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The properties of liposomes produced from milk fat globule membrane material using different techniques

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Abstract – The isolation of milk fat globule membrane (MFGM) material from buttermilk on a commercial scale has provided a new ingredient rich in phospholipids and sphingolipids. In the pharmaceutical and cosmetic industries, highly purified phospholipids extracted from soya oil or egg yolk are used to produce liposomes. Liposomes are spherical structures consisting of one or more phospholipid bilayers enclosing an aqueous core. They may be used for the entrapment and controlled release of drugs or nutraceuticals, as model membranes or cells, and even for specialist techniques such as gene delivery. There are many potential applications for liposomes in the food industry, ranging from the protection of sensitive ingredients to increasing the efficacy of food additives. Our previous work compared the structure and properties of liposomes prepared from a milk fat globule membrane (MFGM) fraction and soya phospholipid material using a high-pressure homogenizer (Microfluidizer). These results identified some potential advantages in the use of MFGM phospholipids for the manufacture of liposomes for use in food systems. This paper compared the general structure and properties of liposomes prepared from the same MFGM phospholipid material using three different techniques – microfluidization, the traditional thin-film hydration and the heating method. The thin-film hydration technique required the use of organic solvents, while the other two methods do not involve any non food-safe chemicals. The liposomes prepared by both microfluidization and the heating method had high entrapment efficiencies. Liposomes produced via microfluidization tended to be significantly smaller than those produced by the other methods, with a narrower size distribution, and a higher proportion of unilamellar vesicles. There did not seem to be any advantages in the use of the thin-film hydration method, opening the door to the use of food-safe methods for liposome production.

liposome / milk fat globule membrane / phospholipid / microfluidization

摘要 – 不同方法从乳脂肪球膜中制备脂质体的性质。从工业生产的酪乳中分离出的乳脂肪球膜 (MFGM) 可作为一种富含磷脂和鞘磷脂的配料。在制药和化妆品工业生产中, 通常以鸡蛋黄或豆油中提取的高纯度磷脂为原料制备脂质体。脂质体是由磷脂双层构成的具有水相内核的脂质微囊。脂质体可以作为药物或活性成分的封装材料及可控释放, 可以模拟膜和细胞, 甚至可以作为基因的载体。脂质体在食品工业中用途广泛, 可以作为敏感性添加物质的保护剂以增加食品添加剂的效率。在过去的研究中, 我们比较了乳脂肪球膜和大豆磷脂用微流态化器制备脂质体的结构和性质。实验证明用乳脂肪球膜磷脂制造的脂质体可以应用于食品加工中。本文比较了同一种脂肪球膜磷脂经微流态化法、传统的薄膜分散法和熔融法三种方法制备脂质体的结构和性质。薄膜分散法需要有机溶剂, 而其他两种方法不使用任何有害的化学试剂。采用微流态化法和熔融法制备的脂质体具有较高的封装效率。由微流态化法制备的脂质体显著地小于其他两种方法制备的脂质体, 且表现出较窄的颗粒分布和较高比例的单室脂质体。从食品安全的角度考虑, 薄膜分散法制备脂质体不具有优势。

