The formation of double emulsions in skim milk using minimal food-grade emulsifiers – A comparison between ultrasonic and high pressure homogenisation efficiencies

Thomas S.H. Leong^{1,2,3}, Meifang Zhou², Darren Zhou³, Muthupandian Ashokkumar^{1,2}, Gregory J.O. Martin^{1,3*}

¹ ARC Dairy Innovation Hub, The University of Melbourne, Parkville, Victoria 3010, Australia

² School of Chemistry, The University of Melbourne, Parkville, Victoria 3010, Australia

³ Department of Chemical & Biomolecular Engineering, The University of Melbourne, Parkville, Victoria 3010, Australia

*corresponding author

Abstract

Double emulsions of W1/O/W2-type were formed in skim milk. Skim milk (W1) was emulsified within sunflower oil (O) using ultrasonication that was in turn emulsified within an external skim milk phase (W2) using ultrasonication or high pressure homogenisation (HPH). The internalised aqueous phase was stabilised within the oil phase using food-grade surfactants: polyglycerol polyricinoleate (PGPR) and/or lecithin. Encapsulation yields of the W1/O emulsion into the double emulsion were between 30-100%, with increased yields achieved with reduced sonication time or HPH pressure, or increased PGPR or lecithin concentration. Ultrasonication was found to form relatively better monodisperse emulsions that showed greater stability to coalescence than those produced by HPH. Ultrasonication and HPH were found to be translatable in the sense that at a similar specific energy density (~ 20 J/g) emulsion droplet sizes with a similar size distribution between 1-10 μm and encapsulation yield (*ca* 37 wt%) could be achieved.

Keywords

Double emulsion; skim milk; ultrasonication; high pressure homogenisation; lecithin; PGPR

1. Introduction

The development of reduced-fat food products that retain the same sensory properties as those of full fat products is of high interest to the food industry. One promising method by which this can be achieved in foods such as sauces and cheese, and in various beverages is by creation of what are known as double emulsions (Muschiolik and Dickinson, 2017). A double emulsion is, in simple terms, an emulsion that is dispersed within another emulsion. For reduced fat products, the double emulsions of interest are typically water-in-oil-in-water type (W1/O/W2). That is, water droplets are emulsified within an oil phase that is emulsified as droplets within an external aqueous phase. The result is an emulsion containing oil droplets partially occupied by an internalised water phase. Fat reduction can be achieved without compromising sensory properties if the emulsion occupies a similar fat phase-volume as a full fat emulsion. The strategy of employing double emulsions in foods for fat reduction has been patented for products such as salad dressings (Gaonkar, 1994), low fat spreads (Okonogi et al., 1994) and previously reported for potential application in reduced fat cheese (Felfoul et al., 2015; Lobato-Calleros et al., 2007; Lobato-Calleros et al., 2006; Lobato-Calleros et al., 2008) and meat (Serdaroğlu et al., 2016). Other promising double emulsion applications in food can be found in a recent comprehensive review (Muschiolik and Dickinson, 2017).

The uptake of double emulsions in the food industry has been limited to date. Emulsions are inherently unstable thermodynamically, and double emulsions are further complicated by having multiple phases that require stabilisation. Large amounts of surfactants are typically required to stabilise both the inner and outer phases of the formed emulsions (Muschiolik and Dickinson, 2017). There is however, growing interest to replacing or reducing the use of synthetic surfactants in emulsions with natural biopolymer emulsifiers such as polysaccharides and milk proteins (Benichou et al., 2002; Muschiolik and Dickinson, 2017; Shanmugam and Ashokkumar, 2014). Some studies have also reported that the interaction of biopolymers with synthetic monomeric emulsifiers, can improve the stability of double emulsions by creating a gel-like barrier that retards water transport across the internal and external aqueous phases (Dalgleish, 2006; Garti, 1997; Oppermann et al., 2015).

