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Design of novel emulsion microgel particles of tuneable size

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Abstract

In this study, we designed a one-step solvent-free route to prepare emulsion microgel particles, i.e., microgel particles containing several sub-micron sized emulsion droplets stabilised by heat-treated whey protein. The heat treatment conditions were optimized using aggregation kinetics via fluorimetry and dynamic light scattering. Emulsions were gelled and microgel particles were formed simultaneously via turbulent mixing with calcium ions using two specific processing routes (Extrusion and T-mixing). By varying the calcium ion concentration and mixing conditions, we identified the optimal parameters to tune the size and structure of the resultant emulsion microgel particles. Microscopy at various length scales (confocal laser scanning microscopy, scanning electron microscopy) and static light scattering measurements revealed a decrease in particle size (100 to 10 µm) with lower turbulent mixing time (ca. 4×10^{-4} s) and lower concentrations of calcium ions (0.1-0.02 M). Larger particle sizes (500-1000 µm) were achieved with an increase in the turbulent mixing time (ca. 2×10^{-2} s) and higher concentrations of calcium ions (1-1.4 M). Using gelation kinetics data (small deformation rheology) and theoretical considerations, creation of smaller sized emulsion microgel particles was explained by the increased flux of calcium ions to the denatured whey protein moieties coating the emulsion droplets, enabling faster gelation of the particle surfaces. These novel emulsion microgel particles of tuneable size formed as a result of complex interplay between calcium ion concentration, heat treatment of whey protein, gelation kinetics and mixing time, may find applications in food, pharmaceutical and personal care industries.

Keywords

Emulsion microgel particles; heat treated whey protein; encapsulation; cold gelation; turbulent mixing

1 Introduction

Lipophilic active molecules such as fat soluble vitamins, flavourings, fatty acids and essential oils pose challenges when incorporated into food, pharmaceuticals or other soft matter applications due to their partial or complete water insolubility. Besides oxidizing rapidly, most of these compounds are difficult to deliver in physiology and are generally only partially absorbed by the skin or via the gastrointestinal regime. Thus, their physiological activity is most often partly or fully lost before reaching the targeted physiological site (McClements, 2015). Consequently, there is a huge need to protect these lipophilic compounds from environmental degradation and tailor their release at particular biological sites (Sung, Xiao, Decker, & McClements, 2015). A wide range of technologies have been developed to encapsulate oil-soluble molecules, such as emulsions, emulsion gels, liposomes, micelles, nanoparticles, etc (McClements, 2011). Each of these has its own specific advantages and disadvantages in terms of degree of protection, delivery, cost, regulatory status, ease of use, biodegradability and biocompatibility (McClements & Li, 2010). Among these, emulsion microgel particles are vehicles that have not been explored as widely.

Emulsion microgel particles are a relatively new class of soft solids (Torres, Murray, & Sarkar, 2016). The particles have a similar structure to emulsion gels, although their physical characteristics and scales differ. In emulsion microgel particles, emulsion droplets are stabilised by an emulsifier and gelling agent that create a soft solid shell around several emulsion droplets, which are then incorporated into a continuous gel matrix (Ruffin, Schmit, Lafitte, Dollat, & Chambin, 2014; Zhang, Zhang, Decker, & McClements, 2015). This soft solid shell has been demonstrated to protect lipophilic compounds such as polyunsaturated fatty acids against oxidation (Augustin &

Sanguansri, 2012; Beaulieu, Savoie, Paquin, & Subirade, 2002; Velikov & Pelan, 2008). Additionally, the microgel particle allows swelling or de-swelling as a function of pH, ionic strength, temperature and enzymatic conditions via tuning the size and/or physicochemical properties (Ballauff & Lu, 2007; Wei, Li, & Ngai, 2016). Hence, these particles have great potential for site-dependent release of lipophilic active compounds in a range of food, pharmaceutical, personal care and other soft material applications (Ching, Bansal, & Bhandari, 2016).

Whey protein isolate (WPI) is widely accepted for research and commercial applications and its versatility as an emulsifier and gelling agent is well recognized (Sarkar, Murray, et al., 2016). Under heat-treatment WPI undergoes conformational changes, exposing its hydrophobic and sulfhydryl groups allowing irreversible aggregation and gel formation under specific conditions of protein concentration, ionic strength and temperature (Roefs & Peppelman, 2001). On addition of calcium (Ca^{2+}) ions, heat treated WPI (HT-WPI) undergoes further aggregation via Ca^{2+} cross-linking of the negatively charged carboxylic groups on the WPI. Protein-Ca^{2+}-protein complexes are formed, reducing the negative charge on the protein (Bryant & McClements, 2000; Hongsprabhas, Barbut, & Marangoni, 1999; Phan-Xuan, et al., 2014).

Several technologies have been developed for the production of WPI stabilised emulsion microgel particles. For instance, multistep emulsion-templating allows the formation of emulsion particles via $O_1/W/O_2$ emulsions (Sung, et al., 2015). The WPI aqueous phase of the $O_1/W/O_2$ emulsion is typically gelled through heat treatment, forming (O_1/W) WPI stabilised emulsion microgel particles suspended in an external oil phase (O_2). The oil phase is then washed away with the use of organic solvents. Although this generates microgel particles of controlled size: the multiple processing

steps causes the technique to be laborious; heat gelation renders it ineffective for the use of heat-sensitive compounds; the use of organic solvents limits its application in certain medical drugs and food products where biocompatibility is a key issue (Beaulieu, et al., 2002). An alternative multistep emulsion-templating method was designed by Egan, Jacquier, Rosenberg, and Rosenberg (2013). The aqueous WPI phase of the $O_1/W/O_2$ emulsion was gelled via a cold set technique. The external oil phase (O_2) was then washed away with surfactants rather than solvents. Although this technique allows the encapsulation of heat-sensitive compounds and does not require the use of solvents, the multiple processing required still causes this method to be time consuming and laborious plus excess surfactant may need to be removed. Extrusion technologies allowing cold external gelation of heat-treated WPI emulsion microgel particles have also been developed (Egan, et al., 2013). In this case, the heat-treated WPI stabilised emulsion was dropped into an ionic bath, allowing the gelation of the continuous phase, which entrapped oil droplets into microgel particles. Although this external gelation method was successful it produced large particles, of 1-2 mm in diameter, limiting their application in food systems. Other processing methods produce emulsion microgel particles by emulsifying the oil phase with WPI or sodium caseinate and gelling the emulsion into microgel particles with alginate or pectin (Ruffin, et al., 2014; Zhang, Zhang, & McClements, 2016). The use of several different biopolymers causes this technique to be not very cost effective. Also, thermodynamic incompatibility between the protein at the interface and the gelling biopolymer might result in uncontrolled release behaviour.

