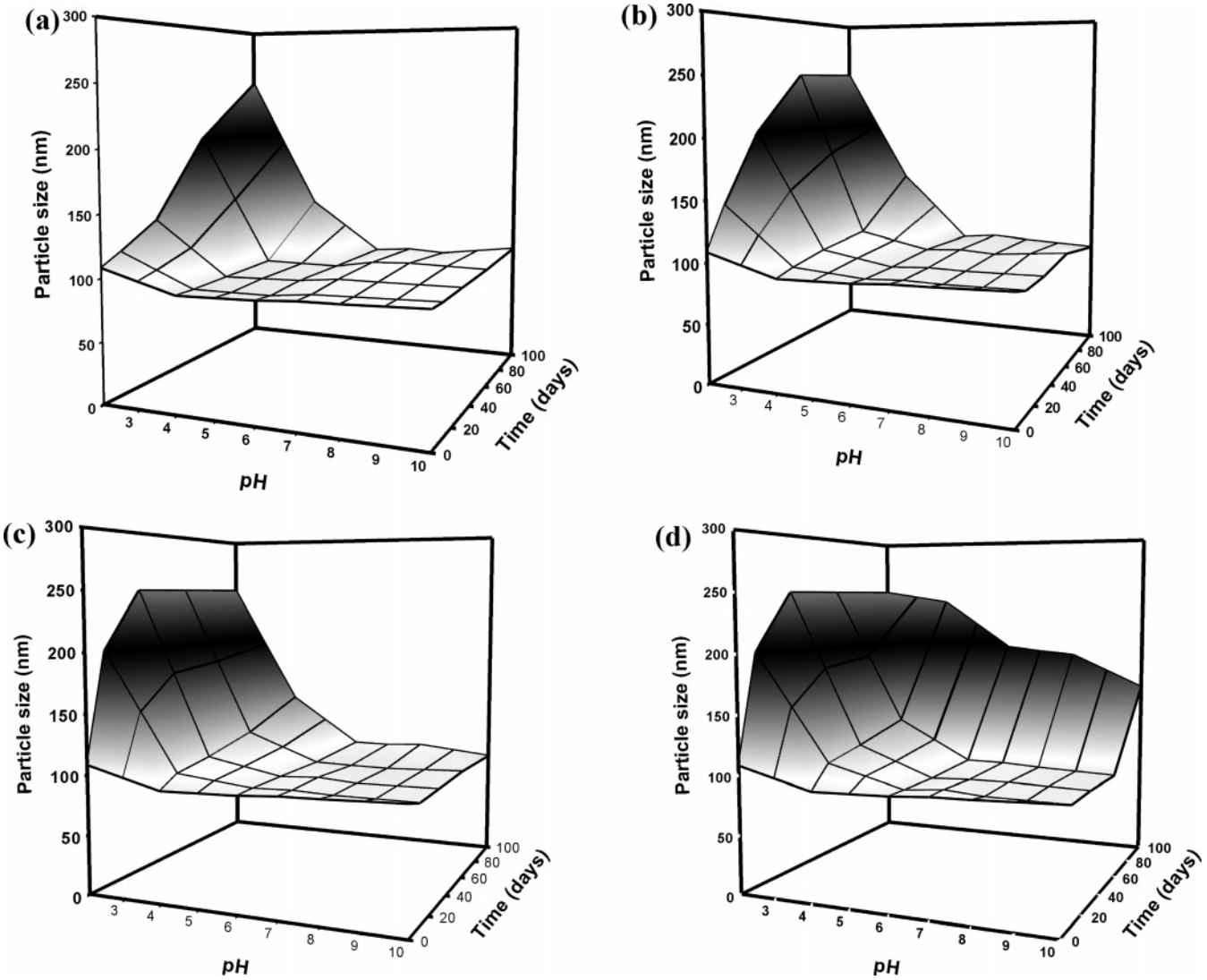

Figure 3. Effect of pH on average hydrodynamic diameter of MFGM phospholipid liposome dispersions during storage at various temperatures: (**a**) 4, (**b**) 20, (**c**) 30, and (**d**) 35 °C.