Recently, Leong et al. (Leong et al., 2016) have shown that double emulsions of W1/O/W2 type could be produced from sunflower oil and skim milk using the synthetic surfactant Span80 to stabilise the inner emulsion, and the milk proteins alone to stabilise the outer emulsion. This has particular promise for application in the dairy industry. The double emulsions were demonstrated to be sufficiently stable to avoid coalescence for up to 7 days. However, due to sub-optimal stabilisation of the W1/O emulsion, a maximum encapsulation yield of only ~ 35% was achieved, even with 20% Span 80 in the oil phase. Reducing the surfactant requirements and replacing Span 80 with fully approved food grade surfactants would represent an improvement to this formulation. Polyglycerol polyricinoleate (PGPR) and soy lecithin, are highly effective food grade lipophilic emulsifiers commonly used in the food industry, and have been successfully used in the formation of double emulsions (Altuntas et al., 2017; Knoth et al., 2005; Muschiolik, 2007; Scherze et al., 2006) with high stability and encapsulation yield. Their usage has yet to be evaluated in the context of forming double emulsions combined with native skim milk proteins in the inner and outer aqueous phases, particularly using high-shear processes that can produce small emulsion droplets.

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47 In addition to the surfactants used to stabilise the water-oil interfaces, the size of both the inner
48 and outer emulsion droplets is an important factor in double emulsion stability. The creation of
49 smaller emulsified droplets by high-shear processing can improve the stability of a double
50 emulsion by increasing the kinetic stability. The preparation of small-sized primary (inner)
51 droplets, has been shown to be important for providing stability to the system as a whole (Guan et
52 al., 2010; Kanouni et al., 2002), whilst the formation of smaller secondary (outer) emulsion
53 droplets, will reduce the rate of creaming and phase separation as well as improving the ‘mouth-
54 feel’ of the emulsion (Kentish et al., 2008).

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56 Various high-shear devices are available that can be used to produce the primary and secondary
57 emulsions including ultrasonication, high pressure homogenisers, high-shear mixers,
58 Microfluidisers, and membrane systems (Leong, 2016). Ultrasonication is of particular interest for
59 the production of the primary emulsion, as it can produce small droplets with a narrow size
60 distribution, and be implemented as a reasonably simple and robust unit operation (Jafari et al.,
61 2007). The second emulsification step is a considerable challenge in double emulsion production,
62 as there is a fine balance between creating smaller emulsion droplets that are beneficial to stability
63 and sensory properties, without causing excessive droplet breakup that will result in loss of
64 encapsulated material. In addition, the second emulsification stage involves a much greater
65 volume of material than the first step (typically 5-10 times greater depending on the level of
66 encapsulation), which makes reducing the energy and capital investment associated with this step
67 important for large scale applications. While ultrasound has the potential to be operated at large
68 scale, it is still considered a reasonably new technology. High pressure homogenisation is another
69 effective technology for creating emulsions for which there are commercially available off-the-
70 shelf units already in established large scale operation in the food and beverage industry. The
71 relative effectiveness of ultrasonication and high pressure homogenisation in the second
72 emulsification stage is yet to be evaluated.

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74 To compare the effectiveness of both systems, the properties of the resultant double emulsions
75 need to be compared in relation to the amount of energy used in their formation. A suitable basis
76 for comparison is the delivered energy density. For ultrasonication, a typical measurement is the
77 calorimetric power delivered to the fluid (Kimura et al., 1996; O’Sullivan et al., 2017), which is
78 the product of energy intensity and processing time. For high pressure homogenisation, the
79 pressure that the fluid is subject to provides a direct indication of the energy density applied to the
80 system. The units for pressure can be directly converted to energy/unit volume i.e., 1 MPa is
81 equivalent to an energy density of 1 J/mL. There is a concern that using high shearing techniques
82 such as ultrasonics and high pressure homogenisation for double emulsion formation may lead to
83 excessive loss of encapsulated material (Lamba et al., 2015). However, this can be minimised
84 provided suitable formulations and operating conditions are used. For example, the use of
85 ultrasonication in the production of stable double emulsions for encapsulation of aspirin has been
86 reported, achieving entrapment yields of up to 99% (Tang and Sivakumar, 2012; Tang et al., 2013).
87 It is yet to be established how to best produce stable, high-encapsulation-yield double emulsions
88 in skim milk while minimising the amount of energy and food grade surfactant used.

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90 In the present study, the creation of double emulsions using the food grade lipophilic surfactants
91 PGPR and/or lecithin in the oil phase, and natural skim milk proteins alone as the main stabiliser

of the secondary droplets in the external phase, is investigated. The use of different high shear techniques, namely ultrasonication and high pressure homogenisation in the second emulsification step are also investigated and compared on the basis of energy applied to the system, to determine their viability for double emulsion production directly in skim milk.

