Preparation of water-in-oil-in-water emulsions by low frequency ultrasound using skim milk and sunflower oil

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Abstract

Double emulsions of water-in-oil-in-water (W1/O/W2) type were prepared in skim milk using 20 kHz ultrasound. Ultrasonic emulsification provides a simple and quick yet effective protocol by which double emulsions can be created using low amounts of surfactant. The fat displacement and shelf stability of the emulsions were found to be dependent on the amount of sonication power delivered during the dispersion of the W1/O emulsion into skim milk. Acoustic intensity was manipulated to control the size distribution of the outer shell of the emulsion droplets to a range similar to that of fat globules in unhomogenized whole milk. The encapsulation yield (proportion of W1/O droplets subsequently encapsulated within the W1/O/W2 double emulsion) varied from 5 to 35 %. The variation could be due to coalescence/aggregation of water phase droplets within the initially formed W1/O emulsion. The resultant double emulsion droplets were found to be relatively stable over 7 days. However, a source of instability was found to be the leakage of entrapped aqueous phase from the inner to the outer phase with storage time. Phase separation was primarily observed for double emulsions prepared using high W1/O loading (20% w/w) and low ultrasonic power delivery (<6W).

Keywords: double emulsion; ultrasound; skim milk

1. Introduction

A double emulsion is an emulsion entrapped within another emulsion. They can be oil-in-water-in-oil (O1/W/O2) or water-in-oil-in-water (W1/O/W2) emulsions (Garti, 1997; Lamba, Sathish, & Sabikhi, 2015). W1/O/W2 double emulsions are of interest for targeted fat reduction of food products such as cheese and butter, in which the influence of fat droplets on the microstructure influences the sensory properties (Goudédranche, Fauquant, & Maubois, 2000). Often, reduced-fat products have compromised sensory properties due to the reduced overall volume of fat in the microstructure. Double emulsions can be used to retain the same perceived volume of fat as in a full fat product, but with reduced calories due to the displacement of some of the internal volume of the droplets. The strategy of employing double emulsions in foods for fat reduction has been patented for salad dressings (Gaonkar, 1994) and previously reported for application in reduced fat cheese (Lobato-Calleros et al., 2007; Lobato-Calleros, Rodriguez, Sandoval-Castilla, Vernon-Carter, & Alvarez-Ramirez, 2006; Lobato-Calleros et al., 2008).

There are several limitations to double emulsions, which have prevented their widespread application in the food industry to date. Emulsions are inherently unstable thermodynamically, relying instead on being sufficiently kinetically stable. The mechanisms underlying the instability of single emulsions are compounded in double emulsions. Leakage of material encapsulated in the inner phase, Ostwald ripening, and the flocculation and coalescence of both internal and external droplets during storage are all problems in double emulsions. Optimising the preparation of double emulsions is therefore much more difficult than for single emulsions. In particular, to enable encapsulation, double emulsions usually consist of relatively large external droplets (~20-100 μm) which have a strong tendency to coalesce, flocculate and cream. Double emulsions also have a tendency to release the entrapped matter in an uncontrolled manner (Garti, 1997). As a result, large amounts of surfactants are typically required to stabilise both the inner and outer phases of the formed emulsions (Matsumoto, Kita, & Yonezawa, 1976).

Double emulsions can be created by first preparing an O1/W or W1/O emulsion using a high or low hydrophilic-lypophilic balance (HLB) surfactant respectively and high shear processing (Lamba et al., 2015). The formed O1/W or W1/O emulsion is then dispersed into an oil or water phase containing either low or high HLB surfactant, but with reduced shear to avoid disruption of the outer droplets of the emulsion. Some of the internal phase is unavoidably lost to the external phase during the second step, regardless of the emulsification method used (Florence & Whitehill, 1982). For double emulsions, non-ionic surfactants are preferred (Matsumoto et al., 1976), with hydrophilic and hydrophobic emulsifiers used to stabilize oil and water droplets respectively. Monomeric emulsifiers tend to produce double emulsions with shorter shelf life and inferior physical stability compared to those made with higher molecular weight polymeric emulsifiers, such as proteins (Garti, Aserin, & Cohen, 1994). Polymeric surfactants migrate much more slowly, and may also form a more sterically bulky, viscoelastic layer around the emulsified droplets, reducing the rates of release of encapsulated material, droplet flocculation and coalescence. Milk contains casein proteins which have surfactant properties even in their native state (Leermakers, Atkinson, Dickinson, & Horne, 1996), and whey proteins that, when partially denatured, can be used to stabilize emulsified oil droplets. Skim milk is widely available and amenable to application in many food products. Oil (7% flax seed oil) has been emulsified directly into skim milk without the addition of surfactants using ultrasound (176 W, 20 kHz) (Shanmugam & Ashokkumar, 2014), to produce emulsions that were stable to phase separation for at least 9 days.

Having smaller emulsified droplets in both inner and outer phases will increase the stability of a double emulsion. The preparation of a W1/O emulsion with small-sized droplets in the first step of W1/O/W2 preparation, has shown to be critical for providing stability to the system as a whole (Kanouni, Rosano, & Naouli, 2002). Various high-shear devices can produce emulsions including ultrasonication, high pressure homogenizers, high shear mixers, microfluidizers, and membrane systems. High pressure homogenisation is proven at large scale, but is limited in its ability to produce very small droplets with a narrow size distribution. In principle, microfluidizers are the most energy efficient and amenable to high throughput processing, however they can be costly to maintain (Jafari, He, & Bhandari, 2006). Ultrasonication can produce small droplet with a narrow size distribution, and can be implemented as a reasonably simple and low cost unit operation (Jafari, He, & Bhandari, 2007).

The ultrasonication of fluids can result in acoustic cavitation, which is the formation, growth and collapse of bubbles (Leighton, 1994) that lead to strong localised shearing forces and temperature increases. The physical and chemical effects created by cavitation bubbles have many practical applications, and are very useful in the intensification of chemical processes (Gogate, 2008; Gogate, Sutkar, & Pandit, 2011). Two mechanisms are responsible for ultrasonic emulsification. First, the application of the sound field produces interfacial waves, which become unstable resulting in the dispersion of the oil phase into the continuous water phase as mid- to large-sized droplets. Second, the shear forces resultant from cavitation break up these initially formed droplets of dispersed oil into droplets of sub-micron size (Thompson & Doraiswamy, 1999). One of the important factors influencing the stability and sensory properties of double emulsions is the size of the secondary droplets of oil. It is well known that ultrasonics can be used to produce very small emulsion droplets (Leong, Wooster, Kentish, & Ashokkumar, 2009) that are exceptionally shelf-stable. Although smaller droplets can improve emulsion stability and sensory properties, excessive size reduction will cause the release of the internal aqueous phase to the external aqueous phase. Application of excessive power levels can also promote droplet-droplet collisions that may result in coalescence. Hence, the secondary oil droplets must be generated at an appropriate size to produce an acceptable yield of aqueous phase entrapment within the double emulsion while creating an emulsion with a size distribution that remains stable with storage. The use of ultrasonication to generate shear for the production of stable double emulsions for encapsulation of aspirin has been reported, achieving entrapment yields of up to 99% (Tang & Sivakumar, 2012; Tang, Sivakumar, & Nashiru, 2013).

The high shear and temperatures generated during acoustic cavitation can also partially unfold and denature proteins (Shanmugam, Chandrapala, & Ashokkumar, 2012) to more effectively stabilize interfaces. In some cases, ultrasonics can facilitate cross-linking of proteins to form aggregates (Cavalieri, Ashokkumar, Grieser, & Caruso, 2008). The protein cross-linking can be reversible, for instance through hydrophobic interactions and hydrogen bonding, or irreversible if covalent links are produced, for example disulphide bonds. In the latter case, this can potentially be facilitated by free radicals generated through ultrasonic cavitation (Cavalieri, Zhou, Caruso, & Ashokkumar, 2011).

The use of double emulsions, ultrasonic emulsification, and the use of dairy proteins as emulsifying agents are topics of developing interest. So far, single emulsions have been generated with ultrasound in dairy systems (Shanmugam et al., 2014), double emulsions have been made using whey protein isolate to stabilise the inner aqueous phase of a W1/O/W2 double emulsion (Oppermann, Renssen, Schuch, Stieger, & Scholten, 2015), and double

emulsions have been generated using ultrasound on non-dairy systems (Tang et al., 2012; Tang et al., 2013).

Here, we attempt for the first time to employ ultrasonication to create food-based double emulsions of W1/O/W2-type directly in skim milk, employing no additional surfactant in the external aqueous phase and a lipophilic surfactant used to stabilise the inner aqueous droplets. Ultrasonication is proposed to provide stability to double emulsions formed in skim milk by simultaneously controlling the size of the inner and outer droplets and partially denaturing whey proteins that contribute to stabilising the oil/water interface of the outer droplets. In this study, the intensity of ultrasonic power delivered and variations in the formulation are assessed for the production of double emulsions, primarily for the purpose of fat displacement. The yield of aqueous phase entrapment and the storage stability of the formed double emulsions are investigated by measuring conductivity changes resulting from the release of sodium chloride from the entrapped inner phase, changes in the oil droplet size during storage, and image analysis of microscopic images. An assessment of the emulsion surface layer was also made using scanning electron microscopy (SEM) and zeta potential measurements.

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 stabilize the inner W1/O emulsion, lipophilic Span 80 surfactant (Sigma Aldrich, USA) was dissolved in the oil, at 10% w/w used unless otherwise indicated. Pasteurised and homogenised skim milk (Paul's brand, Australia) with fat <0.1% purchased from the 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 ~0.03 wt% to each batch of milk to limit microbial growth during storage. Samples of commercially available homogenized full cream milk (Pura milk, Australia) and unhomogenized full cream milk (Paul's milk, Australia) were used for comparative zeta potential measurements.

2.2 Emulsification procedure

A two-step emulsification process was employed for the preparation of the double emulsions (Figure 1). In the first step, the inner aqueous phase (skim milk containing 8% w/w sodium chloride as an entrapment marker) was loaded at a concentration of 10% w/w into a sunflower oil/Span 80 mixture (10% w/w Span 80) and emulsified using a 20 kHz 3 mm microtip ultrasonic horn (Branson 450D, 400 W, Branson Ultrasonics, USA) inside a 15 mL test tube. The total mass of the emulsion was 7.5 g. Sonication was performed at 10 W calorimetric power (an amplitude setting of 30%) and a duration of between 40 to 60 s (specific energy = 53 to 80 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 location approximately 40-50 mm from the bottom of the test tube, so that it was positioned above the oil/water interface. Preliminary tests (results not shown) revealed that it was not necessary to add aqueous phase drop-wise into the emulsion in the first step as previously suggested (Garti et al., 1994), because the shear and mixing forces generated by the ultrasound were sufficient to disperse the aqueous phase homogeneously throughout the oil phase.

In the second step of the emulsification process, the pre-formed W1/O emulsion was added at a loading of 0.375 g, 0.75, and 1.5 g into skim milk to create an emulsion with a total mass of

7.5 g (i.e. 5, 10, 20 % w/w final W1/O loading concentration). Ultrasound was applied at various calorimetric power levels (2 W = 10% amplitude, 6 W = 20%, 10 W = 30%, 18W = 40%, 26 W = 50%) for 5 seconds using a 3 mm microtip horn again inside 15 mL test tubes (specific energy = 1.3 to 17.3 J/g). 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 reported power values were determined by calorimetry. Hand-mixing (i.e., no ultrasound application) was also attempted, however phase separation occurred rapidly with few emulsified droplets formed.

For one set of experiments, Span 80 was varied at a concentration of 2.5, 5, 10 and 20% w/w of the oil phase, whilst keeping the aqueous phase loading in the W1/O emulsion fixed at 10% w/w. In another set of experiments, the aqueous phase loading in the W1/O emulsion was varied at concentrations of 10, 20, 30 and 40% w/w, whilst keeping the Span 80 concentration in the oil phase fixed at a constant 10% w/w. The primary W1/O emulsion was formed using a constant 10 W (calorimetric power) for 60 s. The secondary W1/O/W2 emulsions were formed by dispersing a 5% w/w loading of the primary W1/O emulsion into the skim milk using a constant ultrasonic power of 10 W for 5s.

