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## 25 **Abstract**

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

## 47 **Keywords**

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

50

## 51 **1 Introduction**

52 Lipophilic active molecules such as fat soluble vitamins, flavourings, fatty acids and  
53 essential oils pose challenges when incorporated into food, pharmaceuticals or other  
54 soft matter applications due to their partial or complete water insolubility. Besides  
55 oxidizing rapidly, most of these compounds are difficult to deliver in physiology and  
56 are generally only partially absorbed by the skin or via the gastrointestinal regime.  
57 Thus, their physiological activity is most often partly or fully lost before reaching the  
58 targeted physiological site (McClements, 2015). Consequently, there is a huge need to  
59 protect these lipophilic compounds from environmental degradation and tailor their  
60 release at particular biological sites (Sung, Xiao, Decker, & McClements, 2015). A  
61 wide range of technologies have been developed to encapsulate oil-soluble molecules,  
62 such as emulsions, emulsion gels, liposomes, micelles, nanoparticles, etc (McClements,  
63 2011). Each of these has its own specific advantages and disadvantages in terms of  
64 degree of protection, delivery, cost, regulatory status, ease of use, biodegradability and  
65 biocompatibility (McClements & Li, 2010). Among these, emulsion microgel particles  
66 are vehicles that have not been explored as widely.

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

75 Sanguansri, 2012; Beaulieu, Savoie, Paquin, & Subirade, 2002; Velikov & Pelan,  
76 2008). Additionally, the microgel particle allows swelling or de-swelling as a function  
77 of pH, ionic strength, temperature and enzymatic conditions via tuning the size and/or  
78 physicochemical properties (Ballauff & Lu, 2007; Wei, Li, & Ngai, 2016). Hence, these  
79 particles have great potential for site-dependent release of lipophilic active compounds  
80 in a range of food, pharmaceutical, personal care and other soft material applications  
81 (Ching, Bansal, & Bhandari, 2016).

82         Whey protein isolate (WPI) is widely accepted for research and commercial  
83 applications and its versatility as an emulsifier and gelling agent is well recognized  
84 (Sarkar, Murray, et al., 2016). Under heat-treatment WPI undergoes conformational  
85 changes, exposing its hydrophobic and sulfhydryl groups allowing irreversible  
86 aggregation and gel formation under specific conditions of protein concentration, ionic  
87 strength and temperature (Roefs & Peppelman, 2001). On addition of calcium ( $\text{Ca}^{2+}$ )  
88 ions, heat treated WPI (HT-WPI) undergoes further aggregation via  $\text{Ca}^{2+}$  cross-linking  
89 of the negatively charged carboxylic groups on the WPI. Protein- $\text{Ca}^{2+}$ -protein  
90 complexes are formed, reducing the negative charge on the protein (Bryant &  
91 McClements, 2000; Hongprabhas, Barbut, & Marangoni, 1999; Phan-Xuan, et al.,  
92 2014).

93         Several technologies have been developed for the production of WPI stabilised  
94 emulsion microgel particles. For instance, multistep emulsion-templating allows the  
95 formation of emulsion particles via  $\text{O}_1/\text{W}/\text{O}_2$  emulsions (Sung, et al., 2015). The WPI  
96 aqueous phase of the  $\text{O}_1/\text{W}/\text{O}_2$  emulsion is typically gelled through heat treatment,  
97 forming ( $\text{O}_1/\text{W}$ ) WPI stabilised emulsion microgel particles suspended in an external  
98 oil phase ( $\text{O}_2$ ). The oil phase is then washed away with the use of organic solvents.  
99 Although this generates microgel particles of controlled size: the multiple processing