脂质体 / 乳脂肪球膜 / 磷脂 / 微流态化作用

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Résumé – Propriétés de liposomes produits à partir de matériel membranaire de globule gras du lait. La séparation de matériel membranaire de globule gras du lait (MFGM) à partir de babeurre a fourni à l'échelle commerciale un nouvel ingrédient riche en phospholipides et en sphingolipides. Dans les industries pharmaceutiques et cosmétiques, les phospholipides hautement purifiés extraits d'huile de soja ou de jaune d'œuf sont utilisés pour produire des liposomes. Les liposomes sont des structures sphériques consistant en une ou plusieurs double-couches de phospholipides enfermant un centre aqueux. Ils peuvent être utilisés pour le piégeage et le relargage contrôlé de médicaments ou de nutraceutiques, comme membranes ou cellules modèles, et même pour des techniques spécialisées comme le transport de gènes. À notre connaissance, les phospholipides dérivés de MFGM n'ont pas encore été utilisés dans la fabrication de liposomes. Il y a beaucoup d'utilisations potentielles pour les liposomes dans l'industrie alimentaire, allant de la protection d'ingrédients sensibles jusqu'à l'accroissement de l'efficacité d'additifs alimentaires. Le taux élevé de sphingolipides dans les phospholipides de la membrane des globules gras du lait peut apporter un bénéfice nutritionnel au consommateur, de même qu'une amélioration des fonctionnalités des liposomes dans les aliments. Les liposomes ont été préparés à partir de la fraction phospholipidique de la membrane des globules gras du lait et de phospholipides de soja en utilisant 3 techniques – la traditionnelle hydratation sur couche mince, les méthodes de chauffage et via un homogénéisateur à haute pression (microfluidiseur). Les liposomes préparés à partir de la fraction MFGM avaient une température de transition de phase significativement plus élevée, une membrane plus épaisse et une perméabilité membranaire plus basse que ceux produits à partir de phospholipides de soja. Des investigations ultérieures sur la stabilité relative des deux dispersions de liposomes ont montré que ceux issus de la fraction MFGM étaient plus stables que leurs homologues venant du soja dans une gamme de pH, à différentes températures de traitement et de stockage et en présence de cations monovalents et divalents. Ces résultats illustrent quelques avantages potentiels de l'utilisation des phospholipides de la MFGM dans la fabrication de liposomes pour leur utilisation dans les aliments.

liposome / membrane de globule gras du lait / phospholipide / microfluidisation

1. INTRODUCTION

More than 95% of the total lipid in milk is in the form of globules ranging in size from 0.2 to 15 μm in diameter. These liquid fat droplets are covered by a thin membrane, 8 to 10 nm in thickness, whose properties are completely different from both milkfat and plasma. The total mass of fat globules that is accounted for by membrane material has not been determined with certainty. An estimated mass of the membrane is 2–6% of that of the total fat globules.

Recent developments in commercial isolation techniques have led to the production of a valuable product rich in phospholipids and sphingolipids from waste dairy streams such as buttermilk [2, 7]. Research into the biological functions of phospholipids and sphingolipids has identified a number of health benefits, including liver protection [15], memory improvement [8, 9] and inhibition of cholesterol

absorption [21]. There is much ongoing research in this area, and no doubt there will be many more studies investigating these potential health benefits in future.

Because of its original function in stabilizing the fat globules in whole milk, MFGM material isolated from buttermilk or cream is considered to be an efficient natural surface-active material, with high emulsifying capacity. The emulsifying properties of this material have been previously discussed in a number of publications [6, 24, 34]. Emulsions produced from MFGM fractions have also been successfully used for the delivery of drugs [25, 35].

In the pharmaceutical and cosmetic industries, highly purified phospholipids extracted from soya oil or egg yolk are used to produce liposomes. Liposomes are spherical structures consisting of one or more phospholipid bilayers enclosing an aqueous core [36]. These structures may be used for a wide variety of applications,

including the entrapment and controlled release of drugs or nutraceuticals and as model cells or membranes [16]. There are a large number of potential opportunities for use of liposomes in the food industry [3, 11, 13, 23, 28, 33, 36], but the high cost of the soya and egg phospholipids used by the pharmaceutical industry has limited their commercial application in food systems. To our knowledge, MFGM-derived phospholipids had not been used to produce liposomes by any group before we began our work in 2001 [29].

Comparisons between the liposomes prepared using microfluidization from non-hydrogenated commercial soya phospholipids and the MFGM fraction identified a number of key differences. The MFGM liposomes had a higher phase transition temperature, thicker membrane and lower membrane permeability [31]. MFGM-phospholipid liposomes were found to be more stable than their soya counterparts in a wide range of conditions, including changes in pH or ionic concentration, storage at 4, 20, 30 or 35 °C and during thermal processing conditions such as pasteurization [30].