The effects of pH on the ζ-potential values for the liposome dispersions are also shown in **Figure 1**. The two dispersions have similar shaped curves, with the ζ-potential becoming rapidly less negative at pH values between 2 and 5 and a flat region between pH 5 and 10. The soya liposome dispersions had the highest ζ-potential value, ∼ −80 mV between pH 5 and pH 10 as compared with ∼ −65 mV for the MFGM liposome dispersions. The MFGM liposome dispersion appeared to have a potential of zero at pH 2.6, rising to a positive potential of 15 mV at pH 2.0. The potential for SigP3644 liposomes had not yet reached zero at pH 2.0.

The shape of the curve showing the effect of pH on the ζ-potential of the soya dispersions was very similar to the effect of pH on the average liposome size (**Figure 1**). This suggests that there may be a direct relationship between the surface charge of the soya liposomes and their stability. As the pH decreases, the surface charge decreases (ζ-potential becomes less negative), and there is less electrostatic repulsion between the liposomes. The stability of the MFGM liposomes, however, does not appear to be as dependent on charge repulsion, with the particle size not changing significantly between the pH values of 2 and 10, despite significant changes in ζ-potential. This implies the presence of an additional mechanism responsible for stabilization of MFGM liposomes.

From the average liposome size, it is not possible to distinguish between the different destabilization processes (aggregation, fusion, or rupture). Negative-staining TEM was used to provide a visual indication of the state of the liposome dispersion (**Figure 2**). The effect of pH on the appearance and structure of the liposomes was minimal for the MFGM dispersions (**Figure 2a**,**b**), but the pH change from 7 to 4 resulted in significant loss of structure in the soya liposomes (**Figure 2c**,**d**). These observations suggest that the increase in average liposome size at pH < 5 was not simply due to aggregation of liposomes after surface charge neutralization and that the pH reduction caused a change in the liposome and lipid bilayer structure. These results are in agreement with those of Nacka et al. (*28*), who found that at low pH some vesicles simultaneously aggregate and undergo morphological changes.

The acid- or base-catalyzed hydrolysis of phospholipids has been shown to result in the formation of lysophospholipids and free fatty acids (*29*−*31*). Grit and Crommelin (*32*) reported a v-shaped relationship between the pH and the rate of hydrolysis of saturated soya phospholipids, with a minimum at pH 6.5. Similar relationships have been reported for the hydrolysis of natural soya PC and a variety of other phospholipid materials (*33*, *34*). The hydrolysis products may lead to the destabilization of liposome dispersions (*30*) and morphological changes in the