2. Materials and Methods

2.1 Materials

The oil phase used in this study was sunflower oil (Woolworths Homebrand, Australia) purchased off the shelf. To promote and stabilise the inner W1/O emulsion, the surfactants polyglycerol polyricinoleate (PGPR) and soy lecithin were used. These emulsifiers were kindly provided by a confectionery company located in Australia. Pasteurised and homogenised skim milk (Paul's brand, Australia) with <0.1% w/v fat and a total protein content of 4.2% w/v, purchased from a supermarket, was used for all trials as the basis for both the inner and outer aqueous phase. Sodium azide (Chem Supply, 99 %, Australia) was added at ~0.02 wt% to each batch of milk to limit microbial growth during refrigerated storage.

2.2 Primary emulsification using ultrasound

A two-step emulsification process adapted from Leong et al. (Leong et al., 2016) was employed for the preparation of the double emulsions. In the first step, the inner aqueous phase (skim milk containing 4% w/w sodium chloride as an entrapment marker) was loaded at a concentration of 30% w/w into a sunflower oil/PGPR/lecithin mixture and emulsified using a 20 kHz 3 mm microtip ultrasonic horn (Branson Ultrasonics, USA) inside a 15 mL test tube. The concentration of PGPR used was 1 wt% of the oil phase unless otherwise specified. The concentration of lecithin used varied between 0 to 10 wt% of the oil phase as specified in the text. The total mass of the W1/O emulsion formed was 7.5 g. Sonication was performed at 10 W calorimetric power (an amplitude setting of 30%) and a duration of 90 s (specific energy = 120 J/g), until the emulsion formed was homogenous in appearance without obvious pooled regions of unemulsified aqueous phase. The horn tip was positioned at a fixed position approximately 40-50 mm from the bottom of the test tube, so that it was located above the oil/water interface.

2.3 Secondary emulsification using ultrasound

In the second emulsification step, 0.375 g of the pre-formed W1/O emulsion was emulsified into skim milk using ultrasound to create a double emulsion with a total mass of 7.5 g (i.e., 5 % w/w final W1/O loading concentration). Ultrasound was applied at a fixed calorimetric power level of 6 W (i.e. 20% amplitude setting) for varying durations as specified in the text. The horn tip was positioned at a fixed location near the top of the tube, between 3 to 5 mm from the surface of the sample near the oil/water interface. All emulsions were prepared in triplicate.

2.4 Secondary emulsification using high pressure homogenisation

Emulsions (100 mL) containing a 5 wt% W1/O loading were formulated. The W1/O emulsion consisted of a fixed formulation of 1 wt% PGPR, 2 wt% lecithin and 30 wt% skim milk (containing 4 wt% NaCl). The bulk 100 mL emulsions were first pre-emulsified using an Ultraturrax stirrer (4500 rpm, 2 min) prior to loading into the sample hopper of the high pressure homogeniser (GEA Niro Soavi homogeniser, Panda). Samples were passed once through the 1st stage of the

homogeniser at selected pressures between 30-200 bar with a constant volumetric flow rate of 10 L/hour. Emulsions were prepared in triplicate.

2.5 Conductivity measurements

Sodium chloride (4% w/v) was included in the inner aqueous phase as an entrapment marker, with the release of inner phase into outer phase resulting in an increased conductivity associated with the increased salt concentration. To quantitatively relate changes in conductivity to the release of salt from the emulsions after preparation, standard solutions representing 0, 50 and 100% NaCl release were prepared. Standards for each specific formulation used in the W1/O/W2 emulsion were prepared that included the same concentrations of each component, and which were sonicated with 20 kHz ultrasound for 2 minutes at 50% amplitude using an 11 mm horn (82 J/g specific energy based on calorimetry). The total energy delivered was sufficient to ensure complete homogenisation of the fat droplets and limit phase separation and creaming in the standards. Conductivity was measured in the standard solutions and samples after equilibration to room temperature (~23 °C) for 2 hours, using a k=1.0 laboratory conductivity sensor (TPS, Australia) connected to TPS LabCHEM-Cond conductivity meter (TPS, Australia). The conductivity probe was calibrated using a 2.76 mS standard solution.