Having established that liposomes produced from the MFGM material demonstrated a number of advantages over liposomes produced from soya phospholipids, the next step was to consider the properties of liposome populations produced from the MFGM fraction using three different techniques. There is a great deal of variation between liposome production techniques, particularly in terms of liposome size distribution, lamellarity and entrapment efficiency. Each technique has certain advantages and disadvantages that determine its suitability or otherwise for a specific application. A number of excellent reviews have been published that provide preparation details for the more common currently used preparation techniques and resulting liposome characteristics [10, 14, 32, 36]. This manuscript describes the structure and

Table I. Typical composition of Phospholipid Concentrate 700.

Component	Amount (%)
Total lipids	85
<i>Phosphatidyl choline</i>	23.6
<i>Phosphatidyl serine</i>	2.5
<i>Phosphatidyl ethanolamine</i>	20.2
<i>Sphingomyelin</i>	22.8
Lactose	6.6
Moisture	2.5
Ash	<12

properties of liposomes prepared by microfluidization, thin-film hydration and the heating method.

2. MATERIALS AND METHODS

The MFGM-derived fraction rich in phospholipids (Phospholipid concentrate 700) was provided by the Fonterra Co-operative Group Ltd (New Zealand). A typical composition of Phospholipid Concentrate 700 is shown in Table I.

All other chemicals used were of analytical grade and obtained from Sigma-Aldrich (St. Louis, MO).

2.1. Liposome preparation techniques

2.1.1. Thin-film hydration

This is the standard preparation procedure which has been used to produce liposomes for several decades [12, 22]. The phospholipid material is dissolved in chloroform, and a rotary evaporator used to remove the solvent. A thin phospholipid film on the surface of a round-bottomed flask remains. Addition of imidazole buffer (20 mmol·L⁻¹ imidazole, 50 mmol·L⁻¹ sodium chloride, and 0.02% sodium azide in Milli Q water, adjusted to pH 7, with

1 mol·L⁻¹ hydrochloric acid) and shaking of the flask causes bilayer sheets of the lipid to separate from the bulk and form liposomes.

2.1.2. Microfluidization

The phospholipid powders were dispersed in imidazole buffer (20 mmol·L⁻¹ imidazole, 50 mmol·L⁻¹ sodium chloride, and 0.02% sodium azide in Milli Q water, adjusted to pH 7 using 1 mol·L⁻¹ hydrochloric acid) and thoroughly mixed using a JKA Ultra-Turrax® (JKA, Staufen, Germany) to produce a dispersion with a phospholipid concentration of 10% (w/w). The phospholipid dispersion was then passed through a M-110Y Microfluidizer® (Microfluidics International Corp., MA-Newton, USA) with a 75 µm F12Y-type interaction chamber 5 times at ~ 1100 bar (17 000 psi) [29–31].

2.1.3. Heating method

Liposomes were prepared by the heating method [18] as follows. Each of the phospholipid material was hydrated in 2 mL Milli Q water, or imidazole buffer (20 mmol·L⁻¹ imidazole, 50 mmol·L⁻¹ sodium chloride, and 0.02% sodium azide in Milli Q water, adjusted to pH 7 using 1 mol·L⁻¹ hydrochloric acid), for 2 h under N₂ at room temperature. The lipid dispersion was then mixed with 0.3 mL glycerol and the volume made up to 10 mL with Milli Q water. The mixture was heated to 120 °C while stirring until all the lipids dissolved. The range of possible final phospholipid concentrations were mainly between 1–3% phospholipid, above which the liposomes were in the form of concentrated gel, known as vesicular phospholipid gel [27].

2.2. Characterisation of liposome dispersions

2.2.1. Average liposome hydrodynamic diameter

The average hydrodynamic diameter of the liposome dispersions was measured on a Nano Series ZS Zetasizer (Malvern Instruments Ltd, Worcestershire, UK) using photon correlation spectroscopy (PCS). Each sample was analyzed 13 times at 25 °C with a sampling time of 12 s and a scattering angle of 135°. A medium viscosity of 1.054 cP, aqueous phase refractive index of 1.34 and typical liposome refractive index of 1.45 [1, 4] were used.