Thus, external gelation has considerable potential if it can be made facile, rapid and allow processing of clean emulsion microgel particles. Careful optimization of temperature, shear and WPI and Ca^{2+} concentration might also allow the tailoring of

the size of emulsion microgel particles. The objective of this study was to design and characterize HT-WPI emulsion microgel particles of tailored sizes and examine the complex interplay between whey protein concentration, Ca^{2+} concentration ($[Ca^{2+}]$) and turbulent mixing conditions.

Commercial whey protein isolate was heat treated at different temperatures and times and its unfolding and aggregation rate were monitored using a fluorescent probe method and dynamic light scattering, respectively. The gelation kinetics of HT-WPI stabilised emulsions with different concentrations of Ca^{2+} ions were examined using small deformation shear rheology. These rheological experiments showed the effect of $[Ca^{2+}]$ on the type of gels formed. Finally, two different turbulent mixing processing techniques involving extrusion or T-mixing were tested, hypothesized to offer different mixing times. The emulsion microgel particles were examined using confocal laser scanning microscopy and scanning electron microscopy. Theoretical considerations, such as the Kolmogorov mixing time and the flux of Ca^{2+} ions to HT-WPI interfaces were used to explain the differences in particle size of emulsion microgel particles, obtained with both processing routes.

2 Materials and Methods

2.1 Materials

Whey protein isolate (WPI) powder containing 96.3 wt% protein (Molecular mass: 18.4 kDa) was a kind gift from Fonterra Limited (Auckland, New Zealand). Sunflower oil was purchased from Morrisons supermarket (UK). Calcium chloride, 8–aniline–1–naphthalenesulfonic acid (ANS), sodium hydroxide, hydrochloric acid, sodium chloride, hexane anhydrous, 95% were purchased from Sigma-Aldrich (Gillingham, UK). Silicone oil 350 CST was purchased from VWR international S.A.S (Fontenay-

150 sous-Bois, France). All solutions were prepared with Milli-Q water having ionic purity
151 of 18.2 MΩ·cm at 25 °C (Milli-Q apparatus, Millipore, Bedford, UK). Nile Red was
152 purchased from Sigma-Aldrich (Steiheim, Germany). Dimethyl sulfoxide (DMSO) was
153 purchased from Fluorochem (Hadfield, UK). All other chemicals were of analytical
154 grade and purchased from Sigma-Aldrich unless otherwise specified.
155
156 2.2 Analysis of whey protein aggregation
157 2.2.1 ANS Fluorescence method
158 Different concentrations of WPI (9.6 and 12 wt%) were diluted into Milli-Q water
159 at pH 7. 8–aniline–1–naphthalenesulfonic acid (ANS) (1 mg mL^{-1}) were dissolved into
160 0.1 M NaCl. Spectrofluorimetric measurements were made using a Fluorescence
161 spectrophotometer (Perkin-Elmer, LS-3, Waltham, USA) following the method of
162 Nyman and Apenten (1997). The ANS fluorescence measurements involved a
163 fluorescence excitation wavelength of 280 nm and an emission wavelength of 470 nm.
164 The final concentration of ANS was determined by fluorescent titration of 12 wt% WPI
165 heated at 85 °C for 40 min. Increasing amounts of ANS stock solution were added to
166 WPI samples (3 mL) in a quartz cuvette. Fluorescence emission intensity (ΔF) was
167 recorded in relative fluorescence units (rfu). A graph of volume ANS (x-axis) vs ΔF
168 provided a value for the maximum volume of ANS needed (150 µL) as the curve
169 reached a plateau (result not shown). The concentration of ANS was determined using
170 equation (1):
171 $$[\mathrm{ANS}] = \frac{V_{ANS} \times C_{ANS}}{(V_{ANS} + V_{WPI})} \quad (1)$$
172 where, C_{ANS} is the concentration of ANS stock solution (3.2 mM), V_{WPI} is the
173 volume of protein and V_{ANS} is the volume of ANS added to the protein solution. This
174 final concentration of ANS (0.15 mM) was used for the subsequent measurement.

12 wt% and 9.6 wt% WPI solutions were heated at different temperatures (75, 80 or 85 °C) for different time periods (0, 8, 15, 30, 40, 50 min). Protein solutions were decanted into quartz cuvettes (3 mL) and ANS (150 µL) was then added to each sample. The fluorescence emission intensity of each sample was recorded at the stated temperature.

The data was analysed using the Scatchard eq (2),

$$\frac{LB}{LF} = \frac{nP}{Kd} - \frac{LB}{Kd} \quad (2)$$

where LB is the concentration of ANS bound to the protein, LF is the concentration of unbound ANS, n is the number of moles of ANS bound per mole of protein, P is the concentration of WPI and Kd is the dissociation constant for reaction: ANS + protein = complex.

The LB was determined from ΔF (the fluorescence measurements) using the conversion factor Q as given by eq (3),

$$LB = \Delta F/Q \quad (3)$$

The conversion factor Q was obtained following the method from Nyman, et al. (1997).

The LF was determined from $LF = [ANS] - LB$. The ratio LB/LF was then calculated and plotted against time using eq (4).

$$Relative\ \frac{LB}{LF} = \left(\frac{LB}{LF}\right)_t \Big/ \left(\frac{LB}{LF}\right)_f \quad (4)$$

where $(LB/LF)_t$ is LB/LF at different times and $(LB/LF)_f$ the final value of LB/LF.

All measurements were repeated three times and mean values are reported.

2.2.2. Particle size of protein aggregates

The aggregation rate of the aforementioned 12 wt% and 9.6 wt% WPI solutions were measured at the different time-temperature treatments using dynamic light scattering (Zetasizer, Nano ZS series, Malvern Instruments, Worcestershire, UK). Assuming WPI particles are spherical, the apparent particle diameter is inversely related to the diffusion coefficient (D) via the Stokes-Einstein equation (eq 5) :

$$d_H = \frac{k_b T}{3\pi\eta D} \quad (5)$$

where k_b is the Boltzmann constant, T is the temperature, η is the viscosity of the solution and d_H is the hydrodynamic diameter.

Sizing of WPI particles was conducted based on a relative refractive index of 1.150 (i.e. the ratio of the refractive index of WPI (1.53) to that of the aqueous phase at 1.33). The absorbance value of WPI particles was set at 0.001. Before analysis, samples were diluted to 0.1 wt% WPI with Milli-Q water and filtered through with a membrane of 0.45 μm (PTFE Syringe filters, Perkin Elmer, USA). One mL of solution was injected into a clean cuvette (PMMA, Brand Gmbh, Wertheim, Germany). Particle size was presented as mean hydrodynamic diameter of five readings on duplicate samples.

2.3 Preparation of heat denatured whey protein-stabilised emulsion

Whey protein isolate (12 wt%) was dissolved in Milli-Q water and gently stirred (500 rpm) for 2 h using a magnetic stirrer to allow complete protein hydration. The solution was adjusted to pH 7 using 0.1 M NaOH or HCl. The suspension was then heat treated at 85 °C for 40 min in a water bath and cooled in cold water (10 °C) for 2 h to create heat denatured WPI (HT-WPI).