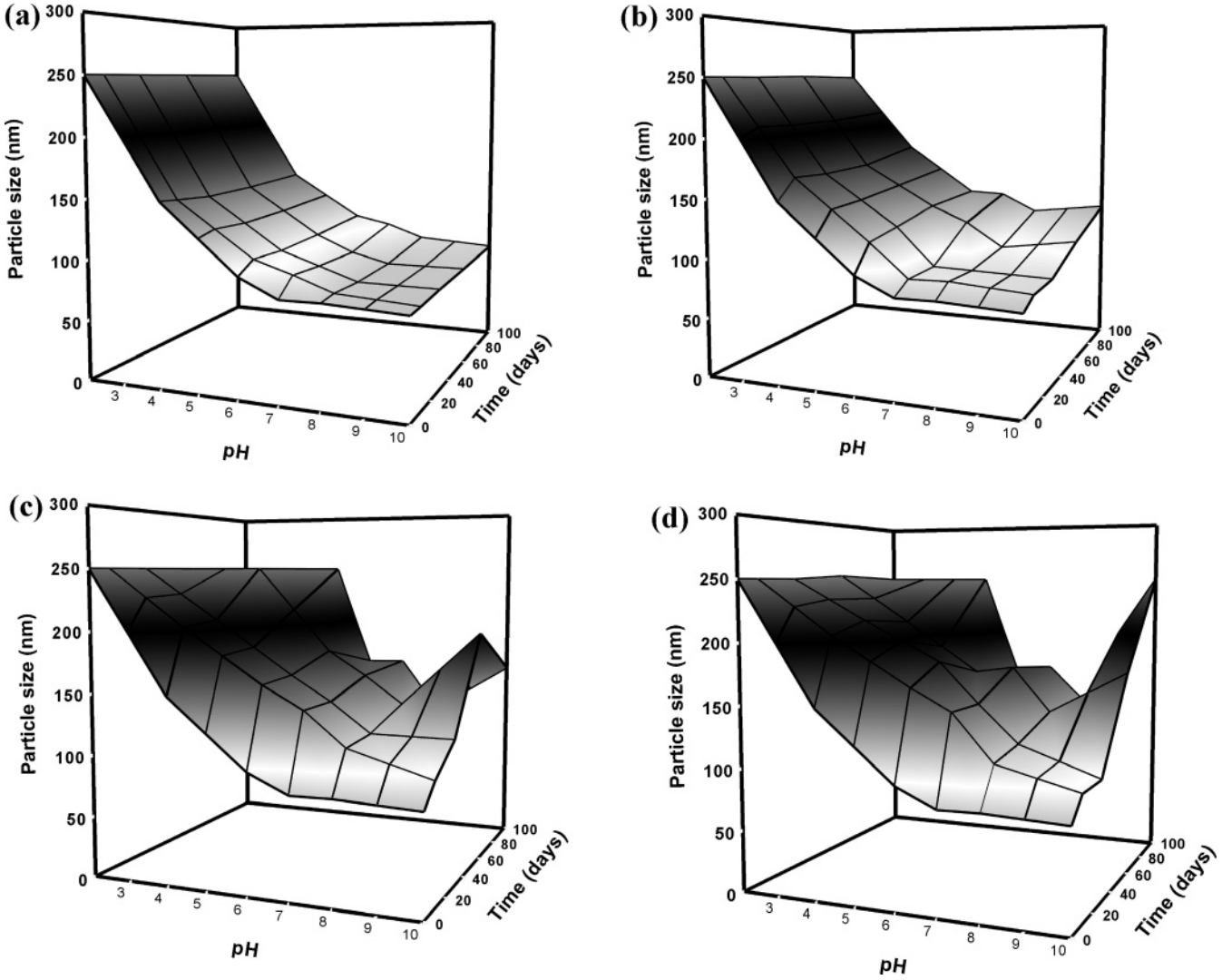

Figure 4. Effect of pH on average hydrodynamic diameter of soya phospholipid liposome dispersions during storage at various temperatures: (**a**) 4, (**b**) 20, (**c**) 30, and (**d**) 35 °C.

liposome structure (*30*, *35*, *36*), although Allen Zhang and Pawelchak (*37*) and Zuidam et al. (*38*) reported that approximately 60% of the phospholipid must be hydrolyzed before significant changes in liposome size were observed. It is possible that the hydrolysis of the soya phospholipids at pH 4 has contributed to the destabilization of these liposomes, resulting in their aggregation and fusion (**Figure 2c**,**d**). Sphingomyelin has been shown to be much more resistant to hydrolysis than phospholipids (*39*), and the presence of high levels of this molecule may have provided some protection for the MFGM liposomes from hydrolytic destabilization.

Stability of Liposome Dispersions during Storage. *Average Liposome Diameter.* **Figure 3** (**a**−**d**) shows the effect of pH, storage time, and temperature on the average liposome diameter of the MFGM phospholipid liposome dispersions. At 4 °C, there was an initial slight increase in diameter for samples at pH 2, but no change was observed for samples at pH 4−10 (**Figure 3a**). The pH 2 sample showed a slight increase in size after 60 days, but samples at pH 6−10 appeared to be relatively stable over the 100 day period. Storage at 20 and 30 °C resulted in a similar pattern, but as the storage temperature increased, the increase in liposome size at pH 4 and below appeared to occur more rapidly (**Figure 3b**,**c**). After 100 days at 30 °C, there was a slight increase in particle size even among the samples at pH > 6, with major changes at pH > 6 for the samples stored at 35 °C (**Figure 2d**). However, samples at pH 6−10 appeared to be stable during storage for up to 60 days even at the higher temperatures used.

The effect of pH, storage time, and temperature on the average liposome diameter of soya phospholipid liposome dispersions is shown in **Figure 4**. These dispersions were not stable at pH < 6 at any temperature, even for short periods. However, when samples at pH 7−10 were stored at 4 °C, there appeared to be very little change in size over the 100 days (**Figure 4a**). At storage temperatures of 20−35 °C, the average liposome diameter of the higher pH samples also increased, demonstrating significant instability after only 10 days (**Figure 4b**−**d**). Although the samples at pH 10 initially had a smaller increase in average diameter, a rapid increase occurred after 40 days. The dispersions at pH 8 had the best long-term stability at all temperatures.