2.2.2. Microscopy

Two different electron microscopy techniques were used to provide information on the microstructure of the liposome dispersions; negative staining transmission electron microscopy (TEM) and atomic force microscopy (AFM).

The negative staining TEM required the liposome dispersions to be diluted ~1:10 with distilled water to reduce the concentration of the liposomes. Equal volumes of the diluted sample and a 2% ammonium molybdate solution were combined and left for 3 min. A drop of this solution was placed on a copper mesh for 5 min before the excess liquid was drawn off using filter paper. The mesh was examined using a Philips 201C Transmission Electron Microscope (Eindhoven, Netherlands).

Atomic force microscopy (AFM) images were recorded in contact mode with a commercial Digital Instruments (Santa Barbara, CA) Nanoscope III AFM fitted with a 15 µm scanner (d-scanner) using standard Si₃N₄ tips. A drop (10–20 µL) of each of liposome suspensions was deposited on mica substrates, dried in air and visualized under AFM at room temperature.

2.2.3. Lamellarity and bilayer thickness

The thickness of the bilayer membrane is likely to affect the rate of release of entrapped bioactives, as is lamellarity, with multilamellar liposomes thought to provide a more gradual and sustained release compared with unilamellar liposomes [19]. The liposome dispersions were concentrated to ~20% phospholipid using Centriscart 119239E centrifugal filters (Sartorius, Goettingen, Germany) at $4000\times g$ for 4 h in a CentraMP4R centrifuge (International Equipment Company, Needam Heights, MA, USA). The samples were transferred into glass capillary tubes (1.5 mm diameter, Charles Supper Company, Natick MA, USA) and the scattering pattern measured during exposure to low divergence $\text{CuK}\alpha$ radiation with a wavelength of 1.54 Å (Rigaku MicroMax007, microfocus rotating anode generator with Osmic multilayer confocal optics; Tokyo, Japan). The exposure time varied from 10 to 15 min, with separate samples of the liposome dispersions analysed at 20 and 40 °C. The diffraction images were recorded on a RAxisIV++ image-plate detector (Rigaku, Fuji; Tokyo, Japan) placed 100–300 mm from the sample. Diffraction patterns were visualised and analysed using CrystalClear software (version 1.3.6SP0, Rigaku-MS; Tokyo, Japan).

2.2.4. Entrapped volume and entrapment efficiency

A common procedure for the determination of liposome entrapment efficiency (with respect to water-soluble agents), release characteristics and entrapped volume is a spectrofluorometric assay using calcein as a water-soluble marker [20]. The encapsulation efficiency (EE) and the entrapped volume (EV) based on calcein

were determined at room temperature as follows. Liposomes were prepared by the methods explained above in the presence of $66.65\ \mu\text{g}\cdot\text{mL}^{-1}$ ($0.1\ \text{mmol}\cdot\text{L}^{-1}$) calcein. Each liposome sample was diluted 1000-fold with imidazole buffer (pH 7.0), and the diluted samples were used to measure the fluorescence intensity of calcein using a fluorometer (Perkin Elmer, Luminescence Spectrometer LS50B) at excitation and emission wavelengths of 460 and 520 nm, respectively (excitation and emission slits 2.5 nm each). Subsequently, 100 μL of Triton X-100 (10%, w/v) was added and the fluorescence was measured again and the resultant fluorescence intensity (F_t) represents total concentration of the free calcein and liposome-entrapped calcein. The EE and EV were calculated, after correction for the dilution factors, from:

$$\begin{aligned}\text{EE (\%)} &= 100 \times (F_t - F_i)/F_t \\ &= \text{EV (\% v/w)}\end{aligned}$$

where F_t is the fluorescence intensity after addition of Triton X-100 and F_i is the fluorescence intensity before addition of Triton X-100.