100 steps causes the technique to be laborious; heat gelation renders it ineffective for the  
101 use of heat-sensitive compounds; the use of organic solvents limits its application in  
102 certain medical drugs and food products where biocompatibility is a key issue  
103 (Beaulieu, et al., 2002). An alternative multistep emulsion-templating method was  
104 designed by Egan, Jacquier, Rosenberg, and Rosenberg (2013). The aqueous WPI  
105 phase of the O<sub>1</sub>/W/O<sub>2</sub> emulsion was gelled via a cold set technique. The external oil  
106 phase (O<sub>2</sub>) was then washed away with surfactants rather than solvents. Although this  
107 technique allows the encapsulation of heat-sensitive compounds and does not require  
108 the use of solvents, the multiple processing required still causes this method to be time  
109 consuming and laborious plus excess surfactant may need to be removed. Extrusion  
110 technologies allowing cold external gelation of heat-treated WPI emulsion microgel  
111 particles have also been developed (Egan, et al., 2013). In this case, the heat-treated  
112 WPI stabilised emulsion was dropped into an ionic bath, allowing the gelation of the  
113 continuous phase, which entrapped oil droplets into microgel particles. Although this  
114 external gelation method was successful it produced large particles, of 1-2 mm in  
115 diameter, limiting their application in food systems. Other processing methods produce  
116 emulsion microgel particles by emulsifying the oil phase with WPI or sodium caseinate  
117 and gelling the emulsion into microgel particles with alginate or pectin (Ruffin, et al.,  
118 2014; Zhang, Zhang, & McClements, 2016). The use of several different biopolymers  
119 causes this technique to be not very cost effective. Also, thermodynamic  
120 incompatibility between the protein at the interface and the gelling biopolymer might  
121 result in uncontrolled release behaviour.

122         Thus, external gelation has considerable potential if it can be made facile, rapid  
123 and allow processing of clean emulsion microgel particles. Careful optimization of  
124 temperature, shear and WPI and Ca<sup>2+</sup> concentration might also allow the tailoring of

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

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

141

## 142 **2 Materials and Methods**

### 143 *2.1 Materials*

144 Whey protein isolate (WPI) powder containing 96.3 wt% protein (Molecular mass: 18.4  
145 kDa) was a kind gift from Fonterra Limited (Auckland, New Zealand). Sunflower oil  
146 was purchased from Morrisons supermarket (UK). Calcium chloride, 8-aniline-1-  
147 naphthalenesulfonic acid (ANS), sodium hydroxide, hydrochloric acid, sodium  
148 chloride, hexane anhydrous, 95% were purchased from Sigma-Aldrich (Gillingham,  
149 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 (Steinheim, 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<sup>-1</sup>) 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):

$$171 \quad [ANS] = \frac{V_{ANS} \times C_{ANS}}{(V_{ANS} + V_{WPI})} \quad (1)$$

172 where, C<sub>ANS</sub> is the concentration of ANS stock solution (3.2 mM), V<sub>WPI</sub> is the  
173 volume of protein and V<sub>ANS</sub> 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.

175 12 wt% and 9.6 wt% WPI solutions were heated at different temperatures (75, 80  
 176 or 85 °C) for different time periods (0, 8, 15, 30, 40, 50 min). Protein solutions were  
 177 decanted into quartz cuvettes (3 mL) and ANS (150  $\mu$ L) was then added to each sample.  
 178 The fluorescence emission intensity of each sample was recorded at the stated  
 179 temperature.

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

181

$$182 \quad \frac{LB}{LF} = \frac{nP}{Kd} - \frac{LB}{Kd} \quad (2)$$

183

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

188 The  $LB$  was determined from  $\Delta F$  (the fluorescence measurements) using the  
 189 conversion factor  $Q$  as given by eq (3),

190

$$191 \quad LB = \Delta F / Q \quad (3)$$

192

193 The conversion factor  $Q$  was obtained following the method from Nyman, et al.  
 194 (1997).

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

197

$$198 \quad \text{Relative } \frac{LB}{LF} = \frac{\left(\frac{LB}{LF}\right)_t}{\left(\frac{LB}{LF}\right)_f} \quad (4)$$