2.3. Conductivity measurements

The addition of sodium chloride in the inner aqueous phase was used as an entrapment marker, with the release of inner phase into outer phase resulting in an increased conductivity associated with the increase salt concentration. To quantitatively relate changes in conductivity to the release of salt from the emulsions after preparation, standard solutions representing 0, 25, 50, 75 and 100% NaCl release were prepared. The release %, was used to determine the encapsulation yield % (on a weight basis), and is calculated simply as the relative proportion of the total salt that was not released to the outerphase:

$$Encapsulation\ yield\ \% \ = \ 100\ \% - \ Release\ \% \qquad (1)$$

The proportion of double emulsion expressed as inner aqueous droplets was further calculated using:

$$Proportion \ = W1/O\ loading\ \% \ \times Water\ loading\ in\ W1/O\ \% \ \times Encapsulation\ yield\ \% \quad (2)$$

Standards for each specific formulation used in the W1/O/W2 emulsion were prepared that included the same concentrations of each component. These standards were prepared by sonication with 20 kHz ultrasound for 2 minutes at 50% amplitude (34 W calorimetric power) using an 11 mm horn in a container holding 50g of the standard. Note that an 11 mm was selected to create these standards rather than using the 3 mm horn, as its larger active area enabled more effective processing of larger volumes. Sonication at these conditions was sufficient to ensure complete homogenization of the fat droplets and limit phase separation and creaming in the standards. Conductivity was measured in the standard solutions and samples within ~3 hours of formation, 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. Each emulsion sample was measured twice.

2.4 Fluorescence microscopy

Fluorescent dyes Nile red (Sigma Aldrich, USA) and fluorescein (BDH, England) were added to the oil and aqueous phases of the formed double emulsions, respectively at concentrations ~ 0.02% w/w. The excitation source was a mercury lamp laser passed through a filter (U-MWIB3, Olympus, Japan) such that the excitation wavelength was in the range 460-495 nm. At this excitation range, fluorescein emits in the green (>500 nm) while Nile Red emits in the yellow (>565 nm) range of the visible spectrum. A microscope (Olympus, Japan) fitted with a 60X oil immersion optical lens was used to visualise the emulsions and the fluorescence emission.

2.5 Scanning electron microscopy

Cryo-scanning electron microscopy (Cryo SEM, FEI Qanta) was used to investigate the surface morphology of the oil-milk double emulsion system. The sample was first transferred into glass tube (1.3 mm × 1.3 mm × 5 mm in size) and then mounted on a copper holder. This fresh sample - copper holder was quickly immersed into liquid nitrogen slush at -210°C. After freezing, the frozen sample was immediately transferred into an attached cryo preparation chamber by 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) followed by etching process (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 nitrogen gas cooled module at -140°C. The detector used for the SEM observation was a solid state backscattered electron detector (SSD).

2.6 Particle size measurements

The particle size of the double emulsion droplets was measured using a Malvern Mastersizer 2000 (Malvern Instruments, UK) with Hydro-G2000 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 initial water-in-oil emulsions was determined using a Zetasizer Nano ZS (Malvern Instruments, UK), with sunflower oil used for dilution. A Zetasizer Nano ZS was used to measure the W1/O emulsions, to avoid flowing large amounts of oil through the Mastersizer 2000 instrument. Each emulsion sample was measured 3 times.

2.7 Reverse Phase High Performance Liquid Chromatography (HPLC)

The concentration of individual milk proteins remaining in the bulk skim phase was determined by reverse phase HPLC following a protocol adapted from Visser, Slangen, and Rollema (1991). The mobile phases employed were water/acetonitrile/trifluoroacetic acid in a 900:100:1 ratio (solvent A) and water/acetonitrile/trifluoroacetic acid in a 100:900:1 ratio (solvent B), using the elution gradient described by Visser et al. (1991). Milk samples (0.1 mL) were dissolved in a 70:30 mixture of A:B (3.7 mL) as per Yüksel and Erdem (2010). Prepared samples (30 μL) were injected into the HPLC (Shimadzu) by an autosampler. The column used was a Jupiter 5u C18 300 A with a length of 300 mm and a diameter of 4.6 mm (Phenomenex, Australia). The column was maintained at a constant temperature of 30 °C inside a column oven. The elution rate was maintained constant at 0.8 mL/min and the UV-Vis spectra was measured at 220 nm.

2.8 Zeta potential

The zeta potential of the particles in the double emulsions was measured using a Zetasizer Nano ZS (Malvern Instruments, UK). Phosphate buffer (0.1 M) at a pH of 6.8 was used as the

dilutant. The double emulsion was diluted approximately 1:1000 and placed inside a disposable polycarbonate folded zeta potential cell cuvette (ATA Scientific, DTS1070). Samples were measured 6 times (with each run consisting of between 10 to 15 measurements automatically determined by the unit).

2.9 Shelf life stability

The visual appearance of the double emulsions was assessed by direct observation and from images obtained by optical and fluorescence microscopy. Conductivity was measured on the day of preparation (day 1). Particle size was measured on days 1, 2, 5 and 7 (the measurement was limited to 7 days since this is typical shelf life range of commercially pasteurised milk). The formed emulsions were stored in the refrigerator at 4 °C between measurements on the specified days. Prior to measuring conductivity, samples were allowed to equilibrate to room temperature for a minimum of 2 hours.

2.10 Statistical analysis

All emulsions were prepared in duplicate 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. The t-test is noted to be acceptable for assessment of the statistical significance for low number of experimental replicates (de Winter, 2013).

For data sets where a trend was apparent, a trend-line regression was fitted using Microsoft Excel 2013 (Microsoft) to ascertain the quality of the relationship, and the R^2 value of these trends is reported where applicable.

3. Results and discussion

3.1 Double emulsion formation

3.2.1 Controlling emulsion droplet size distributions

The sizes of the primary and secondary emulsion droplets are key determining factors in the formation and stability of double emulsions. In particular, the primary droplets (aqueous skim milk droplets stabilised by Span-80 surfactant) must be small enough to allow encapsulation within secondary droplets (oil droplets stabilised by milk proteins) that themselves must be small enough to resist creaming. As the use of ultrasound to produce milk-protein stabilised double emulsions has not yet been investigated, a detailed characterisation of the size of primary water and secondary oil droplets was performed as a function of ultrasonic and formulation parameters.

Primary water droplets

The majority of experiments in this study were performed with a primary water-in-oil (W/O) emulsion consisting of 10 wt% skim milk in 90 wt% sunflower oil/Span-80 formed by application of ultrasound (20 kHz, 10 W for 40-60 s). The size of the primary aqueous droplets in this W1/O emulsion (10% aqueous phase loaded in oil phase) was assessed microscopically and by light scattering (Figure 2A). Microscopic images indicated that, on a numerical basis, most of the aqueous phase droplets dispersed into the oil phase were sub-micron in diameter (~ 0.5 μm). However, much larger and somewhat amorphous regions of aqueous phase were also observed, and can be seen as green fluorescence emanating from the fluorescein loaded into the aqueous phase (see insert in left hand panel of Figure 2A). Light scattering measurements also revealed a bi-modal droplet size distribution of the primary aqueous emulsion droplets (Figure 2A). According to the particle size distribution, the large droplets

comprise over 90% of the total W1/O volume with the smaller sub-micron droplets representing less than 10%. Based on the microscopic images, the larger droplets appear most likely to be aggregates of the sub-micron emulsified droplets, formed during the ultrasonic emulsification process by collisions that occur simultaneously with size reduction in the presence of strong shear forces within the system (Jafari et al., 2007). The localized heating from cavitation may also promote the aggregation of proteins. Size distributions for the W1/O emulsions formed using a range of aqueous phase loadings and surfactant concentrations are available in Supplementary Information Figure S1.

Secondary oil droplets

To verify that secondary oil droplets of sufficient size to encapsulate the primary aqueous droplets, particle size distributions were obtained for emulsions prepared at different secondary loadings of W1/O emulsion at 6 W of ultrasound power (Figure 2B). Secondary loading did not appear to have a major effect on the size distribution of the oil droplets, which were in all cases bimodal, with the larger particles of similar size to that of the larger primary aqueous droplets.

To control the size of the secondary emulsion droplets, the ultrasound power applied was varied. Varying the ultrasound power controls the intensity of cavitation and the size of the resultant emulsion droplets. The influence of ultrasonic power on the size of the secondary oil droplets is shown in Figure 3A (full particle size distributions are available in Supplementary Information Figure S2, photomicrographs in Figure S3). Figure 3B shows a corresponding decline of internalised aqueous phase droplets with increasing ultrasound power. In Figure 3, we observe reasonably strong trends that relate sonication power and emulsion loading concentrations with particle size and aqueous phase loading in the final emulsion. A power law correlation between the particle size and ultrasonic power is observed, with R^2 values of 0.97, 0.96, and 0.93 for 5, 10 and 20% W/O loading respectively. The observation of a power law correlation between the particle size and power delivered is consistent with other ultrasonic emulsification studies [Leong et al, 2009]. A linear correlation between the power delivered and the encapsulation yield is also observed, with R^2 values of 0.97, 0.95 and 0.45 for 5, 10 and 20% W/O loading respectively. Both these trends are expected. Increasing sonication power results in greater shear forces that lead to more disruption of emulsion droplets and decreased retention of internalised aqueous phase droplets. A decline in average diameter (D[4,3]) from between 12 to 18 μm at 2 W to between 2 to 4 μm at 26 W occurred, consistent with microscopic observations.

The loading rate of W1/O emulsion did not appear to influence the average particle sizes formed in the secondary emulsion except for the extreme cases where the highest power setting (26 W) was used to sonicate a small amount of oil (i.e. 5% W1/O loading), or when the lowest power setting (2 W) was used to sonicate a large amount of oil (i.e. 20% W1/O loading). It was observed that smaller and larger droplets were formed at these conditions respectively. The presence of large oil droplets >10 μm at the lowest power (2 W) for 20% W1/O loading was also evident in the particle size distributions (Supplementary Figure S2). The specific energy applied during the emulsification process ranged from 1.3 kJ/kg at 2 W to 17.3 kJ/kg at 26 W. The reduced effectiveness at low energy density and high oil phase volume is consistent with observations of conventional ultrasonic emulsification as reported by Ramisetty, Pandit, and Gogate (2015). It can be explained by the fact that the applied energy becomes more dispersed/distributed among a larger oil volume, resulting in less size disruption per

volume/mass. Another possible explanation is that an increase to the oil phase volume increases the sample viscosity, making sonication less effective.

3.2.2 Encapsulation of aqueous droplets in oil droplets – encapsulation yield

The effectiveness of a process for producing a double emulsion depends on the extent of encapsulation of primary droplets within the secondary droplets, herein referred to as the encapsulation yield. Ideally for this process, all of the Span-80-stabilised skim milk droplets in the primary emulsion (W1/O) would be encapsulated and retained in the milk protein-stabilised oil droplets (i.e. an encapsulation yield of 100%). The encapsulation yield as a function of various processing parameters was assessed by analysis of microscopic images and by gauging the released of solute (NaCl) from the inner aqueous droplets into the bulk aqueous phase.

Micrograph observations

An image of a W1/O/W2 double emulsion, formed by dispersing 5 wt% (secondary loading) of a 10 wt% (primary loading) W1/O emulsion into skim milk using ultrasonication, is shown in Figure 4. The W1/O/W2 emulsion (formed at 6 W ultrasonic power) can be seen to have aqueous phase regions (skim milk) entrapped within the oil phase droplets (sunflower oil). Entrapment appears more prevalent in the larger droplets. Fluorescence microscopic images provide confirmation that the entrapped inner phase is aqueous, due to the absence of fluorescence emanating from the oil-soluble dye. In this case, the darkened regions within the yellow fluorescent oil droplets indicate an oil void space where aqueous phase is entrapped. Those droplets observed to lack an entrapped phase are likely in a different focal plane.

The entrapped aqueous phase appears to be irregularly-shaped, i.e., non-spherical. These irregular shapes appear consistent with the large, amorphous droplets originally seen in the formed primary W1/O emulsion (Figure 2A). It is likely that these large droplets are aggregates of the sub-micron sized droplets (Figure 2A) resulting from the interaction between the Span 80 surfactant used in stabilising the oil droplets and the proteins present in the skim milk aqueous phase. Complexation between Span 80 and milk proteins such as BSA has been found to stabilize the aqueous phase within oil droplets through the formation of a ‘thick’ gelled film that imparts elasticity and resistance to rupture of the inner droplets (Garti et al., 1994). The optimal concentration of BSA in the internalized aqueous phase reported by Garti et al. was 0.2 wt % BSA. As skim milk was used here, the protein concentration of the internalized phase was considerably higher, ~ 4.2 % w/v. Approximately 20% of this protein is whey protein (Farkye & Shah, 2014) which has been shown to be an effective gelling agent that stabilizes the internalized aqueous phase of double emulsions (Oppermann et al., 2015).