Sunflower oil was subsequently mixed with the HT-WPI solutions. The ratio of the aqueous phase to lipid phase in the emulsion was 80:20 (w/w), with a final HT-WPI concentration of 9.6 wt%. This solution was pre-emulsified with a high speed rotor-stator mixer (Silverson, L5M-A, UK) at 8,000 rpm for 5 min. The pre-emulsion was further homogenized in a laboratory scale two-stage valve high pressure homogenizer at 250/50 bar with three passes (Panda Plus, GEA Niro Soave, Parma, Italy). Sodium azide (0.02 wt%) was added as an antimicrobial agent to the emulsion samples stored for 24 h at 4 °C.

2.4 Zeta-potential

The ζ-potential of the emulsion droplets was determined using a particle electrophoresis instrument (Zetasizer, Nano ZS series, Malvern Instruments, Worcestershire, UK). The emulsion was diluted to 0.005 wt% droplet concentration using MilliQ water. It was then added to a folded capillary cell (Model DTS 1070, Malvern Instruments Ltd., Worcestershire, UK). The ζ-potential of the emulsion was measured ten times for each diluted sample. The Smoluchowski approximation was used to calculate the ζ-potential. From Henry's equation ζ-potential can be calculated using the measured electrophoretic mobility of the oil droplets (eq 6):

$$U_{\mathrm{E}} = \frac{2\varepsilon z F(ka)}{3\eta} \quad (6)$$

where U_E is the measured electrophoretic mobility, z the ζ-potential, ε the dielectric constant of the medium, η the viscosity of the solution and F(ka) Henry's function using the Smoluchowski approximation, i.e., F(ka) = 1.5.

2.5 Preparation of emulsion microgel particles

Emulsion microgel particles were produced using two different bottom-up techniques via Ca^{2+}-mediated external gelation: 1. Buchi Encapsulator® or 2. the Leeds jet homogenizer. Table 1. illustrates the key processing conditions for both equipment and Figure 1 illustrates the formation method of emulsion microgel particles.

In the Buchi Encapsulator B-390®, the HT-WPI stabilised emulsion was dropped through a 150 μm vibrating nozzle into a turbulently stirred solution ($Re > 10^5$) of Ca^{2+} ions (1-1.4 M). The Encapsulator nozzle was set to oscillate at a frequency of approximately 260 Hz, with a drive current amplitude of 3 A and generating a differential pressure of 418 mPa. All solutions were at ambient temperature (25 °C) at the time of the experiment. Throughout the "extrusion" process and for 30 min thereafter, the aqueous Ca^{2+} solution was stirred at 500 rpm using a 3 cm magnetic stirrer. The microgel particles were then filtered and washed three times using Milli-Q water to remove residual Ca^{2+} and stored at 4 °C before characterization.

The second method involved the use of the Leeds Jet Homogenizer along the lines described by Pravinata, Akhtar, Bentley, Mahatnirunkul, and Murray (2016). Briefly, the Leeds Jet Homogenizer has two separate chambers of different ratios (45:55 w/w were used in this case) connected via a thin capillary tubing to an outlet via a pinhole (0.5 mm diameter in this work). Essentially, it is a T-mixer capable of

producing very high liquid velocities. A hydraulic ram pushes onto the pistons on top of both chambers forcing the liquids they contain through the pinhole at high velocity, generating highly turbulent conditions depending on the pressure applied (100-400 bar) (Casanova & Higuita, 2011). In this work, HT-WPI stabilised emulsion was added to one chamber and $CaCl_2$ solution (0.02-0.1 M) to the other chamber. A pressure of 250 bar was employed. The turbulent mixing resulted in the formation of emulsion microgel particles. The resulting particles were collected in a beaker and immediately diluted with Milli-Q water and stirred for 30 min at low speed to limit particle aggregation.

The Reynolds number of the Jet Homogenizer was calculated using eq (7):

$$Re = \rho v d/\eta \quad (7)$$

with ρ the solvent density (i.e. water), v the maximum fluid velocity, d the diameter of the nozzle used with the Jet Homogenizer, η the dynamic viscosity of the solution at 20 °C.

In the case of the Jet Homogenizer, the velocity was calculated using the mean velocity of a fluid in a pipe eq (8):

$$v = \frac{4q}{d^2\pi} \quad (8)$$

with q the volumetric flow rate and d the diameter of the nozzle.

In the case of the Encapsulator, the Reynolds number was calculate using the stirred vessel model eq (9):

$$Re = \frac{\rho n d^2}{\eta} \quad (9)$$

with n the rotational speed of the magnetic agitator and d the diameter of the magnetic agitator.

2.6 Small deformation rheology

The dynamic oscillatory viscoelasticity of the HT-WPI and HT-WPI stabilized emulsion gels formed at different [Ca^{2+}] were investigated at low strain and ambient temperature using a Kinexus Ultra, (Malvern Instruments) shear rheometer following the method from Sok, Remondetto, and Subirade (2005) for Ca^{2+}-induced cold gelation of whey protein. The gelation of the protein solution or protein stabilized emulsion were induced by adding different [Ca^{2+}] ions and vortexing the solutions at 23 °C. A 40 mm cone-and-plate geometry (model: CP4/40 SS017SS) was used for all the measurements. About 0.5 mL of sample (HT-WPI solution or HT-WPI-stabilized emulsion (20 wt% oil, 9.6 wt% HT-WPI)) was poured onto the sample plate and sealed with a thin layer of the 350 CS silicone oil to prevent evaporation.

The storage modulus (G') and the loss modulus (G'') were measured firstly on conducting a strain sweep between 0.01 and 100 % strain, at 1 Hz and 25 °C, to determine the linear viscoelastic region. The second test performed on the emulsion gel was the time sweep. This test was carried out in the linear viscoelastic region (0.5 % strain), 25 °C and 1 Hz. Three measurements were performed on individual samples for each of the aforementioned tests.

2.7 Particle size analysis of emulsion and emulsion microgel particles

Static light scattering was used to measure the particle size distribution of the emulsion droplets and emulsion microgel particles via a Malvern Mastersizer 3000E hydro, (Malvern Instruments, Worcestershire, UK). Samples were diluted in distilled water until the instrument gave an obscuration of 4-6%. Sizing of the emulsion oil droplets was conducted based on a relative refractive index of 1.097 (i.e. the ratio of the refractive index of sunflower oil at 1.460 to that of the aqueous phase at 1.33). The absorbance value of the emulsion droplets was set to 0.001. Sizing of the emulsion

microgel particles formed with Leeds Jet homogenizer was conducted based on a relative refractive index of 1.150 (i.e., the ratio of the refractive index of WPI at 1.53 to that of the aqueous phase at 1.33). The absorbance value of the emulsion microgel particles was similarly set to 0.001.