2.6 Scanning electron microscopy

Cryo-scanning electron microscopy (Cryo SEM, FEI Qanta) was used to investigate the surface and internal morphology of the oil-milk double emulsion system. The sample was first transferred into a glass tube (1.3 mm × 1.3 mm × 5 mm in size) and then mounted on a copper holder. The sample and copper holder were quickly immersed into liquid nitrogen slush at -210 °C. After freezing, the frozen sample was immediately transferred into an attached cryo preparation chamber using a vacuum transfer device. The sample was fractured using a chilled scalpel blade within the chamber at -140 °C under high vacuum conditions. The fractured sample was then coated with sputtered gold (6 nm) after etching at -95 °C for 20 min to remove the ice from the surface of the fractured sample. The sample was then transferred under vacuum onto a nitrogen gas-cooled module at -140 °C. The detector used for the SEM observation was a solid state backscattered electron detector (SSD).

2.7 Particle size measurements

The particle size of the double emulsion droplets was measured using a Malvern Mastersizer 3000 (Malvern Instruments, UK) with Hydro-G3000 accessory. Distilled water was used for dilution. A refractive index of 1.462 and absorption of 0.001 were used by the software to determine the size of the droplets. The particle size of the primary water-in-oil emulsions was determined using a Zetasizer Nano ZS (Malvern Instruments, UK), with sunflower oil used for dilution.

2.8 Viscosity measurements

The viscosity of emulsion samples was measured using an AR-G2 rheometer (TA instruments, USA) using a cone and plate configuration with a cone diameter of 40 mm, angle of 2 ° 50'' and a truncation gap of 52 mm (TA instruments, serial number 988134). Approximately 0.5 mL of sample was loaded into the geometry. A flow procedure was employed, where the shear rate was increased from 1 to 100 s⁻¹ step-wise. The samples were maintained at a temperature of 30 °C throughout the measurement.

2.9 Statistical analysis

All emulsions were prepared in triplicate unless otherwise specified. The statistical significance of results were assessed using the Student's t-test (de Winter, 2013) in Minitab 17 (Minitab Pty. Ltd.) where required. A 95% confidence interval was used to assess statistical significance.

3. Results and discussion

3.1 Morphology of primary and secondary emulsion droplets

Lecithin and PGPR were chosen as the food grade lipophilic surfactants to stabilise the inner W1/O emulsion. The effect of using these emulsifiers in combination with the skim milk proteins on the morphology and stability of the primary and secondary emulsions resulting from ultrasonication, is of novel interest. To investigate the performance of the surfactants individually and in combination with each other, W1/O and W1/O/W2 emulsions were formed by ultrasonication using PGPR (1 wt% concentration in oil phase), lecithin (2 wt % in oil phase) and combined PGPR with lecithin (1 wt% PGPR, 2 wt% lecithin in oil phase). Microscopic images of these emulsions are shown in Fig. 1 a-c. The W1/O/W2 emulsions here were formed using 30 s ultrasonication at 6 W power.

The W1/O emulsions, formed using lecithin (Fig 1b), display a crystalline gel-like network, which indicates a strong interaction between the emulsified aqueous phase droplets in the emulsion. This behaviour is consistent with that reported in the study by Knoth et al. (Knoth et al., 2005). The PGPR only W1/O emulsion lacks this structure (Fig 1a), instead consisting of what appears to be nano-sized droplets dispersed throughout, and with a noticeable absence of larger droplets. The W1/O emulsions formed containing PGPR were found to be very stable, undergoing minimal observable phase separation for several weeks when stored in the refrigerator. This is likely due to the smaller nano-sized droplets conferring increased kinetic stability (see section 3.2). The W1/O emulsion formed using lecithin alone however, did result in some observed phase separation after several days, likely due to separation of oil from the gel network. Issues were also reported in the use of lecithin alone to stabilize the W1/O emulsion in the recent study by Altuntas et al. (Altuntas et al., 2017).

In each case, the internal morphology of the formed W1/O/W2 double emulsion droplets, resembled the dispersed phase in the W1/O emulsions. The two emulsions containing lecithin are characterised by the same gel-like network dispersed through the oil droplets. Interestingly, the emulsions where only lecithin was employed (Fig 1b) appear to have incomplete distribution of aqueous phase in the oil phase droplets of the W1/O/W2 emulsion despite the morphology of the W1/O emulsion looking reasonably homogenous. This may be connected with the observation that the W1/O emulsion formed using lecithin alone displayed some separation of oil from the gel network during storage. As reported by Scherze et al. (Scherze et al., 2006), the presence of salt with lecithin may cause coalescence and phase separation in formed emulsions. Another possible contributing factor to the incomplete distribution is the increased viscosity of the W1/O emulsion phase resulting from the presence of lecithin (see section 3.2). By contrast, the internalised water phase for emulsions containing PGPR with lecithin are more thoroughly dispersed in the oil droplets (Fig 1a). Also reported by Scherze et al. (Scherze et al., 2006), salt is noted to promote

the coalescence stability of emulsions formed with PGPR. This may counteract the coalescence/phase separation problems with lecithin in the presence of salt. The presence of PGPR has also been reported to drastically reduce the yield stress of emulsions (Schantz and Rohm, 2005). The implication is that the W1/O emulsions formed with PGPR are able to flow more easily (i.e. have a less rigid structure), and hence the gel-like aqueous phase is more readily able to disperse throughout the oil phase of the W1/O/W2 droplets.