Estimation of encapsulation yield by measurement of released solute

The influence of ultrasonic power (applied during the second emulsification step) on encapsulation yield was assessed by determining the release of sodium chloride from the inner aqueous phase by measurement of conductivity. The encapsulation yield as a proportion of the total salt internalised, generally increased with increasing size of the secondary oil droplets (Figure 5), which results at lower ultrasonic power (Figure 3A). In Figure 5, we observe a trend that relates particle size with the encapsulation yield. The trend (fitted with a logarithmic trend-line) is strongest with a 5% W/O loading ($R2 = 0.97$), but decreases to 0.86 and 0.70 at 10% W/O and 20% W/O loadings. The decline in the correlation is likely due to increased variability of the conductivity measurements with higher W1/O loading. As the encapsulation efficiency was measured using a conductivity probe directly in the emulsions formed, some of the emulsion droplets formed may have reduced the precision of the conductivity measurement i.e. oil droplets sticking to the sensor probe. Since there were more oil droplets present in the

emulsions at higher W/O loading, larger standard deviations were observed for these measurements.

The encapsulation yield was also generally higher at 5% W1/O loading than at 10% or 20%. A maximum encapsulation yield of approximately 35 wt% was achievable under the conditions tested. As the initial loading of skim milk in oil/Span-80 was 10 wt%, this represents up to a 3.5% displacement of the oil phase with skim milk. Although the encapsulation yield was reduced as a function at higher W1/O loadings, the overall amount of encapsulated material did increase at higher loadings. This can be explained by the trend observed in Figure 3B, which shows that the actual amount of internal water phase present in the double emulsion increases with increasing W1/O loading.

The encapsulation yield achieved was somewhat disappointing, considering that (Garti et al., 1994) have reported yields of >80 % when using a similar Span 80/BSA surfactant system for the inner W1/O emulsion formation. The low encapsulation yields can likely be accounted for by the fact that the W1/O emulsions also included large regions of coalesced aqueous phase (Figure 2), that dispersed into the outer aqueous phase upon formation of the secondary emulsion droplets. This highlights the importance of creating a stable primary W1/O emulsion as previously noted (Kanouni et al., 2002).

As NaCl can diffuse both in and out of the oil droplets, it is possible that the conductivity measured will provide either an overestimate or underestimate to the degree of aqueous phase entrapment. In general, water will diffuse across a semi-permeable membrane (in this case the oil and surfactant boundaries) faster than the Na or Cl ions, which tend to diffuse together in order to maintain charge neutrality (Hancock & Cath, 2009). The osmotic pressure difference between the inner and outer aqueous phase, will to a degree govern the direction in which water will diffuse. The general tendency is for water to transfer by osmosis from regions of low osmotic pressure (i.e. low salt concentration) to regions of high osmotic pressure (i.e. high salt concentration). The salt concentration employed in the internal phase is 8 wt% (equivalent to ~1.48 M). The osmotic pressure associated with this salt concentration can be calculated using the van't Hoff Equation (Lang, 1967):

$$\pi = iMRT \tag{3}$$

For NaCl, the van't Hoff factor, *i*, can be approximated as 1.8 (Lang, 1967), resulting in an osmotic pressure of ~ 66 atm. It should be noted that skim milk is present in the internal and external aqueous phase, and this milk also contains lactose, proteins and other minerals. A typical osmotic pressure for skim milk with 9% solids is ~ 7 atm (Heldman, Lund, & Sabliov, 2006). For double emulsions, the internalized aqueous phase droplets are also subject to a Laplace pressure that acts on the droplet interface. The Laplace pressure can be calculated using (Menger, 1979):

$$\Delta P = \frac{2\gamma}{r} \tag{4}$$

Assuming a surface tension that is in the order of 40 mN/m, the Laplace pressure of a 2 μm radius droplet is in the order of 0.3 atm. In this situation, the Laplace pressure is negligible relative to the internal and external osmotic pressure. The osmotic pressure of the internal aqueous phase is larger (~10 times) compared with that of the external phase. There will therefore be a tendency for water to diffuse into the inner phase droplets with time, and diffusion of salt from internal to external. According to (Hancock et al., 2009), the flux of water and reverse flux of salt across a cellulose acetate membrane at a salt concentration of 1.5 M, is in the order of 11 L/m^2.hr and 50 mmol/m^2.hr respectively.

As such, the conductivity likely provides a slight underestimate of the water encapsulation yield. Nevertheless, as the conductivity is measured within a few hours of formation, it can be assumed that any increase in conductivity to the external phase measured is associated with the rupture of droplets leading to a release of the internal aqueous phase to the outer aqueous phase.

The influence of aqueous phase loading (in the initial W1/O formed) and surfactant concentration used for the double emulsion formation on the encapsulation yield was also assessed (Figure 6). The encapsulation yield of NaCl generally decreased with increasing aqueous phase loading. This is not unexpected, since more internal aqueous phase would result in a higher likelihood of it being released to the surrounding aqueous phase due to i) disruption of the oil droplets during sonication and ii) contact of inner aqueous phase into contact with outer aqueous phase to facilitate rapid diffusion of salt from inner to outer phase (Wen & Papadopoulos, 2001) and iii) increased probability of coalescence of the inner aqueous phase during W1/O emulsion formation. The eventual displacement of the oil phase however, regardless of the aqueous phase loading used, was between 2 and 3 % of the oil phase volume (determined by multiplying the encapsulation yield by the primary aqueous loading rate). This suggests that the amount of Span 80 surfactant used, which was kept constant here at 10 %, has a large influence on the eventual entrapment of aqueous phase in the double emulsion.

A further experiment varying the amount of surfactant employed in the double emulsion formation showed wide variation in encapsulation yield as a function of surfactant concentration (Figure S3). The highest yield (*ca* 35% ± 11.2) was obtained at a 10% loading of surfactant. Interestingly, increasing the surfactant concentration to 20% loading appeared to decrease the aqueous phase entrapment according to conductivity measurements. Statistical analysis using the Student's t-test suggested that the result for 10% Span 80-10 % aqueous loading (Figure 6, column 3) was statistically different ($p=0.017$) to 20% Span 80-10 % aqueous loading (Figure 6, column 4). This result could be partly due to excess surfactant creating more aqueous-filled micelles that can move through the oil phase aiding the release of solutes to the exterior bulk phase (Garti, 1997), although in both cases, the Span 80 surfactant is above the critical micelle concentration (Peltonen, Hirvonen, & Yliruusi, 2001). It should be noted that for the 10% Span 80-10% aqueous loading result, a total of 7 emulsions were formed and assessed. These additional emulsions were created across several days using different batches of milks, which may have contributed to the increased variability of the result as indicated by the large standard deviations. A word of caution should be made regarding the statistical significance of these results, since the power of statistical tests is low when small sample replicates (i.e. $n<3$) are used.

It was also attempted to make double emulsions without addition of Span 80 to the oil phase. It was envisioned that the milk proteins may provide sufficient stability to the formed W1/O and subsequent W1/O/W2 emulsion. However, encapsulation yields were lower without the presence of Span 80 in the oil phase (results not shown). Observations made during the formation of the first step W1/O formation indicated uneven product appearance after sonication. Microscopy images of the double emulsion formed also indicated negligible encapsulation compared with samples where Span 80 was used, confirming importance of surfactant in the oil phase to stabilize the double emulsion formed.

While further attempts to improve yields by changing the concentrations of surfactant and aqueous phase achieved limited success in this system (see Figure 6), there remains scope for significant improvement of encapsulation yield by optimising the inner W1/O emulsion, for instance using alternative surfactants.

3.2 Stability

3.2.1 Stabilisation mechanisms

The stability of the oil droplet double emulsions was investigated by examining the mechanism of interfacial stabilisation and size of droplets during storage.

Interfacial stabilisation of oil droplets by milk proteins

In whole milk, native fat globules are stabilised by a milk fat membrane consisting of polar lipids and surface active proteins (Lopez, Madec, & Jimenez-Flores, 2010). During homogenisation, the fat globules are broken into small droplets, increasing their overall surface area. The increased surface area of fat/water interface is largely stabilised by casein micelles (Michalski, Michel, Sainmont, & Briard, 2002). It has previously been shown that ultrasound does not affect the structure of casein micelles (Chandrapala, Martin, Zisu, Kentish, & Ashokkumar, 2012) and has a minor effect on whey proteins in milk (Ashokkumar et al., 2010; Chandrapala, Zisu, Palmer, Kentish, & Ashokkumar, 2011). As such, in this application, as with conventional milk homogenisation, stabilisation of the external O/W interface is likely to be predominantly stabilised by casein micelles present in the skim milk.

The surfactant (Span-80) which is present in the oil droplets may also play a role in stabilising the outer oil-water interface (Leong et al., 2009). To determine whether or not the milk proteins alone could stabilise the sunflower oil droplets, single O/W emulsions were produced with sunflower oil not containing any Span-80. The emulsions formed were similar in size range to those of the double emulsions containing Span 80 (see supplementary Figure S4), although the surfactant-containing emulsion produces slightly smaller droplet size likely due to decrease in surface tension of the oil phase. Minimal phase separation and droplet coalescence occurred during storage over 6 days. This result is consistent with emulsification of flax seed oil in milk using 20 kHz ultrasound by Shanmugam et al. (2014)

In conventional milk homogenisation, approximately ¾ of the milk fat interface becomes covered by casein (Michalski et al., 2002). To test if casein micelles were involved in stabilising the outer droplets of the double emulsions, the protein composition of the bulk milk phase after sonication was determined by reverse phase HPLC (data not shown). The casein concentration decreased with increasing sonication power, consistent with more casein being required to cover the increased interfacial area of the smaller droplets. No noticeable change in the whey protein concentration was observed upon sonication, consistent with previous studies of sonicated whey protein solutions (Zisu et al., 2011). Scanning electron microscopy under cryogenic conditions (cryo-SEM) was also employed to visualise the surface morphology of the double emulsions droplets. As shown in Figure 7, a smooth network of milk protein apparently coats the outer surface of the emulsion droplet. The sub-micron sized circular entities on the surface are consistent with previous observations of casein micelles on the surface of homogenised milk fat droplets (Dalgleish, 2006; Luo et al., 2014).

The zeta potentials of full cream homogenised milk (14.4 ± 0.7 mV), W1/O/W2 double emulsion (14.7 ± 0.7 mV), skim milk (14.2 ± 0.7 mV) were also determined and found to be not statistically different to each other (P=0.528 and P=0.278 between W1/O/W2 and full cream and skim milk respectively). This is consistent with the emulsified oil droplets being stabilised by a similar coating to milk fat in homogenized milks. The zeta potential of the unhomogenized full cream was statistically different (P=0.01) to that of the homogenized milk

(12.8 ± 0.6 mV), as the unhomogenized fat globules are stabilized by a largely intact milk fat globule membrane (Michalski et al., 2002).

Oil droplet size stability

Due to the size of the emulsified droplets formed in the double emulsions (typically between 1 to 20 μm), 'creaming' by gravitational sedimentation occurs within a time scale of several hours. This is evident in the appearance of a cream layer at the top of the emulsion during storage, and appears after the first day of formation in all samples. Further homogenization of the product to prevent creaming is not feasible as it would reduce the encapsulation yield. However, there is a noticeable difference between 'creaming' and 'phase-separation', which was observed as a clear transparent oil phase separated from the skim milk when a 20% W1/O loading double emulsion with sonication at 2 W, 6W or 10 W for 5 s. Power delivery at 18 W or 26 W for 5s was able to prevent phase separation in the emulsion when using a 20 % W1/O phase loading. Notably, phase separation was not observed even at the lowest ultrasonic amplitude after 7 days when a W1/O loading of 5% was employed.

In addition to visual inspections of the emulsion stability, the particle size of the secondary oil droplets was measured as a function of storage time. An increase in particle size would be indicative of droplet flocculation or coalescence and emulsion instability. The particle size increased with storage duration when using 20% W1/O loading, but only when 6 W, 10W, or 20 W ultrasound was employed. This is consistent with visual observations that indicated phase separation within these samples, confirming coalescence of fat droplets.

In Figure 8, we again observe a power-law trend that relates ultrasonication power with the particle size of the emulsions produced. R^2 values of 0.97, 0.96 and 0.93 are observed for W1/O loadings of 5, 10 and 20 wt% respectively. No consistent correlation between the number of days for which the emulsions have been stored i.e. on days 1, 2, 5 and 7, and the particle size could be observed however. A general trend appears to be that emulsion droplets sizes formed with 5 wt% W1/O loading declines with storage, with 10 wt% W1/O it neither increases or declines with storage, and with 20 wt% W1/O it increases with storage.