3. RESULTS

3.1. Average hydrodynamic size and size distribution of liposomes

The average hydrodynamic diameter (Zave) for the various liposome dispersions is shown in Table II, along with the Polydispersity Index (PDI), a measure of the size distribution of the dispersion. It is clear that both composition and preparation method had an effect on the average liposome size, with the largest liposomes produced by the heating method (539 nm) and the smallest by microfluidization (84 nm). Microfluidization also produced dispersions with the smallest

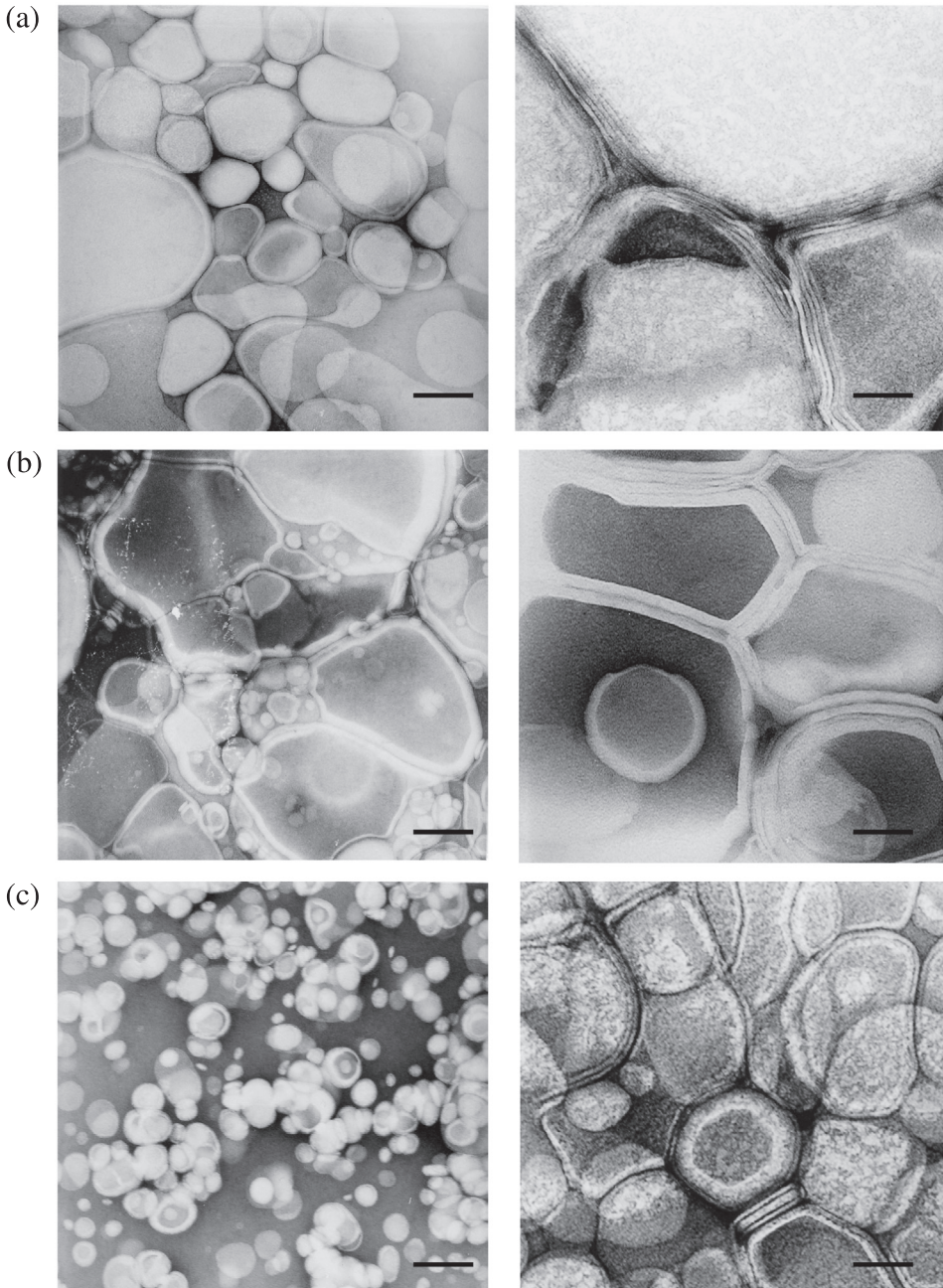


Figure 1. Negative staining TEM micrographs of liposomes produced using the different preparation methods. (a) Heating method, (b) thin film hydration, (c) microfluidization. Left column, bar = 0.5 μm ; right column, bar = 0.1 μm .

Table II. Average hydrodynamic diameter and size distribution for liposome dispersions produced from via different preparation methods. Results are the mean of at least 5 separate samples \pm the standard deviation.

Liposome preparation method	Average hydrodynamic diameter Z_{ave} (nm)	Polydispersity index (PDI)
Heating method	539 \pm 150	0.63 \pm 0.12
Microfluidization	84 \pm 8	0.23 \pm 0.07
Thin-film hydration	338 \pm 130	1*

* The maximum value for the PDI is 1.

PDI (0.23), indicating a narrower range of liposome diameters. Liposome dispersion produced via thin-film hydration consistently had a PDI of 1, indicating these populations had an extremely wide size distribution.

3.2. Microscopy