Lipid Oxidation. The peroxide value of the fresh pH 7 dispersion was measured prior to the pH adjustment and storage of the samples. There was a general increase in peroxide value over time, with a significantly faster rate of increase at the higher storage temperatures. The changes in peroxide value during storage at 20 °C are shown in **Figure 5a**,**b**. The overall increase was much smaller in the MFGM liposome dispersions, with the peroxide values of samples at pH 7 increasing by 100%

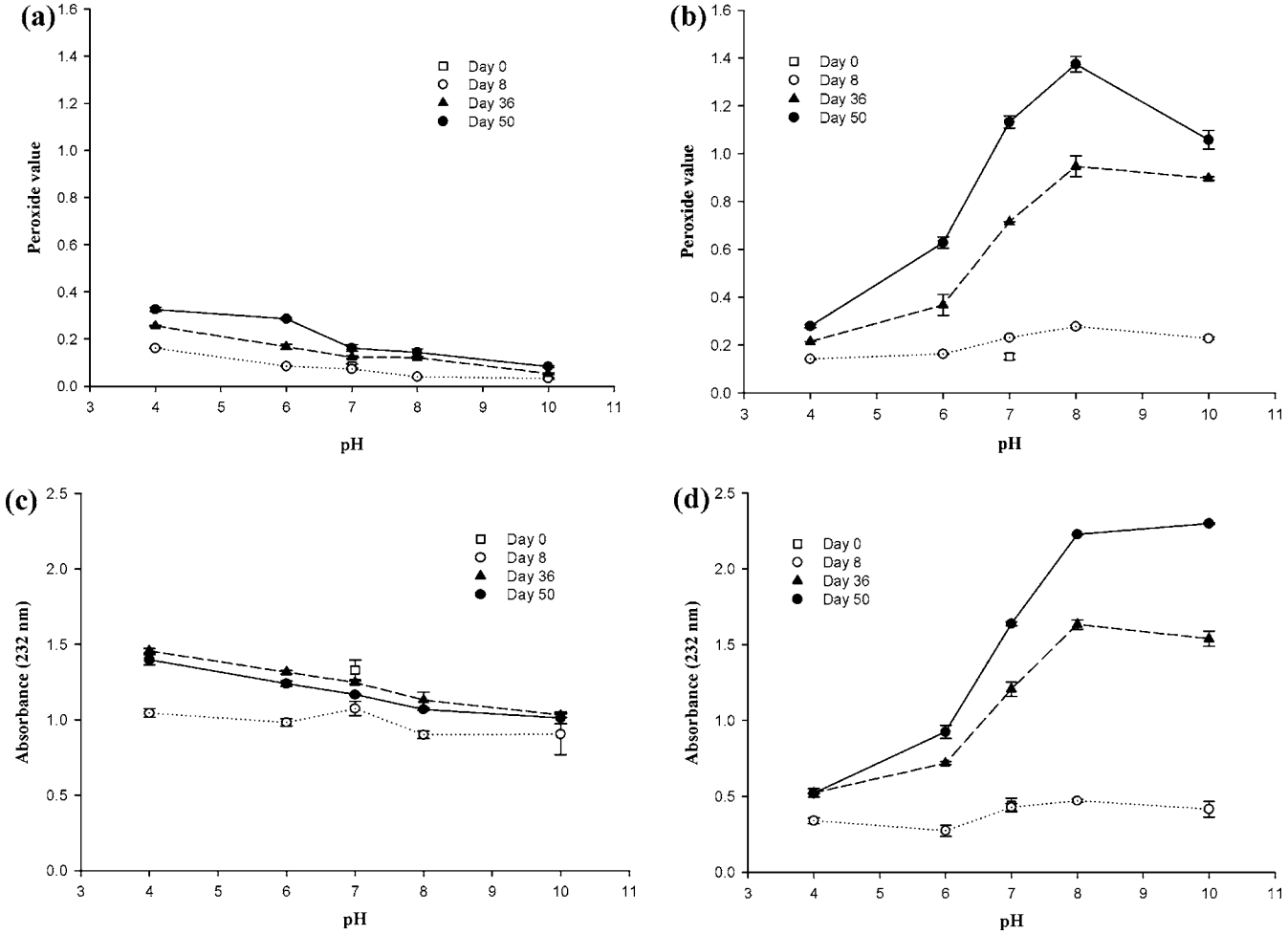

Figure 5. Changes in peroxide value and level of conjugated dienes in liposome dispersions stored at 20 °C. (**a**) Peroxide value of MFGM dispersions, (**b**) peroxide value of soya dispersions, (**c**) conjugated dienes in MFGM dispersions, and (**d**) conjugated dienes in soya dispersions. Each point is the mean of three measurements with error bars ± 1 standard deviation.

over 50 days at 20 °C as compared with 1000% for the equivalent soya liposome dispersions.