199

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

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

202

### 203 2.2.2. Particle size of protein aggregates

204 The aggregation rate of the aforementioned 12 wt% and 9.6 wt% WPI solutions were

205 measured at the different time-temperature treatments using dynamic light scattering

206 (Zetasizer, Nano ZS series, Malvern Instruments, Worcestershire, UK). Assuming WPI

207 particles are spherical, the apparent particle diameter is inversely related to the diffusion

208 coefficient ( $D$ ) via the Stokes-Einstein equation (eq 5) :

209

$$210 \quad d_H = \frac{k_b T}{3\pi\eta D} \quad (5)$$

211

212 where  $k_b$  is the Boltzmann constant,  $T$  is the temperature,  $\eta$  is the viscosity of the

213 solution and  $d_H$  is the hydrodynamic diameter.

214 Sizing of WPI particles was conducted based on a relative refractive index of 1.150

215 (i.e. the ratio of the refractive index of WPI (1.53) to that of the aqueous phase at 1.33).

216 The absorbance value of WPI particles was set at 0.001. Before analysis, samples were

217 diluted to 0.1 wt% WPI with Milli-Q water and filtered through with a membrane of

218  $0.45 \mu\text{m}$  (PTFE Syringe filters, Perkin Elmer, USA). One mL of solution was injected

219 into a clean cuvette (PMMA, Brand Gmbh, Wertheim, Germany). Particle size was

220 presented as mean hydrodynamic diameter of five readings on duplicate samples.

221

222

223        *2.3 Preparation of heat denatured whey protein-stabilised emulsion*

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

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

237

238        *2.4 Zeta-potential*

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

247

248 
$$U_E = \frac{2\varepsilon z F(ka)}{3\eta} \tag{6}$$

249

250 where  $U_E$  is the measured electrophoretic mobility,  $z$  the  $\zeta$ -potential,  $\varepsilon$  the dielectric  
251 constant of the medium,  $\eta$  the viscosity of the solution and  $F(ka)$  Henry's function using  
252 the Smoluchowski approximation, *i.e.*,  $F(ka) = 1.5$ .

253

### 254 *2.5 Preparation of emulsion microgel particles*

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

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

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

273 producing very high liquid velocities. A hydraulic ram pushes onto the pistons on top  
274 of both chambers forcing the liquids they contain through the pinhole at high velocity,  
275 generating highly turbulent conditions depending on the pressure applied (100-400 bar)  
276 (Casanova & Higuaita, 2011). In this work, HT-WPI stabilised emulsion was added to  
277 one chamber and CaCl<sub>2</sub> solution (0.02-0.1 M) to the other chamber. A pressure of 250  
278 bar was employed. The turbulent mixing resulted in the formation of emulsion microgel  
279 particles. The resulting particles were collected in a beaker and immediately diluted  
280 with Milli-Q water and stirred for 30 min at low speed to limit particle aggregation.

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

$$282 \text{Re} = \rho v d / \eta \quad (7)$$

283

284 with  $\rho$  the solvent density (i.e. water),  $v$  the maximum fluid velocity,  $d$  the diameter of  
285 the nozzle used with the Jet Homogenizer,  $\eta$  the dynamic viscosity of the solution at 20  
286 °C.

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

$$289 v = \frac{4q}{d^2\pi} \quad (8)$$

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

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

$$293 \text{Re} = \frac{\rho n d^2}{\eta} \quad (9)$$

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

296

297 *2.6 Small deformation rheology*

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

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

314

### 315 *2.7 Particle size analysis of emulsion and emulsion microgel particles*

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

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

327 Emulsion microgel particles formed using the Buchi Encapsulator B-390<sup>®</sup> were  
328 sized using image analysis of the digitized images captured via a Nikon SMZ-2T  
329 (Nikon, Japan) optical microscope, due to their larger sizes ( $> 500 \mu\text{m}$ ). For comparison  
330 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  
331 Brouckere mean diameter ( $d_{43} = \frac{\sum n_i d_i^4}{\sum n_i d_i^3}$ ) were calculated. Each sample was analysed  
332 ten times and the averages and standard deviations are reported.