Emulsion microgel particles formed using the Buchi Encapsulator B-390® were sized using image analysis of the digitized images captured via a Nikon SMZ-2T (Nikon, Japan) optical microscope, due to their larger sizes (> 500 µm). For comparison of particle size distributions the Sauter mean diameter ($d_{32} = \frac{\sum n_i d_i^3}{\sum n_i d_i^2}$) and the De Brouckere mean diameter ($d_{43} = \frac{\sum n_i d_i^4}{\sum n_i d_i^3}$) were calculated. Each sample was analysed ten times and the averages and standard deviations are reported.

2.8 Microscopy

All emulsion microgel particles were imaged at various length scales via optical microscopy (Nikon, SMZ-2T, Japan), confocal laser scanning microscopy (CLSM) or scanning electron microscopy (SEM).

A Zeiss LSM 700 confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany) with a 10-40× magnification was used. Nile Red (1 mg mL^{-1} in dimethyl sulfoxide, 1:100 v/v) was used to stain oil (argon laser with an excitation line at 488 nm) and Rhodamine B (0.5 mg mL^{-1} in Milli-Q water, 1:100 v/v) was used to stain proteins (argon laser with an excitation line at 568 nm). The microgel particles were mixed with 10 μL of Nile Red (0.1% w/v) and 10 μL of Rhodamine B, stirred for 15 min and placed onto a microscope slide and covered with a cover slip before imaging.

A scanning electron microscope (JEOL 6390 A, JEOL, Japan) was also used to study the structural features of some particles modifying the method of Sarkar, Arfsten,

Golay, Acquistapace, and Heinrich (2016). The emulsion microgel particles were dried in an oven at 37 °C for 72 h and subsequently washed with hexane removing all oil droplets. After removal of the oil, the intact or deliberately fractured particles were mounted onto a chrome coated steel plate with carbon double sided-tape and sputter coated with gold using a JEOL JFC-1600 Auto Fine Coater (JEOL Japan) for 200 s at 30 mA. The SEM images were then obtained at 10-20 kV.

2.9 Statistical analysis

Significant differences between samples were determined by one-way ANOVA and multiple comparison test with Tukey's adjustment performed using SPSS software (IBM, SPSS statistics, version 24) and the level of confidence was 95%.

3 Results and discussion

3.1 Denaturation and aggregation kinetics of HT-WPI solution

ANS fluorescence was used to examine the changes in hydrophobicity of WPI at different heat-treatments, since ANS fluorescence intensity increases when bound to nonpolar hydrophobic groups (Jeyarajah & Allen, 1994). WPI contains globular proteins with their hydrophobic and sulfhydryl groups tending to be buried in the interior of the protein structure. However, during heat-treatment, the WPI proteins unfold, exposing and activating their hydrophobic and sulfhydryl groups towards the outer surface of the protein (Torres, et al., 2016). Therefore, ANS fluorescence can be used to understand the extent to which WPI unfolds at different temperatures and times, initiating aggregation and subsequent gelation (Das & Kinsella, 1990; Kim, Cornec, & Narsimhan, 2005; Nyman, et al., 1997). The temperature at which WPI was heated had a significant effect on the unfolding rate of the protein, regardless of the protein

concentration. It can be observed from Figure 2A that on increasing the temperature by 10 °C (from 75 °C to 85 °C), the relative LB/LF ratio reached a plateau 25 min earlier, irrespective of WPI concentration. The faster unfolding of WPI with increase temperature has also been noticed by Das and Kinsella (1990). In the case of 9.6 wt% WPI, LB/LF reached a plateau at 85 °C after 15 min: approximately 87% ANS was observed to be bound to HT-WPI (Figure 2B). Consequently, it is suggested that after 15 min at 85 °C, no more hydrophobic groups are available for ANS to bind to resulting in almost total unfolding of WPI, in agreement with previous studies (Kim, et al., 2005). In comparison, at the lower temperature of 75 °C, LB/LF reached a plateau only after a longer exposure time of 40 min with 76% ANS bound to WPI (Figure 2A and B). Thus, at 75 °C, the temperature was not high enough to unfold and denature the WPI fully. These results are in agreement with previous studies in the literature (Ruffin, et al., 2014; Wolz & Kulozik, 2015) as well as circular dichroism results (see Supplementary Figure S1).

The concentration of WPI also affected its denaturation and aggregation rate. As shown in Figure 2B, lower WPI concentrations reached a higher LB/LF ratio at any given time and temperature. For instance, 9.6 wt% WPI heat-treated at 80 °C for 30 min had 93% ANS bound whereas, 12 wt% WPI heat-treated at 80 °C for 30 min only had 68% ANS bound. However, the ANS fluorescence method holds limitations. Under prolonged heat treatment WPI aggregates promptly, re-burying the exposed hydrophobic groups which might become inaccessible to ANS. This might reduce the fluorescence intensity of the sample. For that reason, dynamic light scattering and circular dichroism results have been analysed in parallel to the ANS fluorescence measurements.

Analysing ANS results in connection with the aggregation rate of WPI at different times and temperature (Figure 3) highlighted that at higher concentrations, HT-WPI aggregated more easily (Marangoni, Barbut, McGauley, Marcone, & Narine, 2000; Wolz, et al., 2015). As can be observed from dynamic light scattering results, before heat treatment the particle sizes at 12 wt% and 9.6 wt% WPI were similar, i.e. 181 nm and 189 nm, respectively, clearly larger in size than the native constituent proteins of WPI. Eight min after heat treatment, the particle size at both concentrations decreased by approximately 75%. Such a decrease has also been noticed by previous authors (Ju & Kilara, 1998). At high concentration, WPI probably forms oligomers in solution prior to heating, due to its reduced solubility, increasing its particles size. With increasing temperature (> 60 °C), the solubility of the WPI aggregates is likely to increase, allowing the dissociation of these oligomers into dimers and monomers which increases WPI flexibility and mobility as well as decreases the size of the aggregates (Wijayanti, Bansal, & Deeth, 2014; Zúñiga, Tolkach, Kulozik, & Aguilera, 2010).

Interestingly, for 12 wt% at 75 and 80 °C, the particle size after 8 min only decreased by approximately 60% (from 189 nm to 78 and 75 nm, respectively), whereas at 85 °C the particle size decreased by 75%. At high WPI concentration (i.e., 12 wt%), a further 7 min at 80 °C were necessary to break down the oligomers into monomers and reduce WPI particle size by 75%. These results are in agreement with previous studies conducted by Das, et al. (1990). After 15 min of heat treatment, HT-WPI particle size slightly increased. For instance, at 85 °C, 9.6 wt% WPI particles size at 8 min measured 43 nm and after 15 min these measured 48 nm (Figure 3). As previously discussed, 87% ANS was found to be bound to HT-WPI after 15 min at 85 °C, suggesting almost total unfolding. This slight increase in particle size might therefore be explained by the exposure of the hydrophobic groups of the protein upon unfolding

421 which might lead to protein-protein interactions (Beaulieu, et al., 2002; Iametti, Cairoli,
422 De Gregori, & Bonomi, 1995; Jeyarajah, et al., 1994), reinforced by subsequent
423 disulphide and other types of cross-linking.