Interestingly, the average size of the secondary oil droplets in the double emulsions decreased in size with storage duration for the 5% W1/O loading (Figure 8) over the first few days. This can be explained by the difference in salt concentration between the internal and external aqueous phases. This creates a driving force for water to diffuse from the outer to the inner phase, and for salt to diffuse from the inner to the outer phase. As mentioned above, water will generally diffuse across the oil boundaries faster than the Na^+ or Cl^- ions (Hancock et al., 2009). So over time the concentration difference will be diminished predominantly by water diffusing into the inner phase droplet. This will swell the internal droplets until they can no longer be retained in the oil droplets, which will collapse, resulting in a reduction in the size of the secondary emulsion droplets (Wen et al., 2001). This effect is exaggerated by the high salt concentrations of the primary emulsion that are needed in order to use conductivity as a measure of entrapment. Practically, the salt concentration of the inner phase would be much lower. To maximise stability it could set to balance the rate of inward water diffusion (due to the effective osmotic pressure resulting from the salt concentration gradient) to the pressure-induced outwards diffusion resulting from the Laplace pressure of the inner droplets (Menger, 1979).

Additionally, no phase separation was observed in these samples. This implies that the dispersed oil droplets in the double emulsion formed were relatively stable to coalescence (which leads to phase separation) but there might have been a gradual loss of aqueous phase from the oil phase with time, culminating in a shrinking of the oil phase emulsion droplets (Wen et al., 2001). The particle size distributions of these samples (Supplementary Figure S5) are consistent with this revealing generally a shift from larger to smaller oil droplets with time. In some cases, particularly for 20% W1/O loading (Figure 8), after day 7 the emulsion droplet sizes increase, likely indicating onset of inter-droplet coalescence.

Conclusions

Double emulsions were formed in skim milk using ultrasonic emulsification. The maximum encapsulation yield achieved in the W1/O/W2 emulsion when using a Span 80 lipophilic surfactant system to stabilize the initially formed W1/O emulsion was ~35%. Encapsulation yield and hence oil displacement was found to be dependent on the size of the droplets formed in the double emulsion. The emulsion droplets formed were stable to phase separation for 7 days using up to 20 % W1/O loading, provided that sufficient energy input was used in the formation of the outer emulsion. However, increasing energy input lead to greater release of internal aqueous phase to external aqueous phase and consequently decreased encapsulation yields. Characterization of the outer surface suggests that stability was conferred by the milk proteins, particularly casein micelles, similar to emulsified fat droplets in homogenized milks. Leakage of internalized aqueous phase occurs during storage, and this is likely to be the primary source of instability in the formed double emulsions in the absence of coalescence and phase separation.

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767

Figure 1: Schematic of two-step W/O/W double emulsion formation by ultrasonication.

Figure 2: A) Volumetric size distribution of the primary aqueous droplet in a W/O skim-milk (10 wt%) in sunflower oil (90 wt%) emulsion formed by sonication at 20 kHz, 10 W for 40-60s, measured by light scattering. Insert is a fluoromicrograph of the W/O emulsion. B) Volumetric size distributions of secondary oil droplets in W/O/W double emulsions formed by sonication at 20 kHz, 6 W for 5s, measured by laser diffraction. Data are presented for secondary loadings of 5 wt% (red curve), 10 wt% (green) and 20 wt% (purple) W/O emulsion. Each curve is representative of 2 experimental replicates measured 3 times each.

Figure 3: A) Volume-weighted average diameter (D[4,3]) of secondary oil droplets as a function of sonication power and loading of primary W/O emulsion into skim milk during secondary emulsification. B) Overall encapsulation rate of primary skim milk droplets in secondary oil droplets estimated by determining the extent of salt release by conductivity measurements as a function of ultrasonic power for 5%, 10% and 20% W/O loading. Error bars represent the standard deviation of duplicate experiments.

Figure 4: Optical (left) and fluorescence (right) microscopy images of a W/O/W double emulsion (skim milk/sunflower oil/skim milk) formed using sonication (6 W, 5 wt% W/O).

Figure 5: Encapsulation yield of primary aqueous droplets in secondary oil droplet (% w/w) as a function of average diameter of the secondary oil droplets and loading rate of W/O emulsion. Error bars represent the standard deviation of duplicate experiments.

Figure 6: Encapsulation yield of NaCl as a function of aqueous phase loading and Span 80 surfactant concentration used in the formation of double emulsions. A constant sonication power of 10 W, 5s and W/O loading of 5% was used and the encapsulation rate was estimated by conductivity measurements. Alphabetical letters are used to indicate significant differences, as determined by Student’s t-test.

Figure 7: Cryo-SEM image of the external morphology of a double emulsion droplet formed in skim milk. The masses observed to the lower left and right are attributed to regions of frozen liquid milk.

Figure 8: Effect of ultrasound power and loading rate on the initial size and stability of oil droplets.

Figure S1: Particle size distributions of W/O emulsions formed during first step of emulsification at varying (A) aqueous loading and (B) surfactant concentration.

Figure S2: Size distributions of secondary emulsified oil droplets formed using different power intensities and W/O loadings of 5, 10 and 20 wt %.

Figure S3: Photomicrographs of emulsions formed at varying ultrasonic power with a W/O loading of 5 wt%.

Figure S4: Secondary emulsified oil droplet size formed using sonication at 10 W, 5 s in presence and absence of Span 80 surfactant in the oil phase.

Figure S5: Observed change in size distribution with storage time on days 1, 2, 5 and 7 for W/O/W emulsions formed using sonication at 26 W, 5 s with W/O loading of 5, 10 and 20 wt %. Changes appear most prominent for sample with 5 wt % W/O loading.

Figure

Click here to download high resolution image

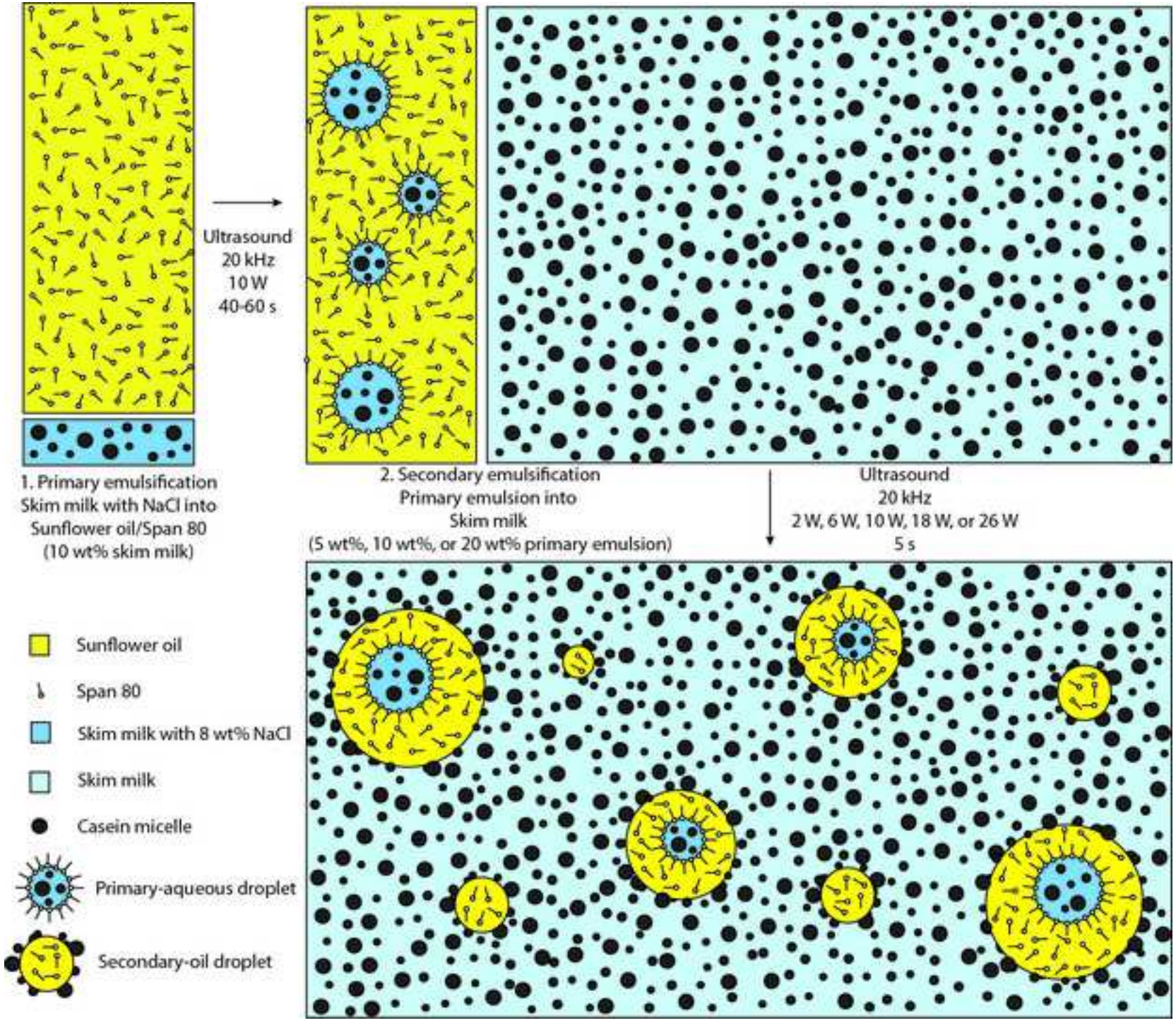


Figure 2

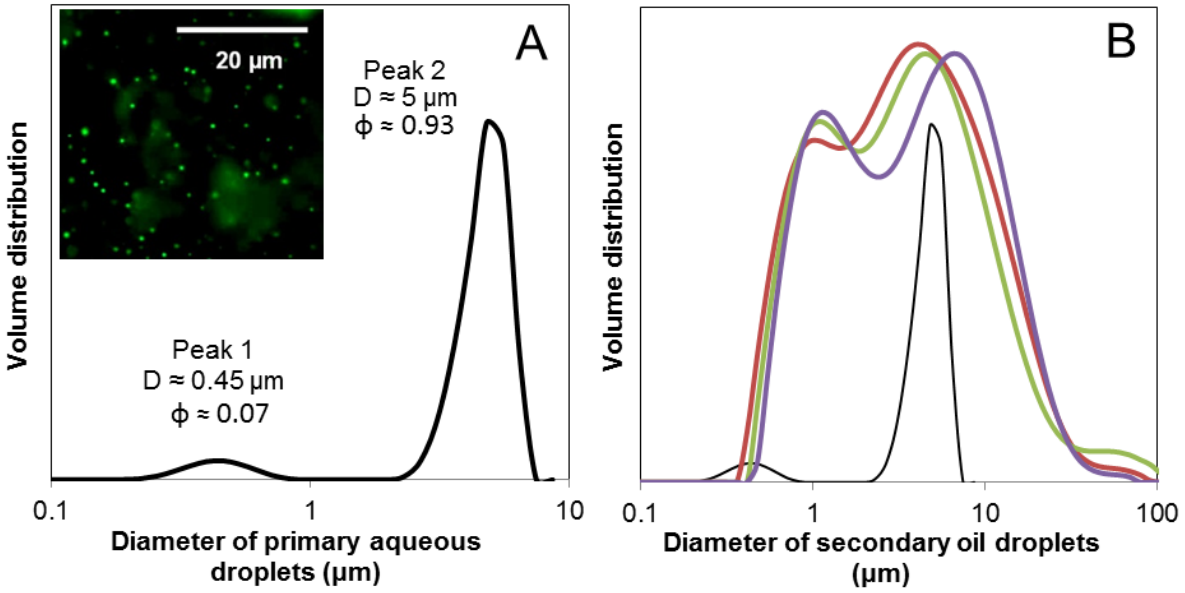

A
20 µm
Peak 2
D ≈ 5 µm
ϕ ≈ 0.93
Peak 1
D ≈ 0.45 µm
ϕ ≈ 0.07
Volume distribution
0.1
1
10
Diameter of primary aqueous droplets (µm)
B
Volume distribution
0.1
1
10
100
Diameter of secondary oil droplets (µm)