333

### 334 *2.8 Microscopy*

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

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

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

347 Golay, Acquistapace, and Heinrich (2016). The emulsion microgel particles were dried  
348 in an oven at 37 °C for 72 h and subsequently washed with hexane removing all oil  
349 droplets. After removal of the oil, the intact or deliberately fractured particles were  
350 mounted onto a chrome coated steel plate with carbon double sided-tape and sputter  
351 coated with gold using a JEOL JFC-1600 Auto Fine Coater (JEOL Japan) for 200 s at  
352 30 mA. The SEM images were then obtained at 10-20 kV.

353

### 354 *2.9 Statistical analysis*

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

358

## 359 **3 Results and discussion**

### 360 *3.1 Denaturation and aggregation kinetics of HT-WPI solution*

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

372 concentration. It can be observed from Figure 2A that on increasing the temperature by  
373 10 °C (from 75 °C to 85 °C), the relative LB/LF ratio reached a plateau 25 min earlier,  
374 irrespective of WPI concentration. The faster unfolding of WPI with increase  
375 temperature has also been noticed by Das and Kinsella (1990). In the case of 9.6 wt%  
376 WPI, LB/LF reached a plateau at 85 °C after 15 min: approximately 87% ANS was  
377 observed to be bound to HT-WPI (Figure 2B). Consequently, it is suggested that after  
378 15 min at 85 °C, no more hydrophobic groups are available for ANS to bind to resulting  
379 in almost total unfolding of WPI, in agreement with previous studies (Kim, et al., 2005).  
380 In comparison, at the lower temperature of 75 °C, LB/LF reached a plateau only after  
381 a longer exposure time of 40 min with 76% ANS bound to WPI (Figure 2A and B).  
382 Thus, at 75 °C, the temperature was not high enough to unfold and denature the WPI  
383 fully. These results are in agreement with previous studies in the literature (Ruffin, et  
384 al., 2014; Wolz & Kulozik, 2015) as well as circular dichroism results (see  
385 Supplementary Figure S1).

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

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

410       Interestingly, for 12 wt% at 75 and 80 °C, the particle size after 8 min only  
411 decreased by approximately 60% (from 189 nm to 78 and 75 nm, respectively), whereas  
412 at 85 °C the particle size decreased by 75%. At high WPI concentration (*i.e.*, 12 wt%),  
413 a further 7 min at 80 °C were necessary to break down the oligomers into monomers  
414 and reduce WPI particle size by 75%. These results are in agreement with previous  
415 studies conducted by Das, et al. (1990). After 15 min of heat treatment, HT-WPI  
416 particle size slightly increased. For instance, at 85 °C, 9.6 wt% WPI particles size at 8  
417 min measured 43 nm and after 15 min these measured 48 nm (Figure 3). As previously  
418 discussed, 87% ANS was found to be bound to HT-WPI after 15 min at 85 °C,  
419 suggesting almost total unfolding. This slight increase in particle size might therefore  
420 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  $\text{Ca}^{2+}$  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  $\mu\text{m}$  as expected

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

450 The emulsion droplets were not flocculated during the homogenization stage, as  
451 confirmed by the  $d_{43}$  value, which was below  $0.5 \mu\text{m}$  and were anionic ( $-43 \text{ mV}$ ) as  
452 expected at pH 7.

453

### 454 *3.3 Rheological properties of cold-set HT-WPI emulsion gels*

455 The gelation of HT-WPI solutions and emulsions was induced by the addition of  $\text{Ca}^{2+}$   
456 ions at different concentrations. Figure 5 shows the storage modulus of the emulsion  
457 gels or protein gels (without oil droplets) at different concentrations of  $\text{Ca}^{2+}$  ions as a  
458 function of time and strain. For all systems,  $G'$  was significantly greater than  $G''$  ( $p <$   
459  $0.05$ ), with  $\tan \delta < 0.3$ , which implied that the gels had an elastic behaviour. Therefore,  
460 in the following, only results for  $G'$  are presented and discussed.