The effect of pH on peroxide value differs between the two types of liposome dispersions. The MFGM liposome dispersions tended to have the highest peroxide value at low pH values, while the peroxide values for the soya liposome dispersions were highest at pH 7−8.

The levels of conjugated dienes formed in each of the liposome dispersions during storage at 20 °C are shown in **Figure 5c**,**d**. The absorbance at 232 nm of the pH 7 dispersion was initially measured prior to pH adjustment. The absorbance values increased with storage time, indicating the formation of conjugated dienes through the oxidation process. The absorbance level for the MFGM liposome dispersion was between 0.8 and 1.5, including after storage at 35 °C for 55 days. The soya liposome dispersion had an initial value of 0.4, but after storage at 20 °C for 50 days, this had increased to 2.4, and it rose to 4.0 after storage at 35 °C for 55 days. The higher initial value for the MFGM liposome dispersion may be due to some nonlipid component present in the fraction that also absorbs at 232 nm, but the relatively small increase as compared with the soya samples indicates that the rate of conjugate diene formation was much lower.

There was a strong correlation between the storage temperature and the rate of increase in conjugated dienes, which is not surprising since the rate of oxidation is known to increase with temperature (*40*). There was some indication that the rate of conjugated diene formation increased at the higher pH values during storage of the soya liposome dispersions, but the absorbance for the MFGM liposome dispersions increased more rapidly at low pH values.

Overall, the peroxide value and conjugated diene results suggest that liposomes prepared from the soya phospholipid fraction are more susceptible to oxidation than the MFGM liposome dispersions. The MFGM liposome dispersions have a much lower rate of formation of conjugated dienes, most likely reflecting its primarily saturated fatty acid profile (*22*).

Stability of Liposome Dispersions during Heat Treatments. Figure 6 shows the effect that a variety of heat treatments had on the average liposome diameter of the dispersions at different pH values. In order to allow comparison between the different liposome dispersions, the results are reported in terms of the percentage change in diameter caused by the heat treatment.

The UHT treatment, which was provided by a pilot-scale plant, had the most severe effect on MFGM dispersions, despite the short exposure to the high temperatures and fast cooling. Heating at 90 °C for 2 min resulted in the most significant increase in liposome diameter for the soya dispersions. In general, heat treatment at high pH appeared to result in a much smaller change in average liposome diameter than the same treatment at lower pH values. The MFGM liposome dispersions were consistently more stable at all pH values than the soya liposome dispersions.