Figure 3

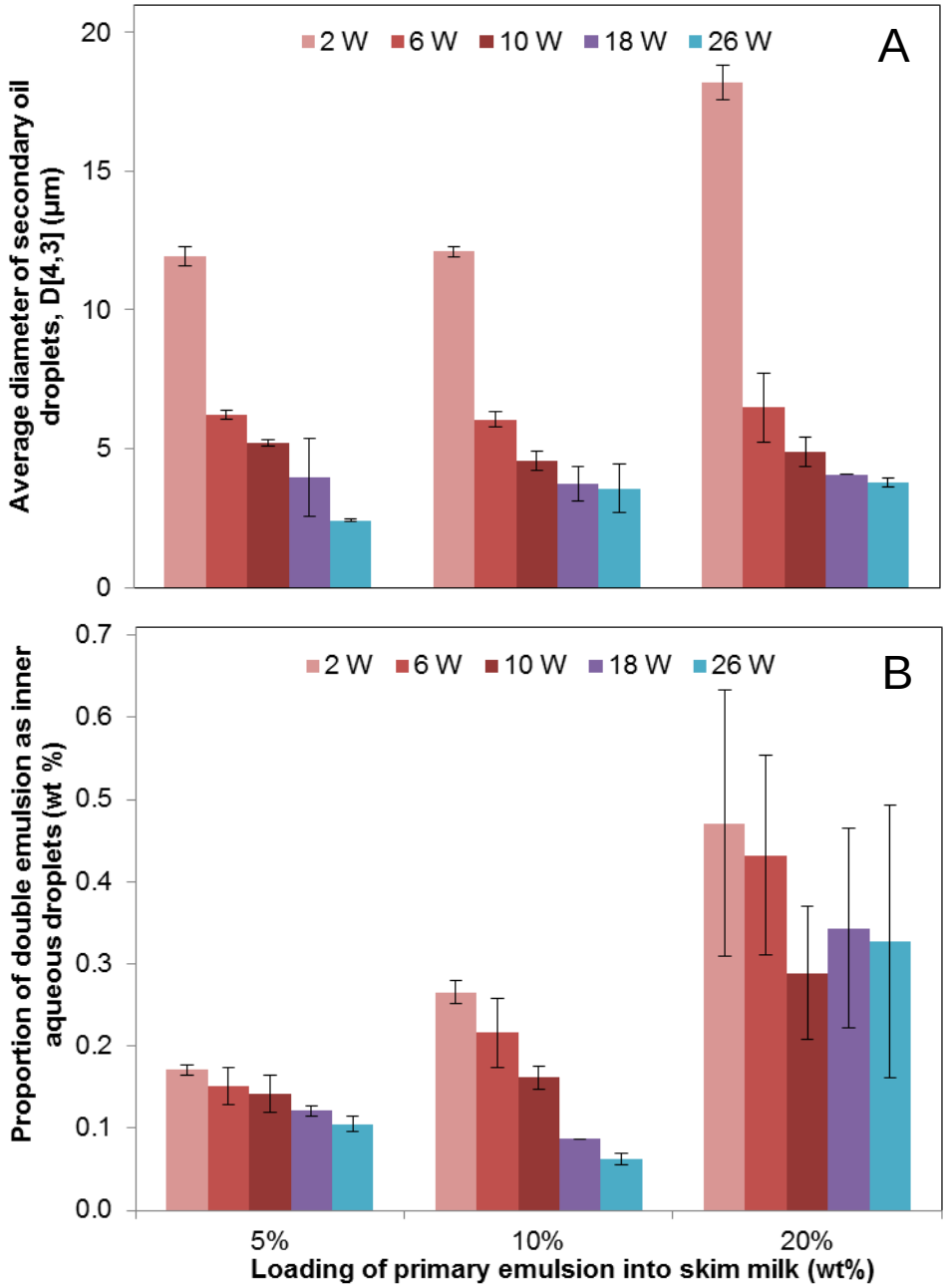

2 W
6 W
10 W
18 W
26 W
A
Average diameter of secondary oil droplets, D[4,3] (µm)
20
15
10
5
0
B
Proportion of double emulsion as inner aqueous droplets (wt %)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
5%
10%
20%
Loading of primary emulsion into skim milk (wt%)

Figure 4

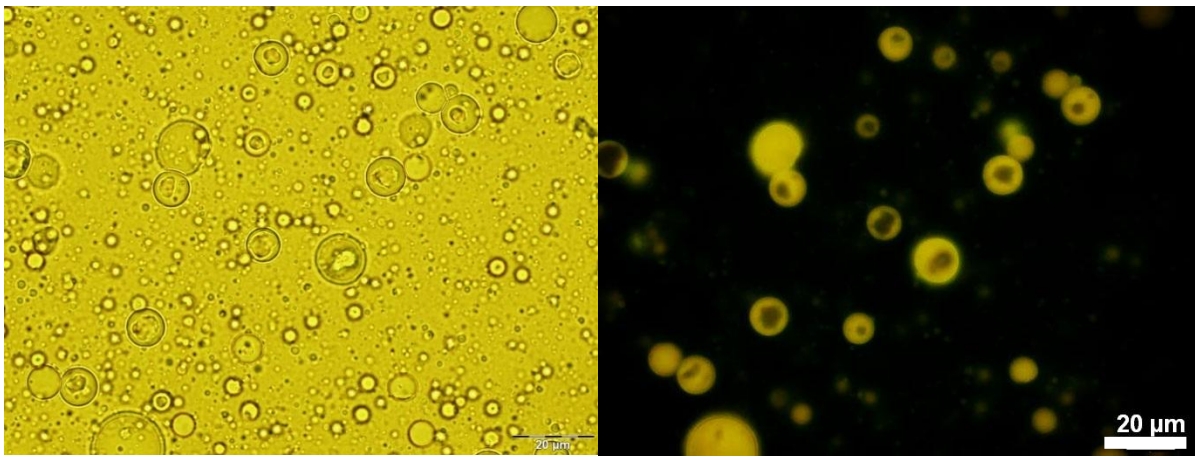


Figure 5

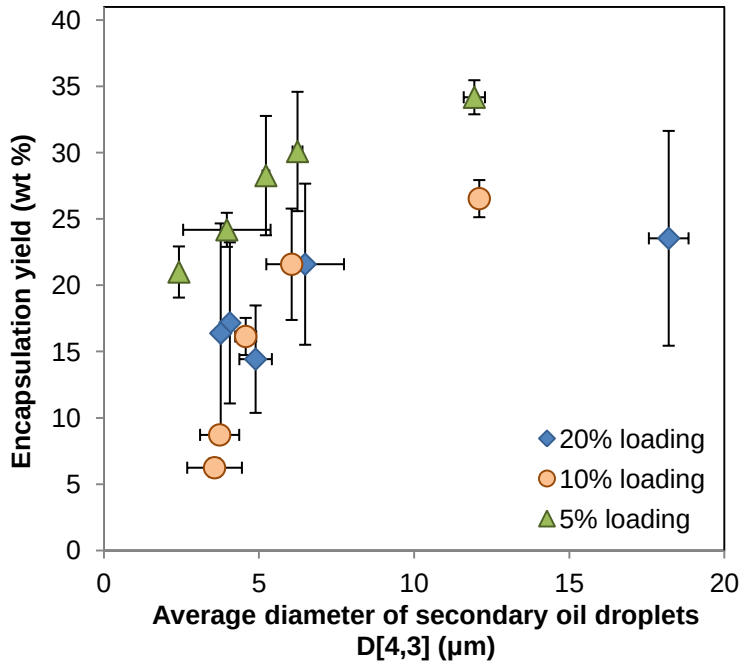

Encapsulation yield (wt %)
40
35
30
25
20
15
10
5
0
0
5
10
15
20
Average diameter of secondary oil droplets D[4,3] (μm)
20% loading
10% loading
5% loading

Figure 6

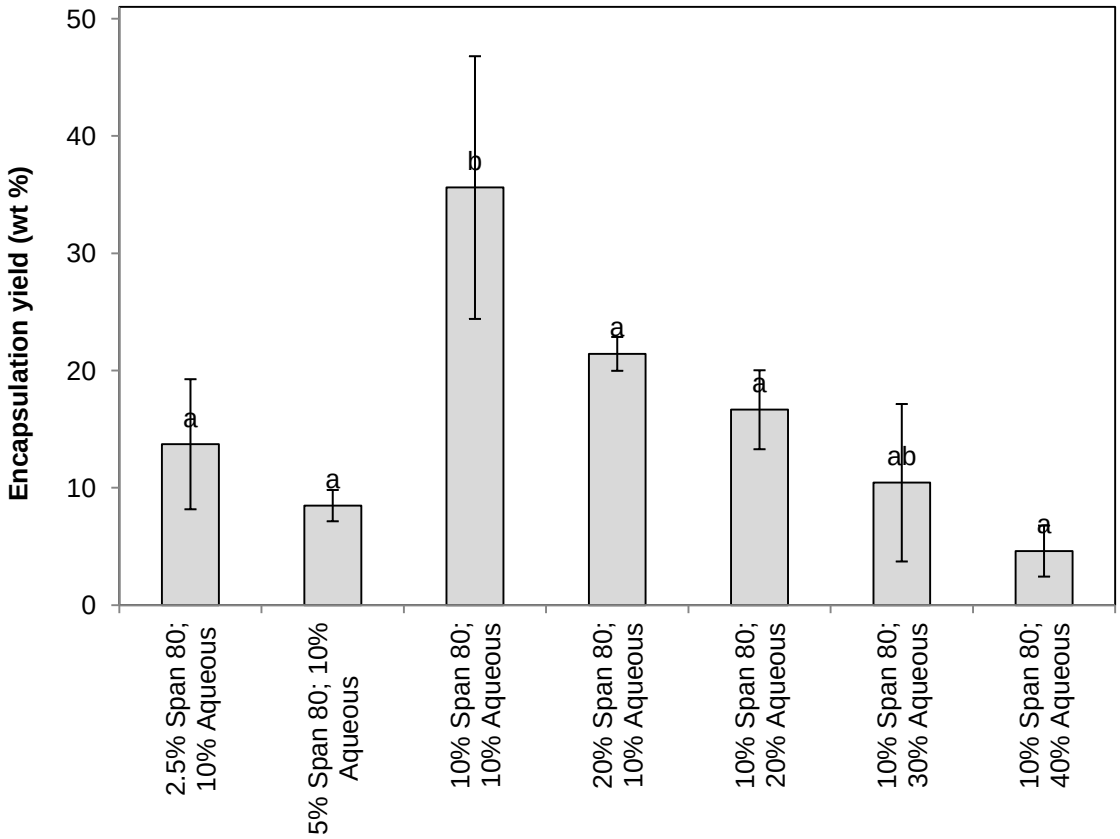

Encapsulation yield (wt %)
50
40
30
20
10
0
a
a
b
a
a
ab
a
2.5% Span 80; 10% Aqueous
5% Span 80; 10% Aqueous
10% Span 80; 10% Aqueous
20% Span 80; 10% Aqueous
10% Span 80; 20% Aqueous
10% Span 80; 30% Aqueous
10% Span 80; 40% Aqueous