Typical negative staining TEM micrographs are shown in Figure 1. The liposomes could easily be identified as discrete particles that were predominantly spherical in shape. The negative staining TEM allowed the outer membrane surrounding the internal aqueous space to be clearly seen in many of the liposomes, and internal membranous structures could also be identified. It is clear that the method used for preparation had a significant effect on the size and lamellarity of the liposomes.

The liposomes produced using the heating method or thin film hydration tended to be considerably larger than those prepared via microfluidization, as well as appearing to have a broader particle size distribution. The dispersions prepared by the heating and film hydration techniques also contained a number of multi-lamellar onion-like structures (clearly shown in the micrographs at 61 000 \times magnification), which were not seen in the microfluidized dispersions.

The liposomes were also imaged by Atomic Force Microscopy (AFM). Typical micrographs are shown in Figure 2.

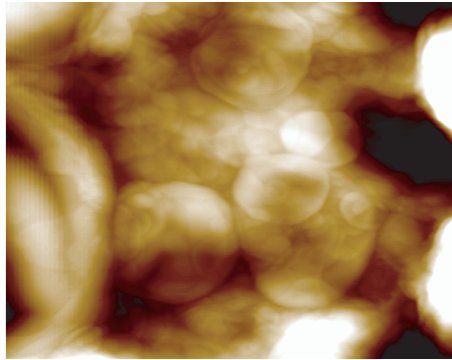
Smooth, spherical vesicles can be clearly seen in all samples. It is possible to measure vertical and horizontal sizes of individual vesicles. However, the sizes of liposomes obtained through AFM do not correspond to the sizes obtained through light scattering method. This is because the vesicles chosen for visualisation and measurement are generally the largest and thus not representative of the sample.

3.3. Bilayer thickness

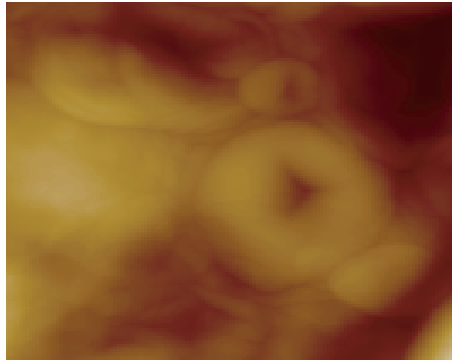
The results from the small angle X-ray diffraction of the concentrated liposome suspensions at 20 °C are shown in Table III. This gives the positions (in Å) of the diffraction maxima for each of the liposome suspensions. These distances provide an indication of the bilayer thickness for the liposomes in each of the dispersions.