424 The concentration of WPI also affected the size of the HT-WPI aggregates. For
425 instance, at 75 °C after 30 min, the particle size of 12 wt% WPI was 35% higher than
426 for 9.6 wt%. This is probably explained by the fact that at higher WPI concentrations,
427 the chances of hydrophobic and sulfhydryl groups from one protein colliding with
428 groups of neighbouring proteins increases, resulting in larger sized particles at all
429 heating times (Barbut & Foegeding, 1993; Hongsprabhas & Barbut, 1997; Ju, et al.,
430 1998). Other non-covalent physical interactions, such as van der Waals attraction,
431 hydrogen bonds and electrostatic attraction, contribute to a lesser extent to the
432 aggregation of HT-WPI during heat-treatment (Roefs & Peppelman, 2001). Therefore,
433 at 12 wt% WPI, HT-WPI might have aggregated completely after 15 min, concealing
434 its hydrophobic and sulfhydryl groups on the inner surface of the protein. These buried
435 hydrophobic groups would be inaccessible to ANS, leading to lower LB/LF ratios as
436 compared to 9.6 wt% WPI (Iametti, et al., 1995). These results suggested that the
437 formation of cold set emulsion microgel particles would only occur if the initial
438 concentration of WPI was high enough and the WPI was largely unfolded and
439 aggregated, allowing spontaneous gelation when contacting Ca^{2+} ions. Based on the
440 above results, further experiments were conducted with an initial concentration of 12
441 wt% WPI heat-treated at 85 °C for 40 min.

442

443 3.2 Droplet size of HT-WPI stabilised emulsions

444 Figure 4 shows the droplet size distribution of the 20 wt% sunflower oil emulsion
445 stabilised by 9.6 wt% HT-WPI. Droplet sizes ranged from 0.1 to 10 μm as expected

from many other studies. The CLSM image (Figure 4) confirms this, showing a uniform size distribution of oil droplets. Additionally, the droplet size distribution was monomodal, narrow and symmetric, suggesting that the emulsion was well homogenized and stable.

The emulsion droplets were not flocculated during the homogenization stage, as confirmed by the d_{43} value, which was below 0.5 µm and were anionic (-43 mV) as expected at pH 7.

3.3 Rheological properties of cold-set HT-WPI emulsion gels

The gelation of HT-WPI solutions and emulsions was induced by the addition of Ca^{2+} ions at different concentrations. Figure 5 shows the storage modulus of the emulsion gels or protein gels (without oil droplets) at different concentrations of Ca^{2+} ions as a function of time and strain. For all systems, G' was significantly greater than G'' ($p < 0.05$), with $\tan \delta < 0.3$, which implied that the gels had an elastic behaviour. Therefore, in the following, only results for G' are presented and discussed.

In comparison to cold set HT-WPI protein gels (without oil droplets), cold set HT-WPI emulsion gels were nearly two orders of magnitude stronger (Figure 5A insert). Since the size of the oil droplets was on average 0.1 µm, the interfacial tension and Laplace pressure means that these droplets can be considered as solid particles (van Vliet, 1988) effectively. Additionally, the HT-WPI adsorbed at the surface of oil droplets may be considered as physically and chemically bound to the HT-WPI in the matrix, via electrostatic and hydrophobic interactions as well as hydrogen bonds. Hence, the oil droplets acted as "active" or "bound" fillers (Torres, et al., 2016), increasing the strength of the gel.

As observed in Figure 5A, all cold set emulsion gels had similar rheological behaviour irrespective of the [Ca^{2+}] (0.02 to 1.4 M). On addition of Ca^{2+} ions, the emulsions gelled instantaneously, as shown by the storage modulus being above 3 kPa at time zero. Over time, all four emulsion gels became slightly stronger: after 1h 40 min, G' of all emulsion gels increased on average by 50%. This might be attributed to a gradual increase in the number density of Ca^{2+}-protein interactions (Marangoni, et al., 2000). Understanding the structure of the emulsion gels with regard to varying [Ca^{2+}] might give valuable insight on the mechanical strength of the emulsion gels. The rubber elasticity theory modified by Flory (Betz, Hormansperger, Fuchs, & Kulozik, 2012; Flory, 1953) for polymers allows a simplistic analysis of the structure of viscoelastic material via their elastic mechanical behaviour. For small deformations (< 2%), the emulsion gels fully recovered to their original dimension in a prompt manner (Peppas, Bures, Leobandung, & Ichikawa, 2000) implying that these emulsion gels were almost perfectly elastic. Therefore, it was of interest to express the results in terms of the theoretical mesh size. The average mesh (or pore) size (ξ) of a cross-linked network is defined as the distance between two crosslinks or macromolecular chains (Peppas, et al., 2000; Sarkar, et al., 2015) and can be calculated using eq (10):

$$\xi^3 = \frac{\kappa_B T}{G'} \qquad (10)$$

where κ_B is the Boltzmann constant, T is the temperature and *G'* the storage modulus. Table 2 highlights the impact of [Ca^{2+}] on the storage modulus and mesh size of the cold set emulsion gels. For instance, 0.1 M Ca^{2+} ions significantly produced the strongest gel (G' = 18.2 kPa) and therefore the smallest calculated mesh size (6.1 nm), whereas 0.02, 1 and 1.4 M Ca^{2+} ions produced the weakest gels (G' = 8.8, 10.6 and 5.7 kPa, respectively), during a corresponding time period of 1 h 40 min. Thus, as expected from eq. (10) and the values of G', calcium plays an important role in the type and

strength of gels formed. Above and below 0.1 M Ca^{2+} values of G' suggest coarser and more porous structures weakening the emulsion gel strength. However, the calculated mesh sizes of all the emulsion gels were nearly an order of magnitude smaller than the oil droplets size (> 80 nm), suggesting the droplets would probably not be able to diffuse out of the gel matrix and further explaining their action as "active" fillers. The chances of them leaking out during the emulsion microgel particles formation is also minimized although possible as cross-linking of the WPI network is not fully complete (Table 2). Emulsion gels produced with 0.02 M Ca^{2+} had gel strengths similar to those formed with 1 M and 1.4 M Ca^{2+}. As explained by several authors, Ca^{2+} ions cross-link with negatively charged carboxylic groups on WPI via electrostatic interactions (Phan-Xuan, et al., 2014). Understanding the minimum concentration of Ca^{2+} required to bind to every free carboxylic groups on WPI may provide further insight into the HT-WPI emulsion gelation. Assuming all the WPI consists of *β*-lactoglobulin molecules, theoretically, this minimum $[Ca^{2+}]$ can be calculating from eq 11:

$$[Ca^{2+}] = \frac{n(COO^{-})m(WPI)_i}{Mw}\frac{1}{2V} \quad (11)$$

where $n(COO^-)$ is the number of free carboxylic groups per *β*-lactoglobulin molecule, $m(WPI)_i$ is the mass of WPI, Mw is the molecular weight of *β*-lactoglobulin and V is the solution volume. In this study, the molecular weight of one *β*-lactoglobulin monomer (18.3 kDa) containing 28 free carboxylic groups (Alexander, et al., 1989) was used, since on heat treatment above 60 °C, *β*-lactoglobulin dimers dissociate into monomers (Zúñiga, et al., 2010). Note that this calculation assumes that all COO^- groups were available for binding, which clearly is an over estimate since some carboxylic groups may still be hidden within the protein structure and unavailable for

binding. From previous studies, the HT-WPI monolayer surface coverage (Das, et al., 1990; Dickinson, 1998) of droplets was estimated at 3 mg/m^2. Therefore, in this study, assuming that the total surface area of the 20 wt% oil emulsion was 1203 m^2 (calculated from the particle size distribution), we calculated that this surface was covered by 3.9 g of HT-WPI.

From eq (11), we then calculated that the minimum [Ca^{2+}] required to bind to all COO^- groups on the β-lactoglobulin molecules absorbed at the oil/water interface would be 0.03 M. On this basis, for the systems gelled at 0.02 M Ca^{2+}, there was not enough Ca^{2+} and this insufficient amount led to slower gelation kinetics of HT-WPI, as well as the formation of a weaker emulsion gel (G' = 8.8 kPa). For systems gelled at 0.1 M Ca^{2+} and above, there would clearly be a significant excess of Ca^{2+} and bridging flocculation might have led to more coarse, porous and non-continuous aggregates, especially for emulsion gels produced at high [Ca^{2+}] such as 1 and 1.4M. These coarser non-continuous aggregates would allow the disruption of the protein network reducing the emulsion gel strength, as seen with the theoretical mesh size calculations (Beaulieu, et al., 2002; Sok, et al., 2005; Westerik, Scholten, & Corredig, 2015).

Figure 5B demonstrates that all emulsion gels tested (0.02-1.4 M Ca^{2+}) had a similar linear viscoelastic region, ranging from 0.1-2.0% shear strain. With increasing strain, emulsion gels became weaker and their storage modulus decreased dramatically. Oil droplets probably acted as weakening points at larger strain (> 10%), allowing the gels to collapse. These results are in accordance with previous studies (Chen & Dickinson, 1999; Dickinson, 2000). Additionally, the concentration of Ca^{2+} ions involved in the emulsion gel formation influenced their behaviour under small deformation. At low [Ca^{2+}] (0.02 and 0.1 M), the structure of the gel was probably more fine stranded (Hongsprabhas, Barbut, & Marangoni, 1999) and able to absorb the energy applied

during shearing, as previously described by Dickinson (2000). For instance, at 0.02 M Ca^{2+} the theoretical initial mesh size is similar to the mesh size at 10% strain (Table 2) and the emulsion gel did not break down (G' = 7.3 kPa at 10% strain). Above this [Ca^{2+}], the emulsion gels broke down readily above 10% strain (G' < 5 kPa). The theoretical mesh size of emulsion gels formed above 0.02 M Ca^{2+} doubled after 10% strain. For instance, the theoretical mesh size of emulsion gels formed at 1.4 M Ca^{2+} ions increased from 9.2 to 20.3 nm. Clearly, this emulsion gel was significantly weaker and less elastic and this could possibly be explained by its higher porosity. In coarser aggregates, zones of higher densities of cross-links act as crack initiators and increase the brittleness of gels (Kuhn, Cavallieri, & Da Cunha, 2010).

3.4 Design of size-tuneable HT-WPI emulsion microgel particles

Two processing methods were used to form different sized and shaped emulsion microgel particles (Figure 6). The first method involved turbulent mixing of the emulsion and Ca^{2+} ions solution via the Leeds Jet Homogenizer at 250 bar and nozzle size 500 µm (Figure 6A). Low concentrations of Ca^{2+} ions (0.02 to 0.1 M) were chosen to create emulsion microgel particles due to the fact that at higher concentrations the gelation happened too quickly, blocking the homogenizer and nozzle. The Leeds Jet homogenizer produced small (around 20 µm) but highly aggregated microgel particles (Figure 6A1). Some oil droplets could also be seen coating the surface of the particles due to the short residence time (Figure 6A2). However, most oil droplets (in red) appeared to be entrapped within the HT-WPI matrix (Figure 6A2) as is emphasized with Figure 6A3, where the protein matrix is in green and the oil droplets are in black. A statistical analysis of the amount of oil found at the surface of the emulsion microgel particles was carried out on Figure A2 using ImageJ software (version 1.48r, National

570 Institute of Health, Bethesda, USA). A colour threshold was applied to segregate oil
571 droplets found at the surface of the particles from oil droplets encapsulated inside the
572 particles and particle analysis was conducted. The number of surface oil droplets, their
573 area and diameter was determined as well as the area of the emulsion microgel particles.
574 The total area represented by the surface oil droplets was only 9,100 μm^2 or 9% of the
575 total area (98,900 μm^2) of the emulsion microgel particles. Although this is purely a 2-
576 dimensional analysis, through a 'cut' across the sample, it suggests that the majority of
577 the oil droplets were effectively incorporated inside the emulsion microgel particles.
578 Further measurements should be conducted for more accurate characterization of the
579 efficiency of emulsion encapsulation. It should also be noted that the oil droplets
580 observed at the surface of the particles tended to be significantly larger (around 4 μm)
581 than the majority of the emulsion droplets entrapped – which appeared to have retained
582 the original mean size (around 0.1 µm) prior to microgel particle formation (Figure
583 6A3). Therefore, it may also be concluded that the formation process did not lead to
584 significant destabilisation and coalescence of the emulsion droplets.

585 The second processing method involved extrusion of the emulsion via the Buchi
586 Encapsulator® at low pressure (0.4 bar) with the smaller vibrating nozzle size (150 µm),
587 as well as turbulent mixing of the Ca^{2+} ions solution (500 rpm stirrer speed; Re = 4.7
588 $\times 10^5$) (Figure 6B). High concentrations of Ca^{2+} ions (1-1.4 M) were required for this
589 method, because at lower concentrations diffusion of Ca^{2+} to the droplets of HT-WPI
590 was not fast enough to produce gelation of the droplets into coherent particles. The
591 Encapsulator method produced large polyhedral particles (< 1000 µm) with a high
592 internal oil volume fraction (Figure 6B2). The protein network produced was well
593 defined (Figure 6B3) with no presence of surface oil. Dark spherical areas of around 10
594 µm can be observed on Figure 6B3 which might suggest minor artifacts, since none can

595 be depicting on Figure 6B2. The encapsulated oil was around 0.1 µm suggesting
596 effective encapsulation of the oil droplets.