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

470 As observed in Figure 5A, all cold set emulsion gels had similar rheological  
471 behaviour irrespective of the  $[Ca^{2+}]$  (0.02 to 1.4 M). On addition of  $Ca^{2+}$  ions, the  
472 emulsions gelled instantaneously, as shown by the storage modulus being above 3 kPa  
473 at time zero. Over time, all four emulsion gels became slightly stronger: after 1h 40  
474 min,  $G'$  of all emulsion gels increased on average by 50%. This might be attributed to  
475 a gradual increase in the number density of  $Ca^{2+}$ -protein interactions (Marangoni, et al.,  
476 2000). Understanding the structure of the emulsion gels with regard to varying  $[Ca^{2+}]$   
477 might give valuable insight on the mechanical strength of the emulsion gels. The rubber  
478 elasticity theory modified by Flory (Betz, Hormansperger, Fuchs, & Kulozik, 2012;  
479 Flory, 1953) for polymers allows a simplistic analysis of the structure of viscoelastic  
480 material via their elastic mechanical behaviour. For small deformations ( $< 2\%$ ), the  
481 emulsion gels fully recovered to their original dimension in a prompt manner (Peppas,  
482 Bures, Leobandung, & Ichikawa, 2000) implying that these emulsion gels were almost  
483 perfectly elastic. Therefore, it was of interest to express the results in terms of the  
484 theoretical mesh size. The average mesh (or pore) size ( $\xi$ ) of a cross-linked network is  
485 defined as the distance between two crosslinks or macromolecular chains (Peppas, et  
486 al., 2000; Sarkar, et al., 2015) and can be calculated using eq (10):

$$487 \quad \xi^3 = \frac{\kappa_B T}{G'} \quad (10)$$

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

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

509

$$510 \quad [Ca^{2+}] = \frac{n(COO^-)m(WPI)_i}{M_w} \frac{1}{2V} \quad (11)$$

511

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

520 binding. From previous studies, the HT-WPI monolayer surface coverage (Das, et al.,  
521 1990; Dickinson, 1998) of droplets was estimated at 3 mg/m<sup>2</sup>. Therefore, in this study,  
522 assuming that the total surface area of the 20 wt% oil emulsion was 1203 m<sup>2</sup> (calculated  
523 from the particle size distribution), we calculated that this surface was covered by 3.9  
524 g of HT-WPI.

525 From eq (11), we then calculated that the minimum [Ca<sup>2+</sup>] required to bind to  
526 all COO<sup>-</sup> groups on the  $\beta$ -lactoglobulin molecules absorbed at the oil/water interface  
527 would be 0.03 M. On this basis, for the systems gelled at 0.02 M Ca<sup>2+</sup>, there was not  
528 enough Ca<sup>2+</sup> and this insufficient amount led to slower gelation kinetics of HT-WPI, as  
529 well as the formation of a weaker emulsion gel ( $G' = 8.8$  kPa). For systems gelled at  
530 0.1 M Ca<sup>2+</sup> and above, there would clearly be a significant excess of Ca<sup>2+</sup> and bridging  
531 flocculation might have led to more coarse, porous and non-continuous aggregates,  
532 especially for emulsion gels produced at high [Ca<sup>2+</sup>] such as 1 and 1.4M. These coarser  
533 non-continuous aggregates would allow the disruption of the protein network reducing  
534 the emulsion gel strength, as seen with the theoretical mesh size calculations (Beaulieu,  
535 et al., 2002; Sok, et al., 2005; Westerik, Scholten, & Corredig, 2015).