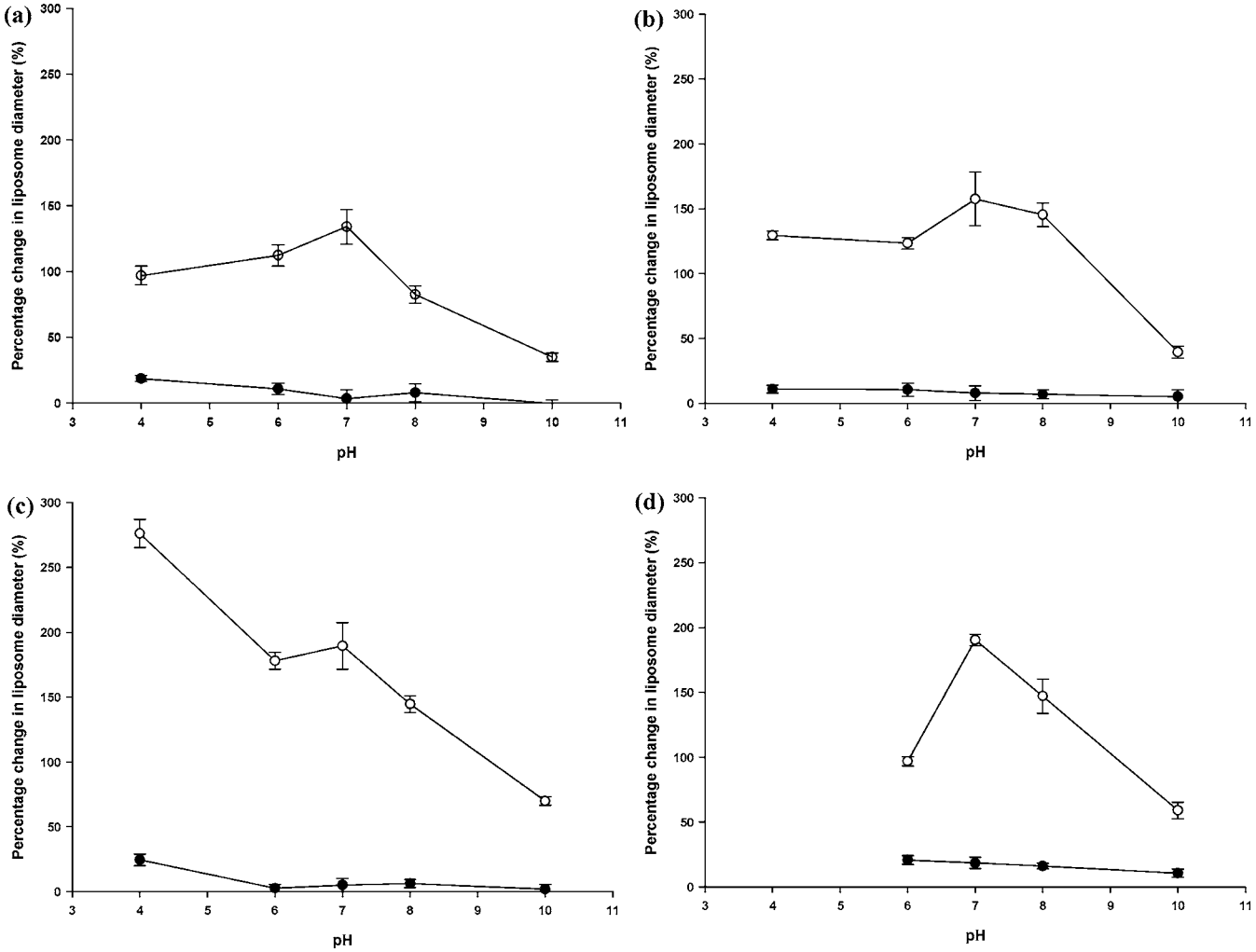

Figure 6. Changes in average diameter of MFGM liposome dispersions (●) and soya liposome dispersions (○) at a range of pH values after various heat treatments. The mean of three measurements is plotted with error bars showing ± 1 standard deviation. Conditions: (**a**) 50 °C for 15 h, (**b**) 72 °C for 2 min, (**c**) 90 °C for 2 min, and (**d**) 141 °C for 15 s.

There is relatively little published material, which considers the heat stability of liposome dispersions, although Chandran et al. (*41*) stated that liposomes are thermolabile and that the lipids are likely to be hydrolyzed during the high sterilization temperatures. Conversely, Arnaud (*42*) reported that heat sterilization could be used to attain microbiological stability of selected liposome dispersions and that it did not appear to affect liposome stability. Zuidam et al. (*43*) also investigated the use of heat for the sterilization of liposomes and found that liposome structure and lipid oxidation values were not affected by autoclaving (121 °C for 15 min).

Stability of Liposome Dispersions during Changes in Ionic Concentration. The effect of changes in ionic concentration on the average liposome diameter of the phospholipid dispersions at pH 7 is shown in **Figure 7**. In order to allow comparison between the different phospholipid fractions, the results are reported in terms of the percentage change in diameter caused by the addition of the sodium or calcium chloride.

Overall, the MFGM liposome dispersions were less affected than the soya liposome dispersions by the changes in ionic concentration. This difference was most clearly seen with the addition of calcium ions. An increase in liposome diameter of less than 10% was exhibited for up to 1.0 M NaCl addition for the MFGM liposome dispersion, as compared with 0.2 M NaCl for the soya liposome dispersions. Calcium ions caused more rapid increase in liposome diameter, with 0.25 M $CaCl_2$ addition resulting in a 10% increase in diameter for the MFGM liposome dispersion and even 0.1 M $CaCl_2$ causing >20% increase for the soya dispersions.