Figure 7

Click here to download high resolution image

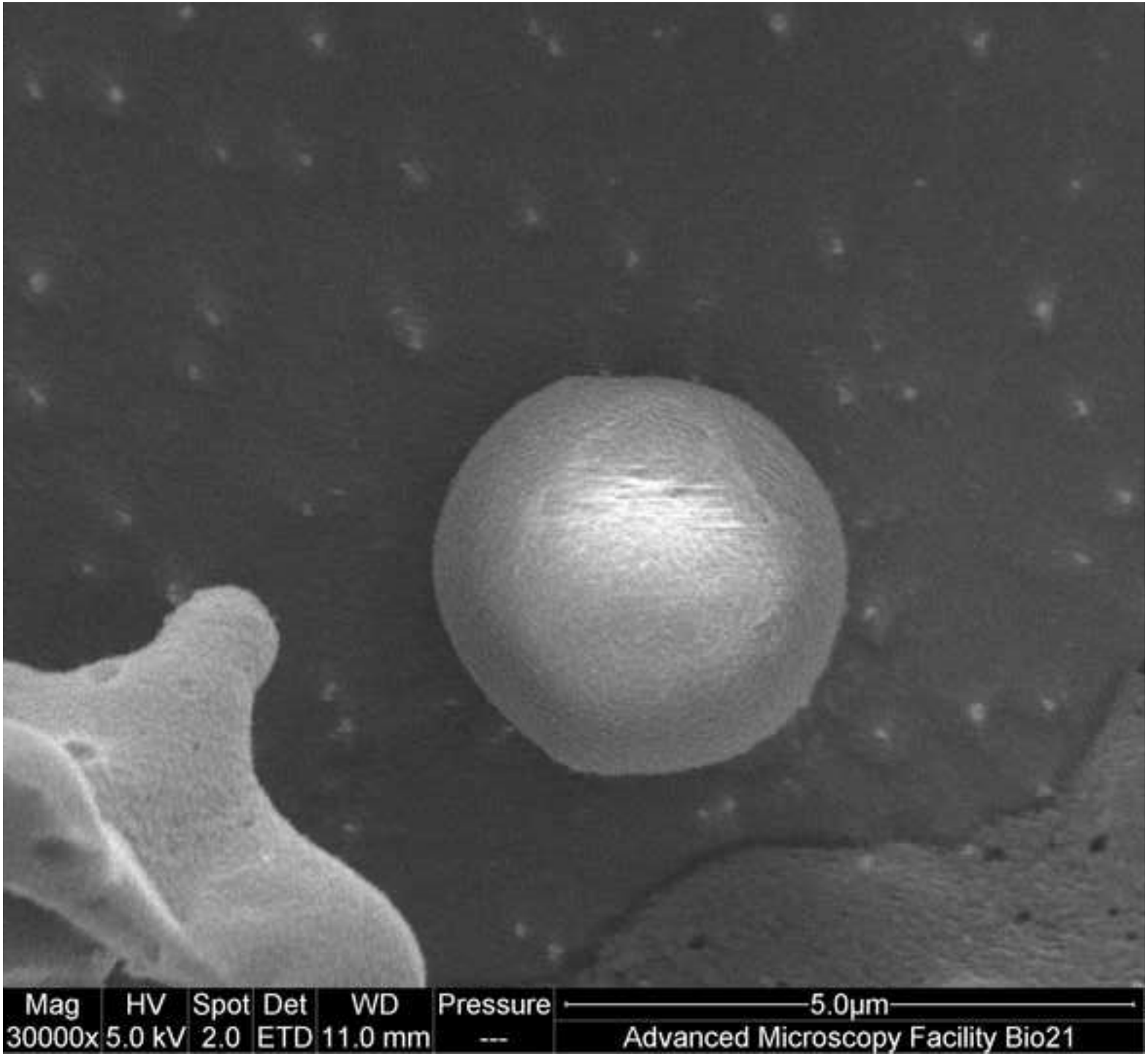


Figure 8

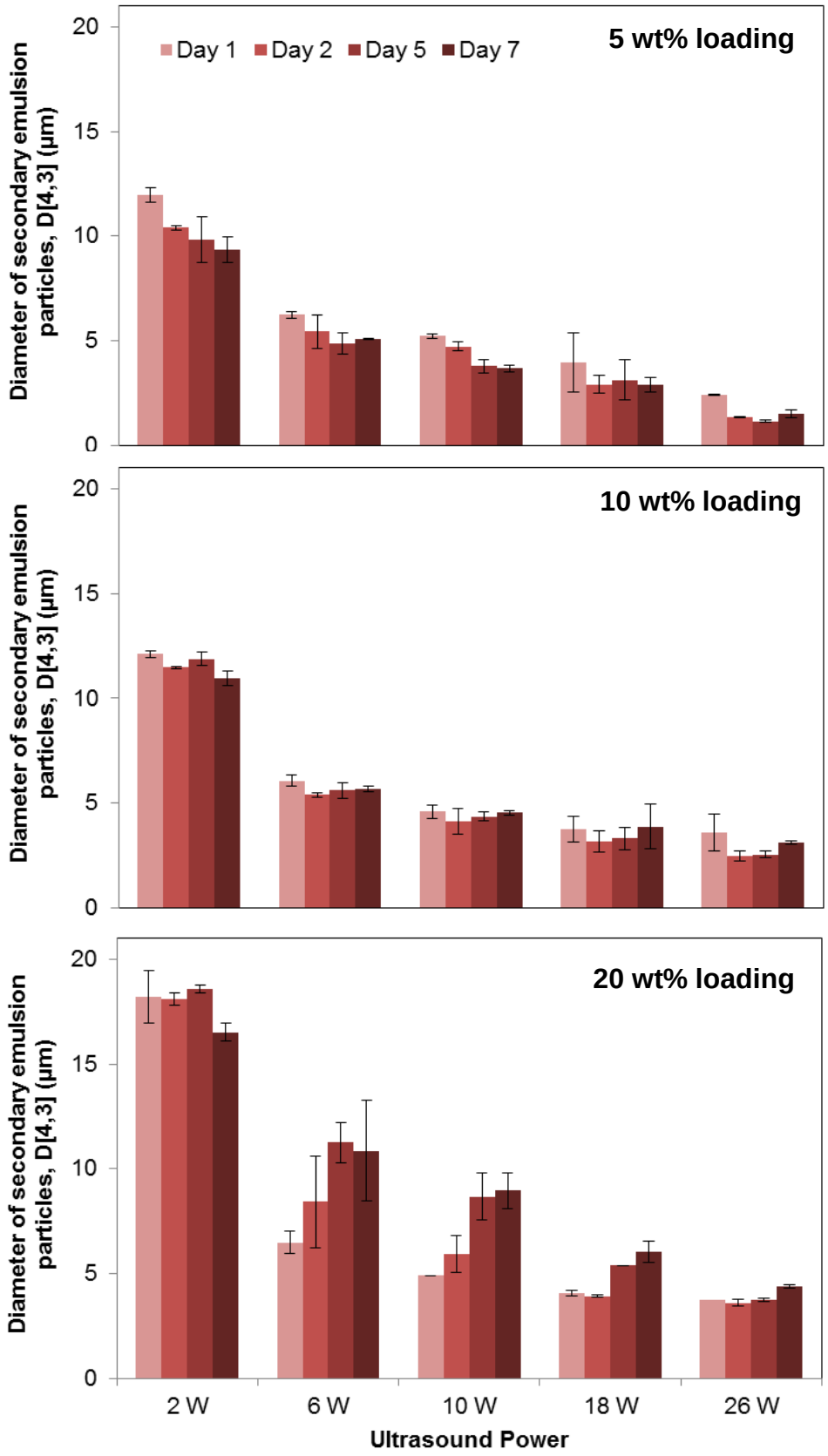
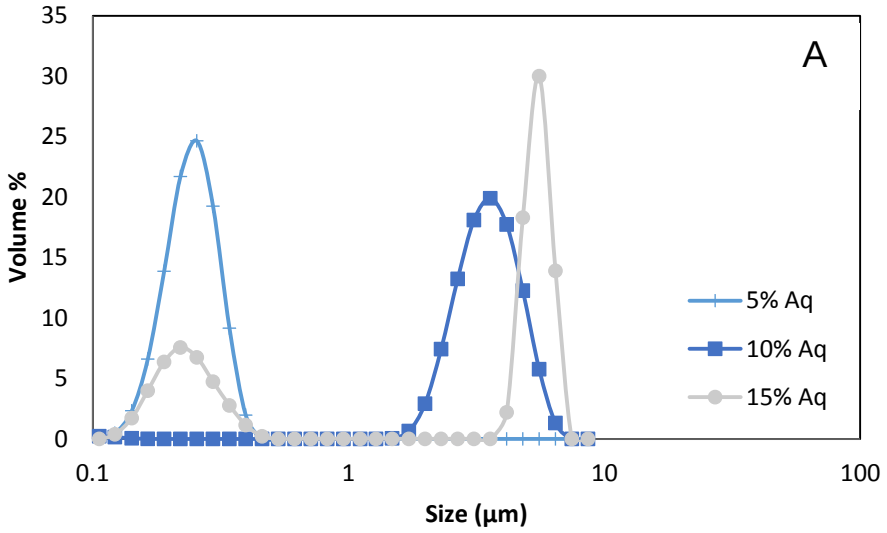

Day 1
Day 2
Day 5
Day 7
5 wt% loading
10 wt% loading
20 wt% loading
Diameter of secondary emulsion particles, D[4,3] (µm)
0
5
10
15
20
2 W
6 W
10 W
18 W
26 W
Ultrasound Power

Figure S1


A
Volume %
Size (µm)
5% Aq
10% Aq
15% Aq
0
5
10
15
20
25
30
35
0.1
1
10
100

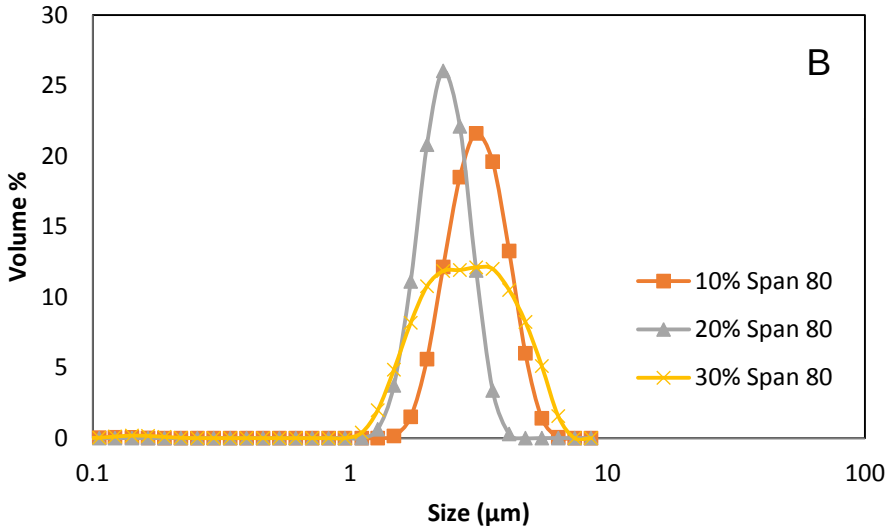

B
Volume %
Size (µm)
10% Span 80
20% Span 80
30% Span 80
0
5
10
15
20
25
30
0.1
1
10
100