536 Figure 5B demonstrates that all emulsion gels tested (0.02-1.4 M Ca<sup>2+</sup>) had a similar  
537 linear viscoelastic region, ranging from 0.1-2.0% shear strain. With increasing strain,  
538 emulsion gels became weaker and their storage modulus decreased dramatically. Oil  
539 droplets probably acted as weakening points at larger strain (> 10%), allowing the gels  
540 to collapse. These results are in accordance with previous studies (Chen & Dickinson,  
541 1999; Dickinson, 2000). Additionally, the concentration of Ca<sup>2+</sup> ions involved in the  
542 emulsion gel formation influenced their behaviour under small deformation. At low  
543 [Ca<sup>2+</sup>] (0.02 and 0.1 M), the structure of the gel was probably more fine stranded  
544 (Hongprabhas, Barbut, & Marangoni, 1999) and able to absorb the energy applied

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

555

#### 556 *3.4 Design of size-tuneable HT-WPI emulsion microgel particles*

557 Two processing methods were used to form different sized and shaped emulsion  
558 microgel particles (Figure 6). The first method involved turbulent mixing of the  
559 emulsion and  $\text{Ca}^{2+}$  ions solution via the Leeds Jet Homogenizer at 250 bar and nozzle  
560 size 500  $\mu\text{m}$  (Figure 6A). Low concentrations of  $\text{Ca}^{2+}$  ions (0.02 to 0.1 M) were chosen  
561 to create emulsion microgel particles due to the fact that at higher concentrations the  
562 gelation happened too quickly, blocking the homogenizer and nozzle. The Leeds Jet  
563 homogenizer produced small (around 20  $\mu\text{m}$ ) but highly aggregated microgel particles  
564 (Figure 6A1). Some oil droplets could also be seen coating the surface of the particles  
565 due to the short residence time (Figure 6A2). However, most oil droplets (in red)  
566 appeared to be entrapped within the HT-WPI matrix (Figure 6A2) as is emphasized  
567 with Figure 6A3, where the protein matrix is in green and the oil droplets are in black.  
568 A statistical analysis of the amount of oil found at the surface of the emulsion microgel  
569 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  $\mu\text{m}^2$  or 9% of the  
575 total area (98,900  $\mu\text{m}^2$ ) 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  $\mu\text{m}$ )  
581 than the majority of the emulsion droplets entrapped – which appeared to have retained  
582 the original mean size (around 0.1  $\mu\text{m}$ ) 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<sup>®</sup> at low pressure (0.4 bar) with the smaller vibrating nozzle size (150  $\mu\text{m}$ ),  
587 as well as turbulent mixing of the  $\text{Ca}^{2+}$  ions solution (500 rpm stirrer speed;  $\text{Re} = 4.7$   
588  $\times 10^5$ ) (Figure 6B). High concentrations of  $\text{Ca}^{2+}$  ions (1-1.4 M) were required for this  
589 method, because at lower concentrations diffusion of  $\text{Ca}^{2+}$  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  $\mu\text{m}$ ) 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  $\mu\text{m}$  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  $\mu\text{m}$  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  $\text{Ca}^{2+}$  ions, the first peak was  
601 approximately in the same region as the emulsion oil droplets (0.1 to 1  $\mu\text{m}$ ), 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  $\mu\text{m}$ ). The ratio  
604 between  $d_{32}$  and  $d_{43}$  at 0.02 M  $\text{Ca}^{2+}$  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  $[\text{Ca}^{2+}]$  required to bind to  
607 every free carboxylic group on HT-WPI adsorbed to oil droplets was  $[\text{Ca}^{2+}]_{\text{min}} = 0.03$   
608 M.