The external and internal morphology of individual double emulsions droplets stabilised by milk proteins in the outer phase, and a combination of milk proteins, lecithin and PGPR in the internal phase, was further characterised using cryo-SEM. Micrographs of these emulsions are depicted in Fig. 2.

The external morphology is consistent with that previously reported for double emulsions formed in skim milk (Leong et al., 2016). In the interior, small aqueous phase droplets can be seen distributed through a rough network, consistent with the gel-like oil phase seen in the light microscopy images in Fig. 1c. It is speculated that this gel-like network could improve stability in regards to loss of the encapsulated water phase, similar to that as reported previously by studies whereby the internal water phase was gelled using whey proteins (Balcaen et al., 2016; Dalgleish, 2006; Oppermann et al., 2015).

A general observation of the morphology from light microscopy shows that PGPR facilitates the production of small W1/O droplets and lecithin is able to produce gel-like W1/O structures (Fig. 1 a and b). However, the PGPR-only emulsions (at 1 wt% concentration of the oil phase here) did not appear to encapsulate a large amount of aqueous phase within the W1/O/W2 droplets, whilst the double emulsions produced with only lecithin did not appear to be stable due to observable oil separation from the gel-like network and incomplete distribution of the network in the W1/O/W2 droplets. By using the two emulsifiers in combination, the gel-like W1/O emulsions could be entrapped within small secondary emulsion droplets, which appeared to be more stable and dispersed more uniformly throughout the oil phase. The encapsulation efficiency of these combined systems is discussed further in the following section.

3.2 Effect of lecithin concentration on encapsulation yield, droplet size and W1/O viscosity

As the lecithin on its own was found to be ineffective at creating stable double emulsions, combinations of PGPR and lecithin were investigated further. The effect of lecithin concentration on the encapsulation yield of PGPR/lecithin double emulsions was evaluated. In Fig. 3, the encapsulation yields are shown for emulsions containing a fixed amount of PGPR (1 wt% of the oil phase) and varying concentrations of lecithin (0-10 wt% of the oil phase) and formed using a constant ultrasonication duration of 30 s, 6 W (24 J/g) are shown. With no lecithin the W1/O emulsion was seen to consist of small droplets (Fig 1a), and the encapsulation yield was only ca 20 wt% (Fig 3). At 2 wt% lecithin the W1/O was seen to consist of a gel-like material (Fig 1c) that was able to be encapsulated to a great extent (ca 35%). Further increases in the amount of lecithin in the presence of PGPR resulted in further increases in the encapsulation yield (and hence displacement of the oil phase with water).

To investigate the reasons for the enhanced entrapment resulting from increased lecithin concentration, viscosity of the W1/O emulsions formed with varying concentration of lecithin in the oil phase was measured as a function of the shear rate (see Supplementary Information Fig. S1). There is a clear trend of increased viscosity as a function of increasing lecithin concentration

(at 30 °C). The viscosity of the W1/O with 2% lecithin was approximately 4-times greater than that of the PGPR-only W1/O emulsion, consistent with the gel-like structure that was observed (Fig. 1c).

The implications of increased viscosity in the context of the second emulsification step are as follows. During emulsification of the W1/O into the W2 phase the resulting droplets will be larger for the more viscous W1/O emulsions. This is confirmed from the particle size distribution of the W1/O/W2 emulsions (Fig. 4 a). Note that the peak at 0.1 μm corresponds to the size of casein micelles (Farkye and Shah, 2014) (see Supplementary Fig. S2). The size distribution data show that the emulsions containing a higher lecithin concentration in the oil phase consist of somewhat larger particles, particularly the emulsion containing 10% lecithin. There is a noticeable decline in the volume fraction occupied by sub-micron sized droplets, and a corresponding increase in the droplets $>1 \mu\text{m}$ in diameter with increasing lecithin concentration. This apparent decline in the casein micelle peak at 0.1 μm with increasing lecithin concentration is an artefact of the measurement, due to the larger sized droplets diffracting more light, and hence contributing disproportionately to the signal.