Figure S2

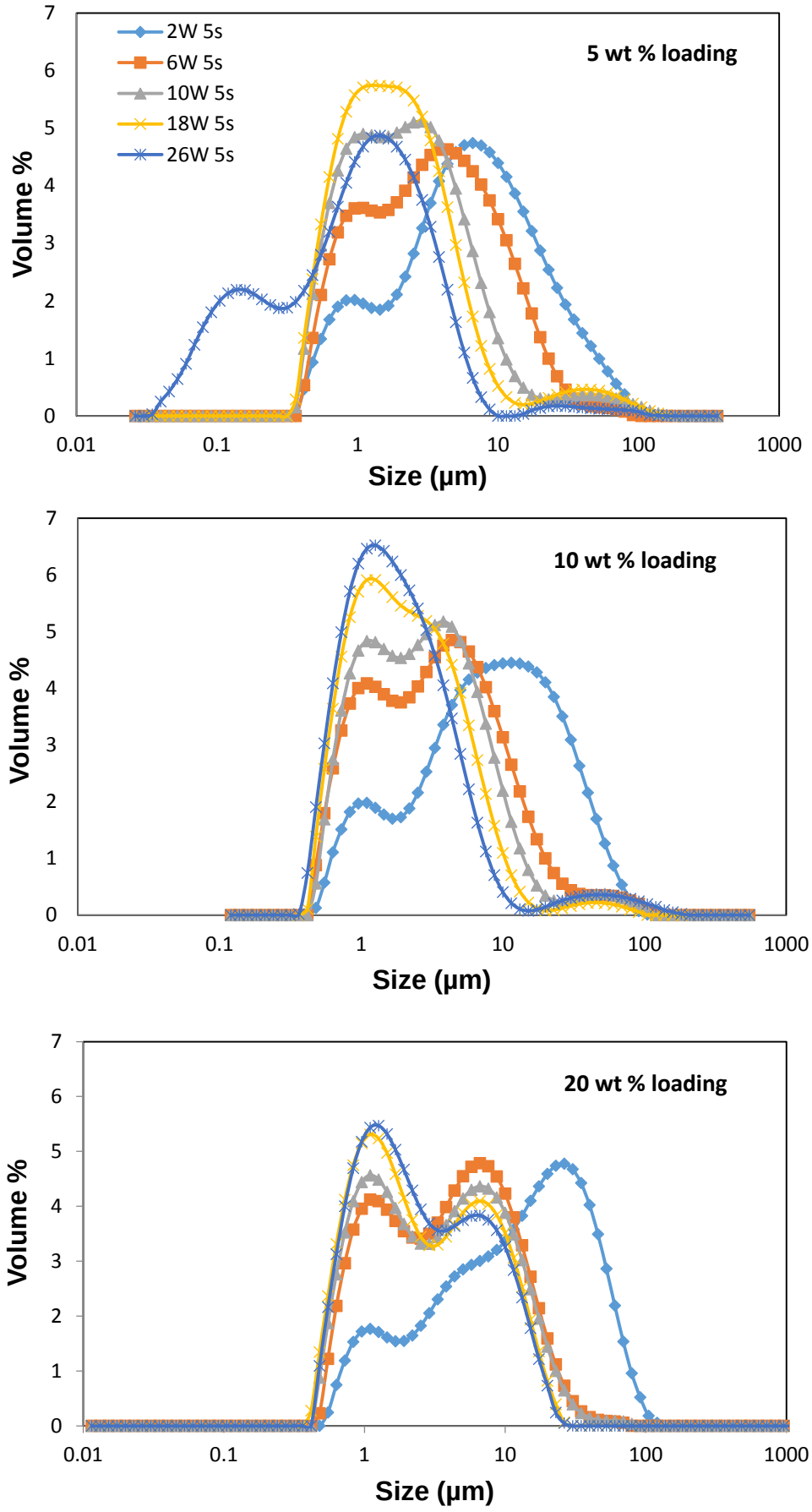
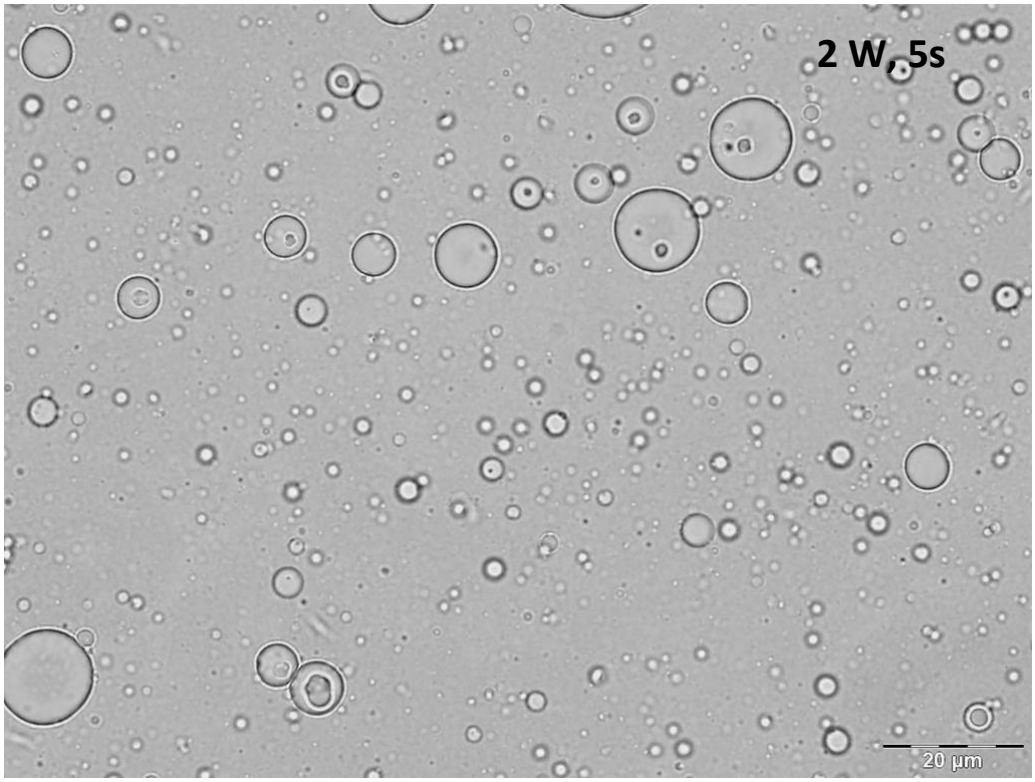
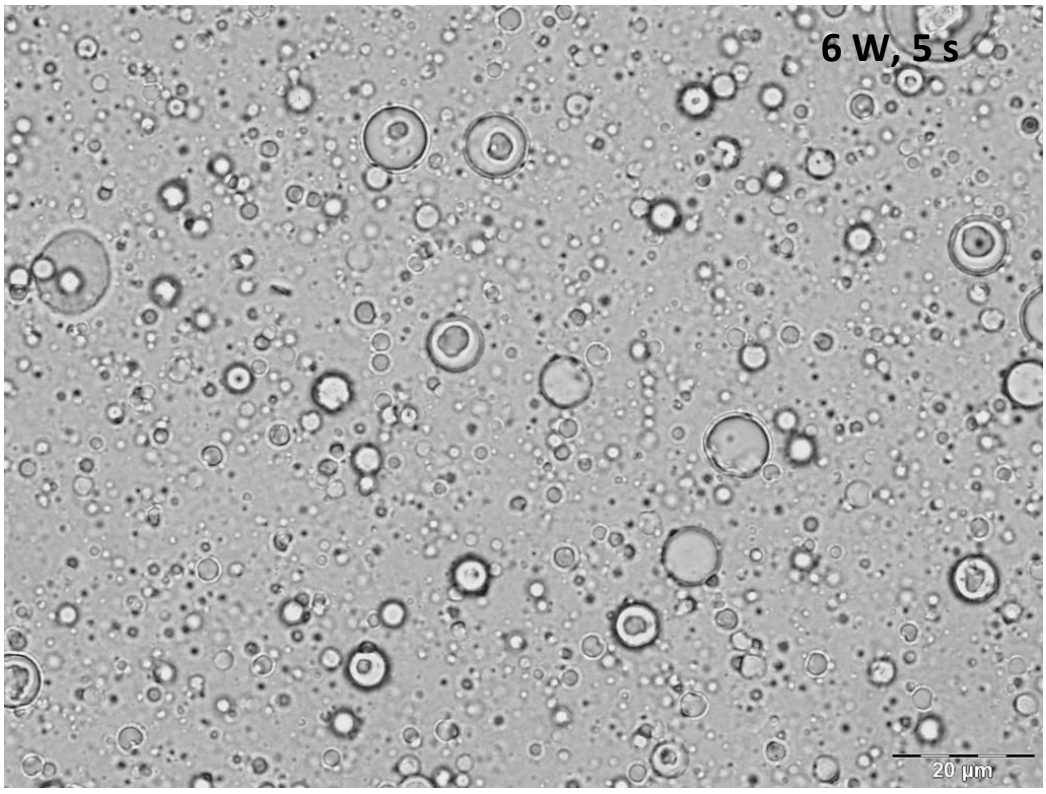
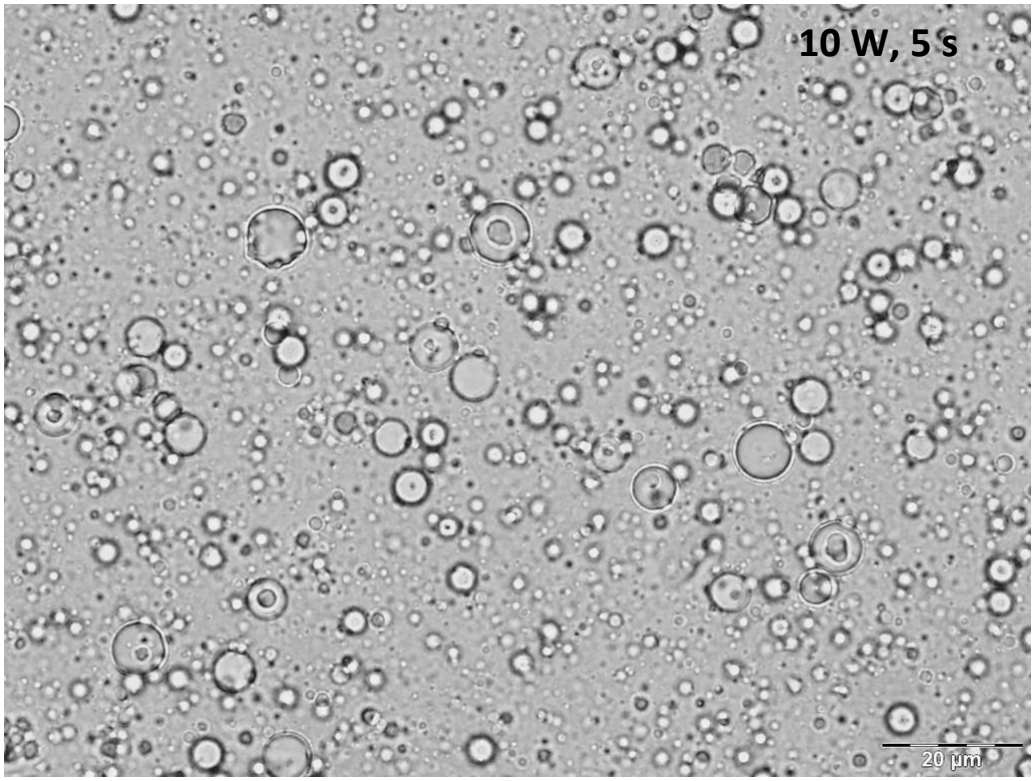
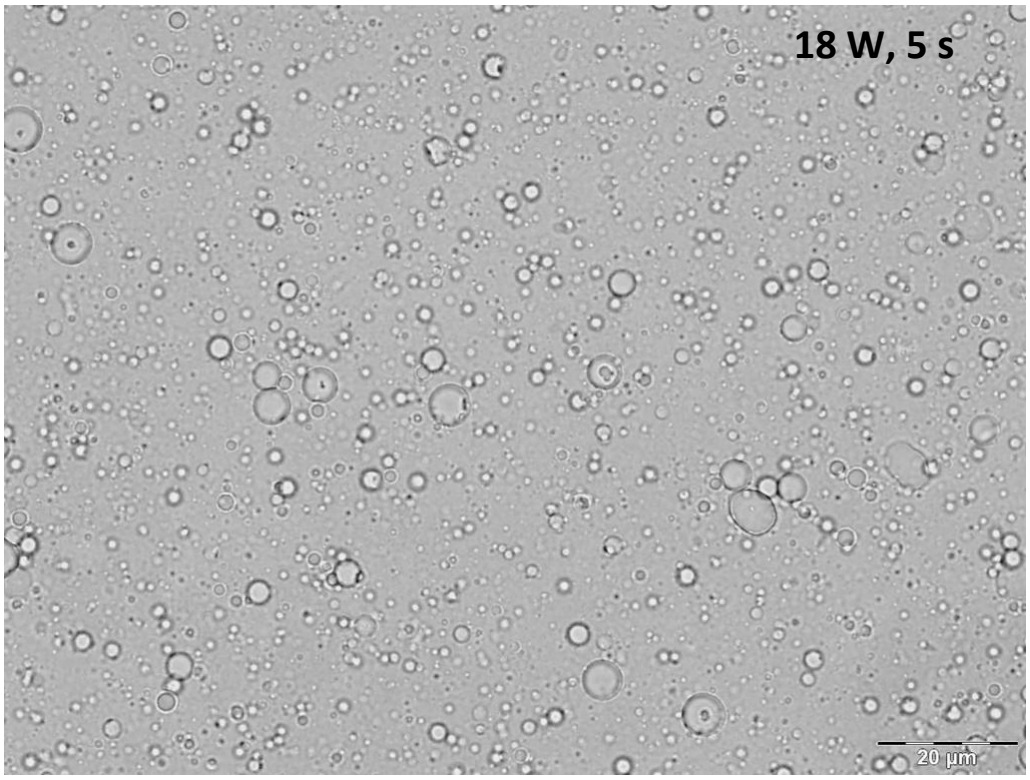

2W 5s
6W 5s
10W 5s
18W 5s
26W 5s
5 wt % loading
10 wt % loading
20 wt % loading
Volume %
Size (µm)
0
1
2
3
4
5
6
7
0.01
0.1
1
10
100
1000