609 Increasing the concentration of  $\text{Ca}^{2+}$  ions to 0.1 M led to smaller microgel  
610 particles with an 80% reduction in mean  $d_{43}$  value (306  $\mu\text{m}$ ). The first peak of the  
611 particle size distribution then shifted to 1 to 30  $\mu\text{m}$  (Figure 7A). This suggested that  
612 emulsion droplets that were not encapsulated into the emulsion microgel particles at  
613 0.02 M  $\text{Ca}^{2+}$  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  $\mu\text{m}$   
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  $\text{Ca}^{2+}$  ions was approximately  
619 in the same region as the second peak for particles formed with 0.02 M  $\text{Ca}^{2+}$  ions,

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

626 In comparison, emulsion microgel particles formed via the Encapsulator had a  
627 monomodal size distribution - though they were much larger - from 0.5 to 1 mm (Figure  
628 7B). The emulsion microgel particles produced at higher concentrations of  $\text{Ca}^{2+}$  (1.4  
629 M) were 10% larger compared to those formed at 1 M (Figure 6B1). As previously  
630 demonstrated by (Jeyarajah, et al., 1994), the addition of salt to heat-treated WPI  
631 solution increases the hydrophobicity of the protein as well as its reactive SH content.  
632 SH groups found in proximity of  $\text{Ca}^{2+}$  ion cross-bridges might form additional covalent  
633 bonds more easily, strengthening the aggregation of WPI (Jeyarajah, et al., 1994).  
634 Therefore, increasing the concentration from 1 to 1.4 M may enhance various protein-  
635 protein interactions resulting in further aggregation and larger particle sizes.

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

645 could be found at the exterior surface which could be attributed to air bubbles entrapped  
646 at the surface prior to drying. The top of the particle was fractured to observe the  
647 interior distribution of the emulsion microgel particle. All oil droplets associated with  
648 the oil droplets within the microgel particle had been previously washed away with  
649 hexane. Figure 9B shows the protein network (white) around the hollow pockets where  
650 the oil droplets previously resided (darker colour) (as observed by Beaulieu, et al.,  
651 2002; Chen, et al., 1999). The white protein layer noticed around the hollow pockets  
652 suggested that the oil droplets were physically bound to the WPI gel matrix, confirming  
653 the rheological results (Rosenberg, et al., 2004). The micrographs also indicated that  
654 the oil droplets were evenly distributed throughout the WPI matrix. Some hollows had  
655 been distorted and did not retain their spherical shape upon drying of the particles.  
656 However, the sizes of the hollows were in the same size range of the original emulsion  
657 droplets (0.1 to 1  $\mu\text{m}$ ). These observations confirm very little oil droplet coalescence  
658 occurred during processing and hollows were left by oil droplets rather than pores of  
659 the protein gel (previously estimated at 7.9 nm).

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

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

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

678

$$679 \quad t_{mix} = \left(\frac{\nu}{\varepsilon}\right)^{\frac{1}{2}} \quad (12)$$

680

681 where  $\nu$  is the kinematic viscosity of the solution and  $\varepsilon$  is the energy dissipation.

682 The emulsion behaved as a non-Newtonian shear-thinning fluid and its viscosity  
683 was estimated at the shear rate of the Jet Homogenizer and the Encapsulator. The shear  
684 rate of both instruments was defined by  $\gamma \dot{=} 8v/d$  where  $v$  is the velocity of the emulsion  
685 and  $d$  the diameter of the nozzle. The energy dissipation produced by the Leeds Jet  
686 Homogenizer at 250 bar has been previously calculated (Casanova, et al., 2011) and  
687 was found to be  $\varepsilon = 3.1 \times 10^6$  W kg<sup>-1</sup>. Following eq 10, the corresponding mixing time  
688 was  $4 \times 10^{-4}$  s.

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

693

$$694 \quad \varepsilon = \frac{P}{V} \quad (13)$$

695

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

697

$$698 \quad P = N_p \rho N^3 d^5 \quad (14)$$

699

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

702 The energy dissipation produced by the Encapsulator was thus calculated as 4.8  
703  $\times 10^4 \text{ W kg}^{-1}$ , where the power number had previously been reported (James R. Couper,  
704 2005) for Reynolds numbers of the same order of magnitude ( $N_p = 4$ ). Following eq 12,  
705 the mixing time in the Encapsulator was therefore  $2,6 \times 10^{-2} \text{ s}$ . Consequently, it is  
706 proposed that the mixing time in the Leeds Jet Homogenizer was at least two orders of  
707 magnitude faster than that in the Encapsulator. This faster mixing time allowed  
708 emulsion microgel particles to form by reactive precipitation (Casanova, et al., 2011)  
709 and explains why considerably smaller emulsion microgel particles were formed  
710 compared to those formed with the Encapsulator, even at much higher  $[\text{Ca}^{2+}]$  in the  
711 Encapsulator.