To determine whether or not these differences were due to the phospholipids present rather than the differences in the ionic composition of the original material, samples were dialyzed against the other dispersion to exchange the ionic profiles. These samples then had varying amounts of NaCl and $CaCl_2$ added as previously described. The soya liposome dispersion dialyzed against the MFGM dispersion was less stable in the presence of Na^+ ions than the same dispersion with its original ionic profile, but there was no change in the MFGM liposome dispersion dialyzed against the soya liposome dispersion (results not shown). This indicates that the natural mineral content of the fractions was not responsible for the observed differences in the susceptibility of the liposome dispersions to increasing ionic concentrations.

Conclusions. As reported in a previous publication (*22*), the most significant difference between the phospholipid composition of these two fractions was the high level of sphingomyelin in the MFGM fraction (approximately one-third of the polar lipid present). Sphingomyelin has a more structured gel phase than phosphatidyl choline (the primary component of the soya phospholipid fraction), and sphingomyelin membranes seem to be more stable than phosphatidyl choline bilayers (*44*). Experiments comparing the stability of the liposome

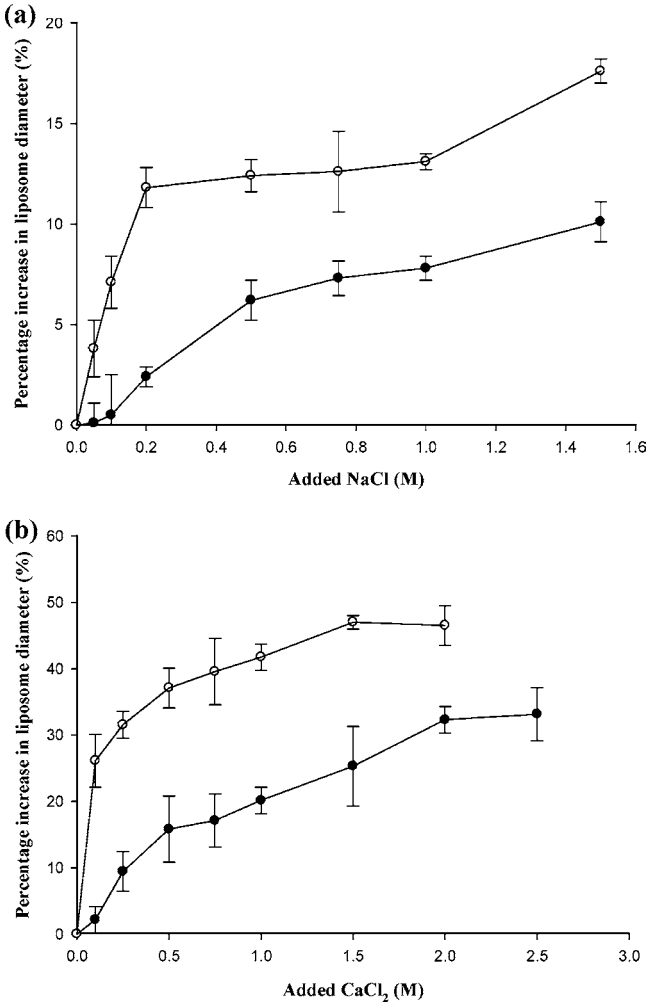

Figure 7. Effect of increasing ionic concentration on the average diameter of MFGM liposome dispersions (●) and soya liposome dispersions (○) at pH 7. (**a**) Addition of NaCl and (**b**) addition of $CaCl_2$. Each point is the mean of three measurements with error bars ± 1 standard deviation.

dispersions found that the MFGM liposome dispersions showed significantly better stability during storage at temperatures in the range 4−35 °C. The MFGM dispersions were also more stable during a wide range of heat treatments. Increases in ionic concentration resulted in much more rapid aggregation and/or fusion among the soya liposome dispersions than in the MFGM liposome dispersions, independent of the natural mineral balance of the fraction. These results all support the claimed increase in stability due to the presence of sphingomyelin and suggest that the high proportion of sphingomyelin in the MFGM fraction may provide significant advantages in terms of the resistance of the liposomes to environmental stresses.

So far, our work has demonstrated that it is possible to make liposomes from MFGM material (*12*), that these liposome dispersions have thicker membranes and reduced permeability than their soya counterparts (*22*), and that this appears to translate into an increase in stability for the MFGM liposome dispersions. The next step in determining the potential for use of such dispersions for the delivery of bioactive material in food systems is to investigate the encapsulation and release of hydrophobic and hydrophilic materials within the liposome dispersions.

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