The size distributions of the aqueous phase droplets loaded within the corresponding W1/O emulsions formed also display a trend of increasing size with increasing lecithin concentration (Fig. 4 b). At 5% and 10% lecithin these distributions were bi-modal, presumably a consequence of the highly viscous gel-like interaction. For all lecithin concentrations the peak diameter of the W1/O/W2 droplets (Fig. 1a) was considerably greater than that of the corresponding W1/O droplets (Fig. 1b), suggesting that these W1/O/W2 droplets are sufficiently large to accommodate the W1/O.

There is not a completely clear relationship between W1/O viscosity, W1/O droplet size, and W1/O/W2 encapsulation rate as the increase in encapsulation rate is reasonably linear as a function of lecithin concentration (Fig. 3), whereas there is relatively minor difference in viscosity between the 5% and 10% lecithin W/O1 emulsion and a much more apparent difference in the W1/O and W1/O/W2 droplet size (Fig. 4). The exact mechanisms are likely quite complex, but it is clear from these results that encapsulation yields can be improved by using lecithin in combination with PGPR. Also, the increased encapsulation yield with higher lecithin concentration, would also need to be considered in practical terms. The high viscosity of W1/O emulsion containing 10wt% lecithin would likely present challenges for production in terms of increasing pumping and cleaning requirements.

3.3 Effect of secondary emulsification method on encapsulation yield and droplet size distributions

The dispersion of W1/O emulsions into skim milk was further investigated by comparing the effectiveness of ultrasonication and high pressure homogenisation (HPH) on the basis of specific energy density delivered to the fluid. W1/O emulsions (formulated with 1 wt % PGPR and 2 wt % lecithin in the oil phase) were emulsified into skim milk using either ultrasonication for varying durations (5-60 s) or a HPH operated at varying pressures between 30 to 200 bar. The size distributions of the resulting W1/O/W2 emulsions formed using ultrasonication or HPH at select

conditions are shown in Fig. 5b. Prior to entering the HPH, a coarse emulsion was formed using an Ultraturrax mixer (4500 RPM, 5 min) (for reference, the size distribution of this pre-emulsion is also shown in Fig. 5b). Note that the peak between 0.01 to 1 μm is present in every sample measured and as noted earlier, represents contribution from casein micelles present in the milk and possibly some smaller sub-micron droplets that are formed in the emulsification process. It can be seen that the Ultraturrax (rotor-stator) mixing generated mostly large droplets in the range between 10-100 μm . These droplets were not stable to phase separation. These coarse emulsions were further processed using HPH to yield emulsion droplets in the range between 1 to 50 μm . An increase to the pressure in the HPH, or increase in sonication duration, both resulted in smaller diameter droplets (i.e. a shift to the left in the size distribution), as expected.

A size range between 1 to 10 μm , similar to native fat droplets in whole milk, was achievable using a homogenisation pressure of 150 bar, or sonication duration of 30 s (6 W power). For these two processing conditions, the shape of the size distributions and the encapsulation yield (38% for HPH and 37% for US) were statistically the same ($P=0.69$). The specific energy required for the two treatments was also similar: ~ 15 J/g for the HPH and ~ 24 J/g for the ultrasonication. The results therefore indicate that double emulsions with droplets of similar size, and with similar extents of encapsulation can be formed using either ultrasound or HPH for a given energy load.

At lower specific energy inputs, the size of the double emulsion droplets increased (Fig. 5b), however the encapsulation yield was seen to decrease for HPH and increase for US (Fig. 5a). The higher encapsulation yield achieved with US at low specific energy (e.g. 4 J/g; 5 s) could be due to a high degree of encapsulation occurring in the large droplets (i.e. >20 micron) that were present in the double emulsions from low-specific energy US but not in the double emulsions produced using HPH at a low specific energy (5 J/g, 50 bar) (Fig. 5b). The HPH is more efficient at creating exclusively small emulsion droplets (Schultz et al., 2004), since the mechanism of the HPH is such that droplets larger than the valve gap in the disruption chamber should not be produced. By comparison, the mechanism of ultrasonication is primarily acoustic cavitation (Kentish et al., 2008; Leong et al., 2009), which is due to the formation and collapse of microbubbles in the fluid. The size reduction of droplets caused by exposure to these collapsing bubbles is inherently stochastic, since the high shear is confined to a localised region near the horn tip and near the surface of collapsing bubbles, resulting in a broader size distribution. In this case the presence of larger droplets may have resulted in higher encapsulation yields, but this may also reduce the stability of these double emulsions.