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

716

$$717 \quad J = 4\pi D_t r_i [\text{Ca}^{2+}] \quad (15)$$

718

719 where  $r_i$  is the radius of oil droplets,  $[Ca^{2+}]$  the concentration of  $Ca^{2+}$  ions and  
720  $D_t$  the turbulent diffusion (Deberdeev, Berlin, Dyakonov, Zakharov, & Monakov,  
721 2013) coefficient given by  $D_t = Q \times d$  where,  $Q$  is the flow rate and  $d$  is the diameter  
722 of the nozzle or stir bar. Of course a key limitation of using Fick's first law is that it  
723 does not take into account the role of chaotic advection taking part during turbulent  
724 mixing (Nguyen, 2012). Further numerical simulation including the impact of chaotic  
725 advection might give additional understanding of the effect of turbulent mixing  
726 conditions on the formation of emulsion microgel particles.

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

740 With regard to the Encapsulator, 1.4 M  $Ca^{2+}$  ions had a 70% faster flux than 1  
741 M  $Ca^{2+}$  ions, leading to slightly faster gelation, in agreement with HT-WPI emulsion  
742 gelation kinetics (Figure 5A). Therefore, emulsion microgel particles formed at 1.4 M  
743  $Ca^{2+}$  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 ( $d_{32} = 1.2 \text{ mm}$ ) as  
745 compared to lower  $[Ca^{2+}]$  ( $d_{32} = 0.9 \text{ mm}$ ) even though the  $Ca^{2+}$  flux was significantly  
746 faster. As demonstrated by Hongprabhas, 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; Hongprabhas, 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 ( $D_t > 10^{\cdot}$   
754  $^{11} \text{ m}^2 \text{ s}^{-1}$ ) was three orders of magnitude larger than in the Encapsulator ( $D_t > 10^{-8} \text{ m}^2 \text{ s}^{-}$   
755  $^1$ ).

## 756 **4 Conclusions**

757 Findings from this study have demonstrated that emulsion microgel particles of  
758 tuneable size can be designed using simple bottom-up approaches and solvent-free  
759 turbulent mixing techniques. This is driven by the ability of heat-treated WPI to  
760 stabilise oil droplets as well as gel in presence of divalent cations, creating a soft solid  
761 network encapsulating several oil droplets into one particle. This study has also  
762 demonstrated the effect of different  $\text{Ca}^{2+}$  concentrations and turbulent mixing  
763 techniques on the gelation kinetics as well as their effect on particle size. Low  $[\text{Ca}^{2+}]$   
764 (0.02 to 0.1 M) in T-mixing devices allowed the formation of small (10 to 100  $\mu\text{m}$ )  
765 aggregated emulsion microgel particles. High  $[\text{Ca}^{2+}]$  (1 to 1.4 M) and extrusion stirrer  
766 mixing devices allowed the formation of large (500 to 1000  $\mu\text{m}$ ) non-aggregated  
767 emulsion microgel particles. These differences in size were explained by the fact that  
768 the T-mixer (Leeds Jet Homogenizer) allowed for more rapid flux of  $\text{Ca}^{2+}$  ions to HT-  
769 WPI, which in turn led to faster mixing times and faster gelation of HT-WPI stabilised  
770 emulsions. In comparison, the Encapsulator gave much slower mixing times and  $\text{Ca}^{2+}$   
771 ions flux, leading to slower gelation of HT-WPI stabilized emulsions. Further  
772 experiments on these emulsion microgel particles such as, encapsulation efficiency,  
773 stability and gastro-intestinal digestibility are required for full characterisation.

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

780

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788

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