Figure S3






10 W, 5 s
20 µm


18 W, 5 s
20 µm

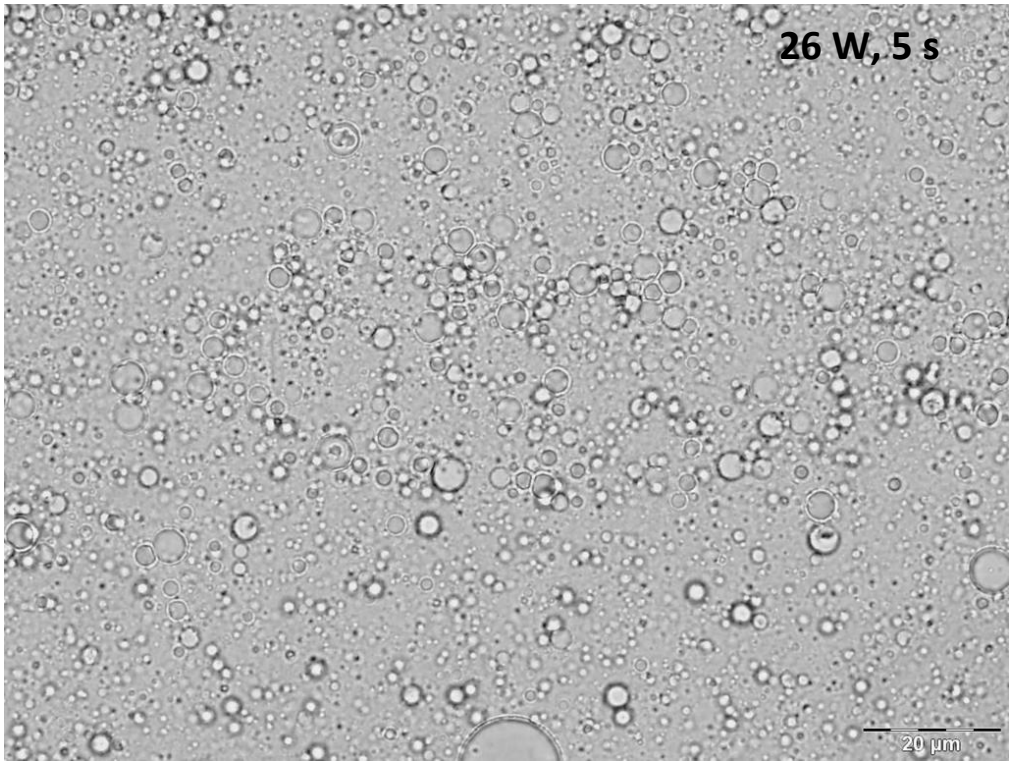

26 W, 5 s
20 µm

Figure S4

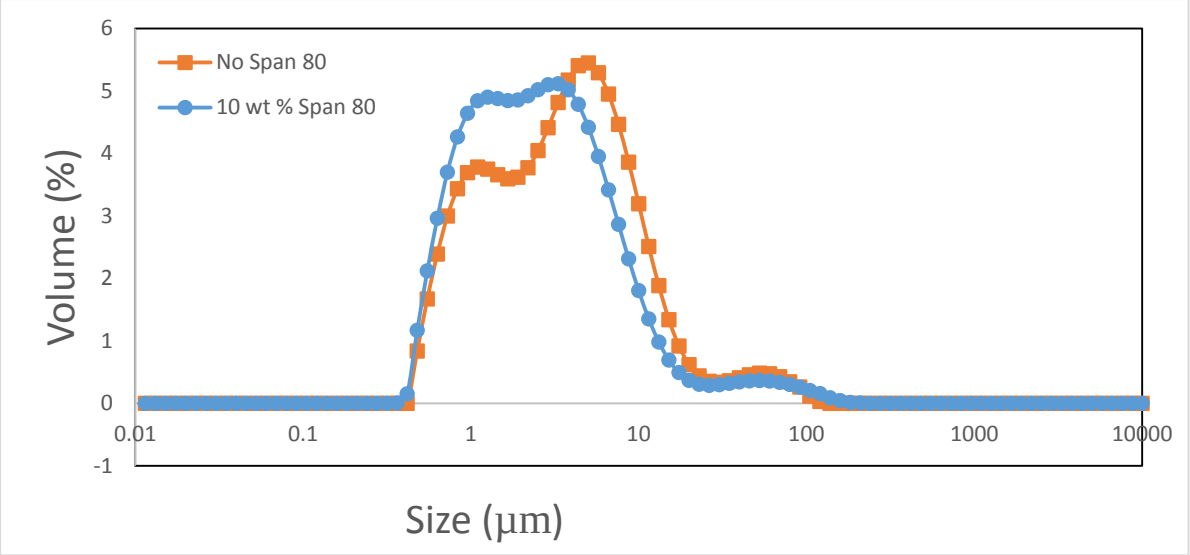
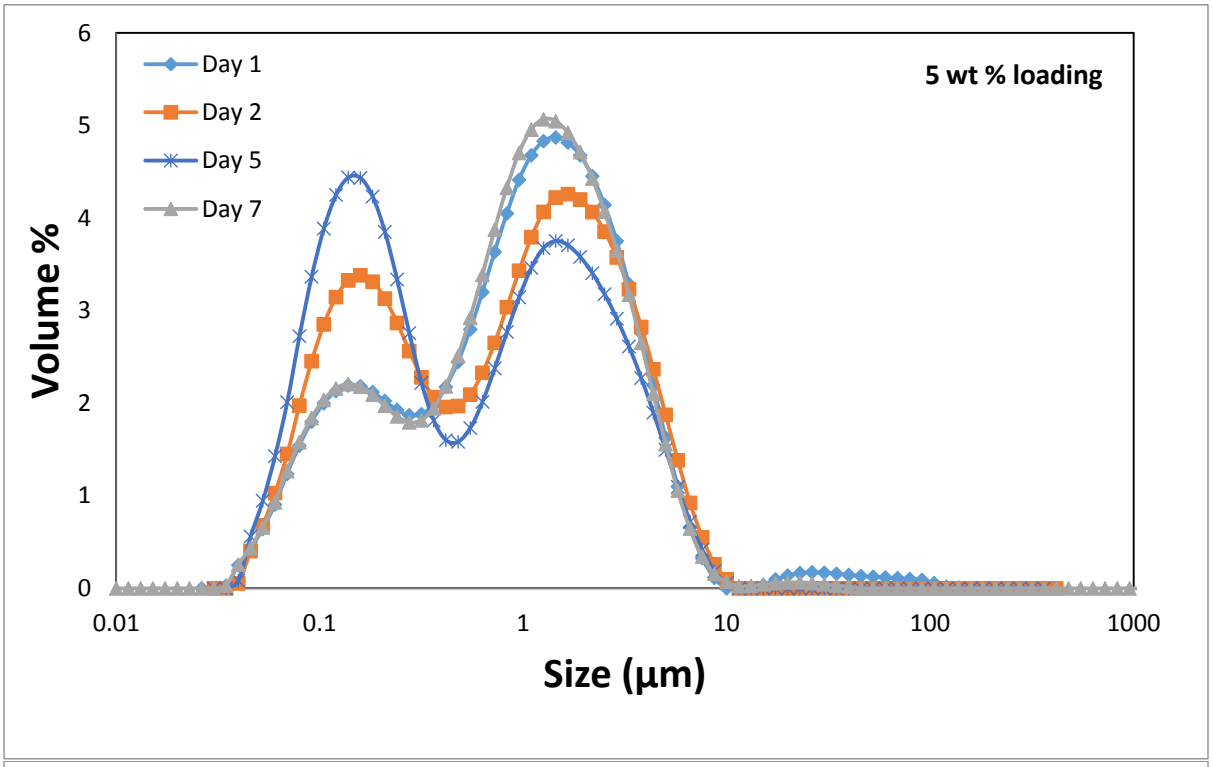
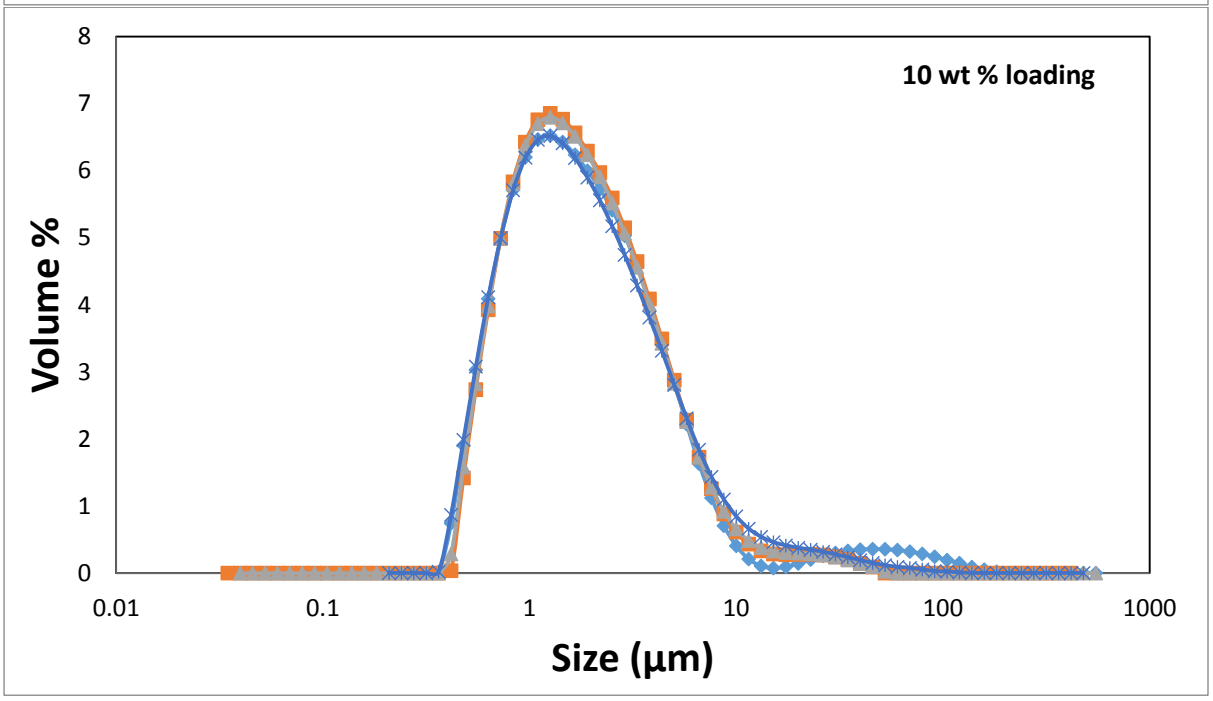
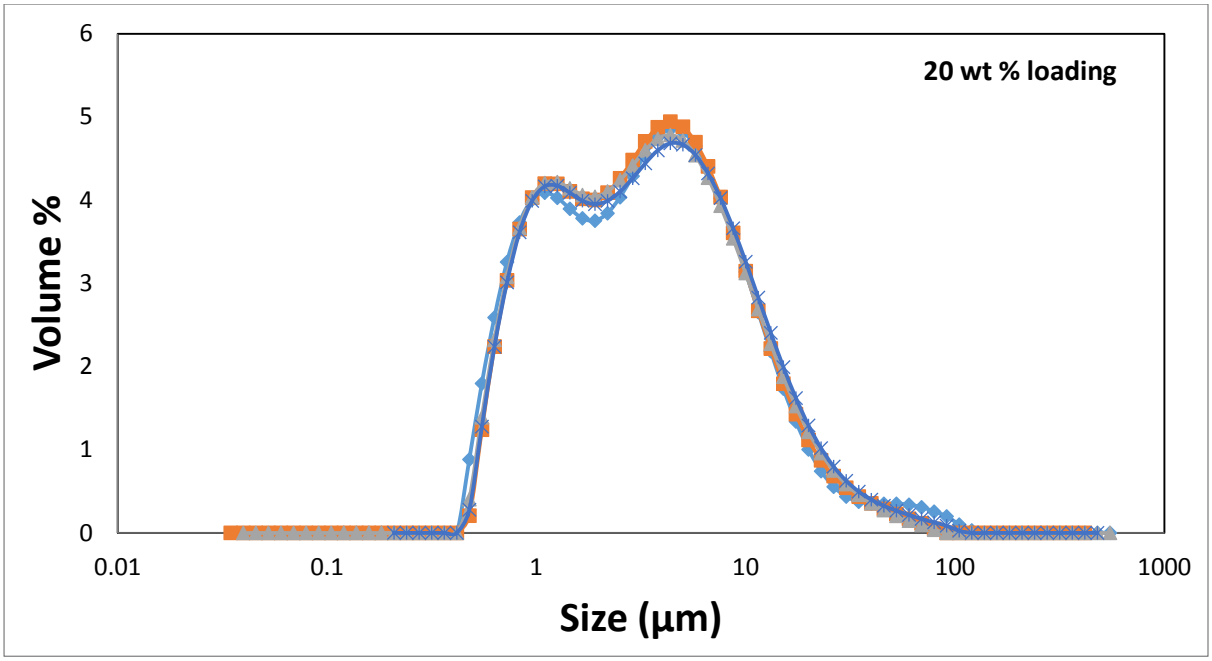

No Span 80
10 wt % Span 80
Volume (%)
6
5
4
3
2
1
0
-1
0.01
0.1
1
10
100
1000
10000
Size (µm)

Figure S5


Day 1
Day 2
Day 5
Day 7
5 wt % loading
Volume %
Size (µm)
0
1
2
3
4
5
6
0.01
0.1
1
10
100
1000


10 wt % loading
Volume %
Size (µm)
0
1
2
3
4
5
6
7
8
0.01
0.1
1
10
100
1000


20 wt % loading
Volume %
Size (µm)
0
1
2
3
4
5
6
0.01
0.1
1
10
100
1000

Highlights

- Water-in-oil-in-water double emulsions were made with skim milk and oil.
- Ultrasound (20 kHz) was used to create the inner and outer emulsion droplets.
- Entrapment of water varied with amount of ultrasonication and lipophilic surfactant.
- Milk proteins alone, in particular casein micelles, stabilised the outer oil droplets.
- Water-containing oil droplets of similar size to milk fat globules stable for 7 days.

*Graphical Abstract

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