3.3 Stability of encapsulation over time

To investigate the issue of stability, emulsions prepared using both shearing methods were assessed and compared in regards to the loss of salt encapsulation as well as their propensity to coalesce/phase separate over 7 days (i.e. typical shelf life of a pasteurised milk).

Particle size measurements of the emulsions formed using selected ultrasonication or HPH conditions can be found in Supplementary Information (Fig. S3). For the ultrasonically prepared samples, coalescence was detected only in the size distributions for emulsions processed using 5 s sonication time (6 W power) where the presence of large droplets in the range 10-100 μm were formed. This coalescence was indicated by an increase in the particle size distribution at day 7 compared with day 1. Emulsions sonicated for longer duration at the same power displayed no

353 coalescence. Instead, the emulsions generally resulted in a small decline in the measured size,
354 indicated by a small shift to the left after day 7. This shrinkage of droplet size would suggest loss
355 of encapsulated material with time, consistent with observations made previously by Leong et al.
356 for emulsions created by ultrasonication in skim milk (Leong et al., 2016).

357 Similar to the double emulsions produced using US for 5s, the larger droplets produced using HPH
358 displayed coalescence instability with storage. The size distributions of the droplets formed in the
359 size range $< 10\mu\text{m}$ however, were found to shift to the left, similar to the shrinkage of droplets as
360 observed in the case of the ultrasonically produced emulsions. The reason could be the loss of
361 encapsulated material that leads to shrinkage. However, the combination of these smaller emulsion
362 droplets with the larger droplets (i.e. Ostwald ripening) present in the emulsions cannot be ruled
363 out as it is another potential source of instability. Another possible reason for this increase in size
364 is swelling of the droplets due to osmotic pressure.

365 To evaluate further, microscope observations were made over the 7 day storage period to visually
366 check for loss of encapsulated material over time. Selected images for emulsions prepared using
367 10 s ultrasonication duration and HPH pressure of 150 bar are presented in Fig. 6. An obvious
368 trend that can be observed is that the HPH emulsions have a more poly-disperse range of droplets
369 (especially a lot more sub-micron sized droplets), consistent with the size distributions obtained.
370 It can also be observed that there is a visual decline in the number of encapsulated droplets within
371 the secondary W1/O/W2 droplets that is more prominent in the US produced samples compared
372 with HPH. However as can be observed in the salt-release measurements with time (Fig. 7 a and
373 b), the HPH produced emulsion (150 bar) had a significantly greater ($P<0.05$) release of salt
374 compared with US produced using 10s, 6 W power in the initial few days of storage. The HPH
375 sample also appears to approach a plateau in salt loss ($\sim 65\%$) after the 2nd day, as compared with
376 the US samples which do not reach an equivalent degree of salt loss until the 7th day. Note that the
377 salt released on day 1 for these samples are different to those presented in Fig. 5, as there were
378 made using a different batch of milk.

379 The release of salt with time has some limitation in regards to quantifying the retainment of
380 encapsulated water phase, which should be addressed. This is because the NaCl used as an
381 entrapment marker can diffuse both in and out of the oil droplets with storage time and so the
382 conductivity measured will provide either an overestimate or underestimate to the degree of
383 aqueous phase entrapment. In general, water will diffuse across a semi-permeable membrane (in
384 this case the oil and surfactant boundaries) faster than the Na^+ or Cl^- ions, which tend to diffuse
385 together in order to maintain charge neutrality (Hancock and Cath, 2009). In this case, as the
386 osmotic pressure is higher in the internal phase due to salt loading, the tendency is for water to
387 transfer into the internal water droplets with time. This will lead to some swelling of the internal
388 droplets until they can no longer be retained in the oil droplets, which will collapse, resulting in a
389 reduction in the size of the secondary emulsion droplets (Wen and Papadopoulos, 2001). As
390 mentioned above, it appears that for these samples, the encapsulation yield of salt plateaus once it
391 reaches a release of $\sim 65\%$, indicating possibly a balance of the osmotic pressure such that transport
392 of salt comes to an equilibrium. This is supported by the microscopy images that indicate minimal
393 change in the encapsulation morphology of the HPH samples after day 2, suggesting encapsulation
394 stability.