The bilayer thickness is the same for both the heating method and the thin-film hydration method, but appears to be slightly thicker (+5 Å) for liposomes produced via microfluidization. This seems unusual given that these dispersions are produced from the same phospholipid material. In addition, the liposome dispersions produced via thin-film hydration had a darker section on one side of the beam-stop, corresponding to an ordered system of approximately 70–75 Å in length.

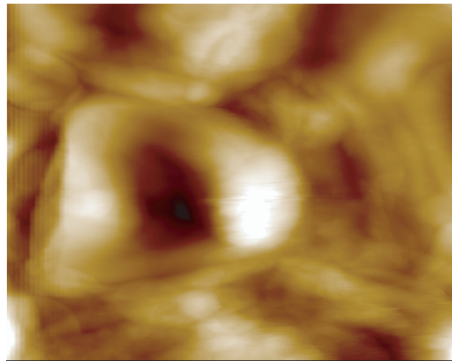
It was suggested that the latter observation could be due to a population of very small liposomes co-existing with larger



(a)



(b)



(c)

Figure 2. Atomic Force Microscopy image of liposomes prepared by the microfluidization technique. (a) Heating method, (b) thin film hydration, (c) microfluidization. Each image is $5\ \mu\text{m} \times 5\ \mu\text{m}$.

Table III. Diffraction maxima (corresponding to bilayer thickness) as determined by small angle X-ray diffraction.

Liposome preparation method	Diffraction maxima (Å)
Heating method	54 ± 2
Microfluidization	60 ± 2
Thin-film hydration	53 ± 2, additional dark section 75 ± 1

liposomes. These small liposomes would exhibit extreme membrane curvature, leaving gaps for more molecules to insert into the exterior leaf of the membrane, thus making it appear thicker (Fig. 3). This suggestion is supported by the negative staining electron microscopy image of one of these dispersions (Fig. 4), which clearly shows a population of large liposomes around 500 nm in diameter, as well as a population of much smaller ones with diameters of between 20–100 nm. The theoretical minimum diameter possible for a liposome is 0.02–0.025 µm, limited by the surface curvature (resulting in crowding of the phospholipid headgroups) and the thickness of the phospholipid bilayer (about 4 nm or 40 Å). As the extra length (40 Å bilayer thickness plus an additional 30–35 Å to give a total of 70–75 Å) corresponds to approximately 70–80% of the width of one leaf of the membrane, these extra molecules would not need to be inserted very far into the exterior leaf.

It may also be possible that the smaller average diameter of the liposomes produced via microfluidization (80–100 nm, Tab. II) could be opening up small spaces between the molecules in the outer leaf of the membrane, although the smaller increase in width (only ~5 Å) would suggest that any molecules which become incorporated into the liposome to fill these gaps are more completely inserted into the outer leaf than in the thin-film counterparts.

3.4. Lamellarity

The intensity maxima were taken as the centre of a very broad and intense ring for each of the liposome suspensions, similar to those reported for previous MFGM liposome dispersions [31]. As discussed in our previous work, X-ray scattering curves from dispersions containing only unilamellar liposomes are typically very broad and flat with a single symmetric peak, while multilamellar liposomes exhibit diffraction peaks at regular intervals. If the majority of the multilamellar liposomes present are only bi- or tri-lamellar, they need to comprise >20% of the total liposome population before they will have an obvious effect on the X-ray scattering curve [5]. Multilamellar liposomes with different lamellar spacings either within the same vesicle or between different vesicles will have different scattering curves, and the overall diffraction pattern for the system may average out to a broad smoother peak [17]. In addition, multivesicular liposomes will be recognised as unilamellar structures by the diffuse small angle X-ray diffraction [26]. Therefore, although the scattering curves are similar to those observed from primarily unilamellar and multivesicular systems, this does not rule out the possibility that there are still a significant proportion of multilamellar liposomes within the dispersions, as seen by the electron micrographs (Fig. 1).