597 More quantitative particle sizing was performed via static light scattering
598 (Figure 7A) and image analysis (Figure 7B). Figure 7A shows the emulsion microgel
599 particle size distribution formed with the Leeds Jet Homogenizer. The particle size
600 distribution was bimodal. In presence of 0.02 M Ca^{2+} ions, the first peak was
601 approximately in the same region as the emulsion oil droplets (0.1 to 1 µm), suggesting
602 that some emulsion droplets had not been incorporated into microgel particles. Second
603 and third peaks indicated particles in a higher size range (100 to 3000 µm). The ratio
604 between d_{32} and d_{43} at 0.02 M Ca^{2+} ions, suggested that most of particles were
605 aggregated and confocal microscopy confirmed the highly aggregated nature of the
606 sample (Figure 8A). As discussed previously, the minimum [Ca^{2+}] required to bind to
607 every free carboxylic group on HT-WPI adsorbed to oil droplets was $[Ca^{2+}]_{min}$ = 0.03
608 M.

609 Increasing the concentration of Ca^{2+} ions to 0.1 M led to smaller microgel
610 particles with an 80% reduction in mean d_{43} value (306 μm). The first peak of the
611 particle size distribution then shifted to 1 to 30 µm (Figure 7A). This suggested that
612 emulsion droplets that were not encapsulated into the emulsion microgel particles at
613 0.02 M Ca^{2+} ions were now immobilized into small microgel particles. Interestingly, it
614 can be observed in Figure 8B that some oil droplets (black) were individually stabilized
615 by a layer of HT-WPI aggregates (green), forming particles of approximately 2 µm
616 diameter. These singly encapsulated oil droplets can be compared to Pickering
617 emulsions stabilized by whey protein microgels (Sarkar, Murray, et al., 2016). The
618 second peak of the size distribution in the case of 0.1 M Ca^{2+} ions was approximately
619 in the same region as the second peak for particles formed with 0.02 M Ca^{2+} ions,

suggesting that some microgel particles remained aggregated. Previous experiments have reported such aggregation when using T-mixing devices (Casanova, et al., 2011). The highly turbulent mixing processes generated in T-mixers can lead to the precipitation of the emulsion and Ca^{2+} ions. This precipitation has been demonstrated to reduce particle surface charge, increasing electrostatic attraction and aggregation before gelation of the particles can be completed (Casanova, et al., 2011).

In comparison, emulsion microgel particles formed via the Encapsulator had a monomodal size distribution - though they were much larger - from 0.5 to 1 mm (Figure 7B). The emulsion microgel particles produced at higher concentrations of Ca^{2+} (1.4 M) were 10% larger compared to those formed at 1 M (Figure 6B1). As previously demonstrated by (Jeyarajah, et al., 1994), the addition of salt to heat-treated WPI solution increases the hydrophobicity of the protein as well as its reactive SH content. SH groups found in proximity of Ca^{2+} ion cross-bridges might form additional covalent bonds more easily, strengthening the aggregation of WPI (Jeyarajah, et al., 1994). Therefore, increasing the concentration from 1 to 1.4 M may enhance various protein-protein interactions resulting in further aggregation and larger particle sizes.

The SEM imaging allowed further understanding of the structure of the emulsion microgel particles as well as the oil distribution inside the particles. Preparation of the emulsion microgel particles for SEM resulted in some shrinkage of the particles. Prior to drying and washing, the particle size was between 0.5 to 1 mm. Upon drying the particle size seem to have reduced by 50% (Figure 9A). However, no surface indentations could be noticed suggesting that drying did not induce uneven shrinkage of the particles. Therefore, particles retained their initial internal structure upon drying (Rosenberg & Lee, 2004). Figure 9A shows the smooth exterior surface of an emulsion microgel particle produced with the Encapsulator. Small spherical voids

could be found at the exterior surface which could be attributed to air bubbles entrapped at the surface prior to drying. The top of the particle was fractured to observe the interior distribution of the emulsion microgel particle. All oil droplets associated with the oil droplets within the microgel particle had been previously washed away with hexane. Figure 9B shows the protein network (white) around the hollow pockets where the oil droplets previously resided (darker colour) (as observed by Beaulieu, et al., 2002; Chen, et al., 1999). The white protein layer noticed around the hollow pockets suggested that the oil droplets were physically bound to the WPI gel matrix, confirming the rheological results (Rosenberg, et al., 2004). The micrographs also indicated that the oil droplets were evenly distributed throughout the WPI matrix. Some hollows had been distorted and did not retain their spherical shape upon drying of the particles. However, the sizes of the hollows were in the same size range of the original emulsion droplets (0.1 to 1 µm). These observations confirm very little oil droplet coalescence occurred during processing and hollows were left by oil droplets rather than pores of the protein gel (previously estimated at 7.9 nm).

In summary, the two methods produced different sized and shaped emulsion microgel particles. The Leeds Jet Homogenizer produced aggregated, but smaller (around 20 µm), particles whereas Buchi Encapsulator formed well defined emulsion microgel particles but of a much larger size (around 900 µm). In order to fully understand the reasons for the microstructural differences between the two systems, several theoretical aspects were considered regarding particle formation, such as pressure, flow velocity, Reynold number and [Ca^{2+}].

The Leeds Jet Homogenizer is effectively a T-mixer in which the HT-WPI emulsion comes into contact with Ca^{2+} ions in a turbulent flow ($Re > 10^5$). The Buchi Encapsulator involved the extrusion of the HT-WPI stabilised emulsions through a

nozzle at a transitional flow (Re ≈ 4 ×10^3) into a Ca^{2+} ions bath. However, the bath had stirring which provided turbulence (Re > 10^5). In the latter, since the gelation of the HT-WPI emulsion occurred as soon as the HT-WPI came into contact with Ca^{2+} ions, the flow influencing the particle size was assumed to be the shear rate in the Encapsulator bath. Thus, both systems effectively had turbulent flow, though their mixing dynamics differed significantly. We calculated theoretical mixing time in both methods using Kolmogorov (Kolmogorov, 1991; Peters, et al., 2016) microscale theory of energy dissipation. Kolmogorov theory defines the mixing time shown by eq (12):

$$t_{mix} = \left(\frac{v}{\varepsilon}\right)^{\frac{1}{2}} \quad (12)$$

where v is the kinematic viscosity of the solution and ε is the energy dissipation.

The emulsion behaved as a non-Newtonian shear-thinning fluid and its viscosity was estimated at the shear rate of the Jet Homogenizer and the Encapsulator. The shear rate of both instruments was defined by $\dot{\gamma} = 8v/d$ where v is the velocity of the emulsion and d the diameter of the nozzle. The energy dissipation produced by the Leeds Jet Homogenizer at 250 bar has been previously calculated (Casanova, et al., 2011) and was found to be ε = 3.1 ×106 W kg-1. Following eq 10, the corresponding mixing time was 4 ×10^{-4} s.