One of the motivations in this present work was to limit the amount of surfactants used to stabilise the double emulsions. It is possible to significantly improve the encapsulation stability by increasing the amount of surfactant used. An increase of PGPR from 1 wt% to 5 wt% of the oil phase (without lecithin), produced emulsions (formed using US 10 s, 6W) that were able to retain ~ 100% of the salt marker, even after 7 days of storage (Fig. 7c). These results show there is a trade-off between surfactant use and encapsulation stability that will need to be considered when formulating these double emulsions for particular applications.

4. Conclusion

Double emulsions were formulated in skim milk using ultrasonication or high pressure homogenisation in the secondary emulsification stage. Displacement yields were found to be dependent on the viscosity of the internalised W1/O phase, which in this study was controlled by increasing the relative amount of lecithin in the oil phase. Fat displacement yields of between 15 to 30 % (i.e. 50 to 100 % encapsulation) could be achieved, using minimal amounts of surfactant in the inner oil phase and no additional surfactants in the external aqueous phase. Encapsulation stability with storage can be improved by adding more emulsifier to the oil phase. The use of 5% PGPR in the oil phase maintained an encapsulation yield of ~ 100% in the emulsion over 7 days.

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Fig. 1. Micrographs for W1/O and W1/O/W2 emulsions formed using a) PGPR (1 wt% of oil phase) b) lecithin (2 wt% of oil phase) and c) PGPR (1 wt% of oil phase) and lecithin (2 wt% of oil phase) combined. The scale bars represent 20 μm .

Fig. 2. Cryo-SEM images of the a) external and b) internal morphology of a double emulsion droplet stabilised by milk proteins in the exterior and milk proteins/PGPR/lecithin surfactant in the interior. The ‘flake-like’ masses surrounding the oil droplet is the frozen liquid milk phase of the emulsion.

Fig. 3. Encapsulation yield and proportion of oil phase displaced as water phase as a function of lecithin concentration in the oil phase. The emulsions also contain PGPR at a concentration of 1 wt% of the oil phase. All emulsions were processed at ultrasonic amplitude of 20% for 30s duration (24 J/g). Error bars represent the standard deviation for the encapsulation yield of triplicate emulsions.

Fig. 4. Size distributions of the a) secondary W1/O/W2 emulsion and b) primary W1/O emulsion droplets as measured by a Malvern Mastersizer. The W1/O/W2 droplets were all formed using 30 s ultrasonication at 6 W. The primary W1/O droplets were all formed using 90 s ultrasonication at 10 W. The data are the average of triplicate measurements for each emulsion and are representative of the trends from triplicate emulsion samples.

Fig. 5. a) Encapsulation yield as a function of specific energy for ultrasonication and high pressure homogenisation. Error bars represent the standard deviation of measurements of triplicate emulsions. b) Particle size distributions of W1/O/W2 droplets at select ultrasonication and HPH processing conditions. The data are the average of triplicate measurements for each emulsion and are representative of the trends from triplicate emulsion samples. All emulsions are formulated with emulsifier concentrations of 1 wt % PGPR and 2 wt % lecithin in the oil phase.

Fig. 6. Micrograph images of W1/O/W2 emulsion droplets formed using 10 s sonication compared with HPH formed using 150 bar on days 1, 2, 5 and 7 (scale bar represents 20 μm).

Fig. 7. Salt released expressed as a percentage of the amount loaded into the W1/O emulsion for emulsions formed using a) ultrasonication 10 s and b) HPH 150 bar. c) The salt encapsulation

yield in emulsions formed using surfactant concentrations of 1% PGPR, 2 % lecithin compared with 5 % PGPR, formed using 10 s sonication. Error bars represent the standard deviation of measurements of triplicate emulsions.

Fig. S1: Shear viscosity as a function of shear rate at a temperature of 30 °C for W1/O emulsions formed with varying concentrations of lecithin. Error bars represent the standard deviation of duplicate measurements.

Fig. S2: Size distribution of casein micelles in skim milk measured using Mastersizer3000

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576 Fig. S3: Particle size distribution change from day 1 to day 7 for emulsions created selected
 577 sonication and high pressure homogenisation conditions. The data are the average of triplicate
 578 measurements for each emulsion and are representative of the trends from triplicate emulsion
 579 samples.

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