3.5. Entrapment efficiency

The entrapped volume depends on liposome size, composition, and lamellarity, and allows the prediction of aqueous encapsulation efficiency for any hydrophilic bioactive material. Results of the evaluation of encapsulation efficiency (EE) and entrapped volume (EV) of 1% phospholipid liposome dispersions prepared by the

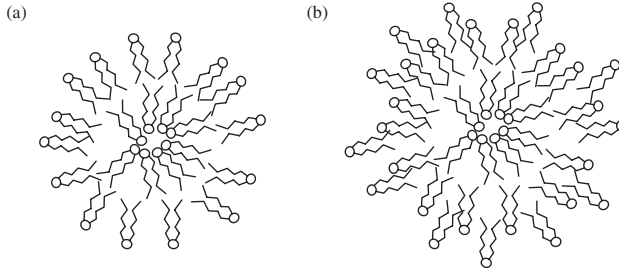


Figure 3. Schematic of small liposome with extreme membrane curvature (a), demonstrating possible insertion of extra molecules into leaf of membrane (b).

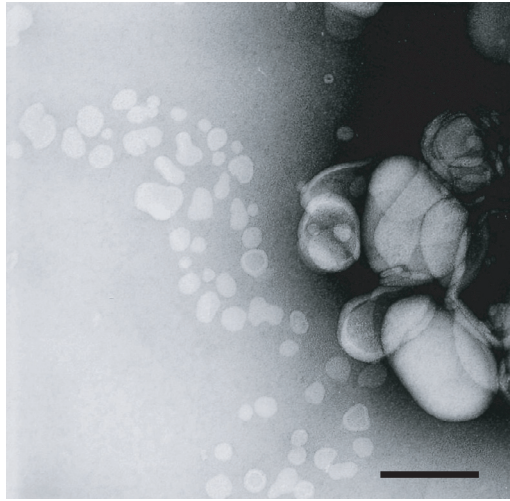


Figure 4. Negative staining TEM micrograph of liposome dispersion produced via thin film hydration showing population of very small liposomes. $31\,000\times$ magnification, bar = 500 nm.

different methods are shown in Table IV. These results suggest that the preparation method used does not have a large effect on either the encapsulation efficiency or the entrapped volume of the dispersions. This is surprising given the large difference in mean diameters between the populations. In fact, the liposomes prepared via microfluidization have the smallest mean size but possessed the largest encapsulation efficiency and entrapped volume. This seems unusual given the geometric relationship between the diameter and volume of a sphere, but part of the explanation

may be due to the thin film and heating method liposome populations containing a large number of multilamellar and multivesicular structures. This would result in part of their internal volume being occupied by lipid bilayer structures, leaving less aqueous spaces for the entrapment of the hydrophilic marker.

4. CONCLUSION

The traditional thin-film hydration method requires utilisation of potentially

Table IV. Encapsulation efficiency and entrapped volume of liposome dispersions containing 1% phospholipid prepared by different methods.

Preparation method	Encapsulation efficiency (%)	Entrapped volume ($\mu\text{L}\cdot\text{mg}^{-1}$)
Heating method	64	6.4
Thin-film hydration	68	6.8
Microfluidization	72	7.2

toxic solvents (i.e. chloroform and methanol) for the preparation of liposomes. Although these solvents may be removed, this requires additional processing steps and subsequent increase in processing costs, and some residues are likely to remain. Thin-film hydration does not appear to offer any significant advantages over microfluidization or the heating method, neither of which required the use of solvents.

Liposome dispersions produced via microfluidization and the heating method had encapsulation efficiencies of 72 and 64%, respectively. The particular product application will determine which technique is the most suitable. The smaller particle size and narrower size distribution of the liposome populations produced using microfluidization may make this method the most suitable for beverage applications with low viscosity, although either method may be used in semi-solid products such as yoghurts.

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