Regarding the Encapsulator, the energy dissipation was calculated following models developed for stirrer tanks using an impeller (Hortsch & Weuster-Botz, 2010; Sánchez Pérez, Rodríguez Porcel, Casas López, Fernández Sevilla, & Chisti, 2006; Villermaux & Falk, 1994):

$$\varepsilon = \frac{P}{V} \quad (13)$$

where V the solution volume and P is the power input given by eq (14):

$$P = N_p \rho N^3 d^5 \quad (14)$$

where, N_p is the power number, ρ the density of the solution (kg m^{-3}), N the agitation speed (min^{-1}) and d the diameter of the stir bar (m).

The energy dissipation produced by the Encapsulator was thus calculated as 4.8 $\times 10^4$ W kg^{-1}, where the power number had previously been reported (James R. Couper, 2005) for Reynolds numbers of the same order of magnitude ($N_p = 4$). Following eq 12, the mixing time in the Encapsulator was therefore 2,6 $\times 10^{-2}$ s. Consequently, it is proposed that the mixing time in the Leeds Jet Homogenizer was at least two orders of magnitude faster than that in the Encapsulator. This faster mixing time allowed emulsion microgel particles to form by reactive precipitation (Casanova, et al., 2011) and explains why considerably smaller emulsion microgel particles were formed compared to those formed with the Encapsulator, even at much higher [Ca^{2+}] in the Encapsulator.

The above calculations do not take into account the different [Ca^{2+}]. Therefore, it was of interest to calculate the theoretical flux of Ca^{2+} ions to the WPI layer absorbed to the oil droplet surface. As a first approximation, the diffusive molecular flux of Ca^{2+} to the HT-WPI surface was calculated from Fick's first law:

$$J = 4\pi D_t r_i [\mathrm{Ca}^{2+}] \quad (15)$$

where r_i is the radius of oil droplets, [Ca^{2+}] the concentration of Ca^{2+} ions and D_t the turbulent diffusion (Deberdeev, Berlin, Dyakonov, Zakharov, & Monakov, 2013) coefficient given by $D_t = Q \times d$ where, Q is the flow rate and d is the diameter of the nozzle or stir bar. Of course a key limitation of using Fick's first law is that it does not take into account the role of chaotic advection taking part during turbulent mixing (Nguyen, 2012). Further numerical simulation including the impact of chaotic advection might give additional understanding of the effect of turbulent mixing conditions on the formation of emulsion microgel particles.

Table 3 summarizes the flux of Ca^{2+} to HT-WPI (J) absorbed on the oil droplet surface depending on the [Ca^{2+}] and turbulent diffusion coefficient (D_t). Noticeably, in both systems [Ca^{2+}] did not affect the flux in the same manner. In the Jet Homogenizer, increasing [Ca^{2+}] from 0.02 M to 0.1 M should increase the Ca^{2+} ions flux by a factor of ten, suggesting Ca^{2+} ions should bind to WPI more rapidly at 0.1 M, increasing the gelation kinetics. This was observed during measurement of the small deformation rheology (Figure 5A). The increase in flux might also help explain the formation of individually encapsulated oil droplets in HT-WPI (Figure 8B). At 0.1 M Ca^{2+} ions, the excess and high flux of Ca^{2+} ions to HT-WPI led to prompt gelation of WPI adsorbed at the oil-water interface and a higher probability of individually encapsulated oil droplets rather than emulsion microgel particles. Additionally, the lower flux of Ca^{2+} ions, as well as the insufficient amount of Ca^{2+} ions (0.02 M), led to slower gelation of HT-WPI resulting in a higher probability of fractal aggregates.

With regard to the Encapsulator, 1.4 M Ca^{2+} ions had a 70% faster flux than 1 M Ca^{2+}ions, leading to slightly faster gelation, in agreement with HT-WPI emulsion gelation kinetics **(**Figure 5A). Therefore, emulsion microgel particles formed at 1.4 M Ca^{2+} ions should theoretically be smaller than the ones formed in presence of 1 M Ca^{2+}

744 ions. However, high [Ca^{2+}] led to larger emulsion microgel particles (d_{32} = 1.2 mm) as
745 compared to lower [Ca^{2+}] (d_{32} = 0.9 mm) even though the Ca^{2+} flux was significantly
746 faster. As demonstrated by Hongsprabhas, et al., (1997) and Jeyarajah, et al., (1994)
747 the addition of Ca^{2+} increases the hydrophobicity and sulfhydryl group reactivity of
748 WPI, enhancing protein-protein interactions and aggregation through Ca^{2+} ion cross-
749 linkage and covalent bonds (Beaulieu, et al., 2002; Hongsprabhas, et al., 1997;
750 Jeyarajah, et al., 1994).

751 Overall, the main factor influencing the flux of Ca^{2+} is the turbulent diffusion
752 coefficient, leading up to a 10 fold difference between both systems (Jet homogenizer
753 and Encapsulator). The turbulent diffusion coefficient in the Jet Homogenizer ($D_t > 10^{-11}$
754 $m^2 s^{-1}$) was three orders of magnitude larger than in the Encapsulator ($D_t > 10^{-8} m^2 s^{-1}$
755 ^{1}).

4 Conclusions

Findings from this study have demonstrated that emulsion microgel particles of tuneable size can be designed using simple bottom-up approaches and solvent-free turbulent mixing techniques. This is driven by the ability of heat-treated WPI to stabilise oil droplets as well as gel in presence of divalent cations, creating a soft solid network encapsulating several oil droplets into one particle. This study has also demonstrated the effect of different Ca^{2+} concentrations and turbulent mixing techniques on the gelation kinetics as well as their effect on particle size. Low [Ca^{2+}] (0.02 to 0.1 M) in T-mixing devices allowed the formation of small (10 to 100 μm) aggregated emulsion microgel particles. High [Ca^{2+}] (1 to 1.4 M) and extrusion stirrer mixing devices allowed the formation of large (500 to 1000 μm) non-aggregated emulsion microgel particles. These differences in size were explained by the fact that the T-mixer (Leeds Jet Homogenizer) allowed for more rapid flux of Ca^{2+} ions to HT-WPI, which in turn led to faster mixing times and faster gelation of HT-WPI stabilised emulsions. In comparison, the Encapsulator gave much slower mixing times and Ca^{2+} ions flux, leading to slower gelation of HT-WPI stabilized emulsions. Further experiments on these emulsion microgel particles such as, encapsulation efficiency, stability and gastro-intestinal digestibility are required for full characterisation.

Thus, stable emulsion microgel particles with tuneable sizes and mechanical properties can be produced as long as there is a strong understanding of the interplay between concentration of WPI, heat treatment of WPI, [Ca^{2+}], gelation kinetics and the mixing time. Such emulsion microgel particles made may find applications for delivery of lipophilic molecules in various soft matter applications in food, pharmaceutical and allied sectors.

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