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Overcoming in vitro gastric destabilisation of emulsion droplets using emulsion microgel particles for targeted intestinal release of fatty acids

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Abstract

Whey protein based emulsion microgel particles (9.6 wt% whey protein – 20 wt% sunflower oil) were produced via cold set precipitation using calcium ions (0.1 M) and their behaviour under in vitro gastrointestinal digestion was investigated with conventional oil-in-water emulsions (9.6 wt% whey protein – 20 wt% sunflower oil) as a control. The droplet size distribution, zeta-potential, microstructure and hydrolysis of interfacial whey protein during in vitro gastric digestion and free fatty acid release during in vitro intestinal digestion were compared for both samples. During in vitro gastric digestion, emulsions flocculated and coalesced (d_{32} ~ 0.13 μm to ~ 12 μm after 120 min) due to pepsinolysis of the adsorbed protein layer, as evidenced by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis). This destabilisation led to uncontrolled and limited release of free fatty acids (44 % FFA) during subsequent intestinal digestion, largely due to the reduction in interfacial area. In comparison, emulsion microgel particles were noticeably more stable during in vitro gastric digestion, with only a slight decrease in particle size (d_{32} ~ 50 μm to ~ 20 μm after 120 min). The protection of emulsion droplets against gastric coalescence in emulsion microgel particles was controlled by physicochemical interactions between calcium ions and whey protein in the particles, limiting both pepsin-diffusion and cleavage at the pepsin active site. Under subsequent in vitro intestinal digestion, the microgel particles degraded due to the action of intestinal proteases, releasing fine emulsion droplets, which then gave significantly higher release of free fatty acids (54 % FFA).

Keywords: Emulsion microgel particles; gastric stability; in vitro digestion; free fatty acid release; pepsin; bile salts

1. Introduction

Lipophilic bio-active molecules, such as fat soluble vitamins, fatty acids, essential oils and drugs pose substantial challenges when incorporated into food, pharmaceuticals and other soft matter applications. Most of these lipophilic compounds are difficult to deliver to physiological sites (i.e., via the intestinal phase) due to the physical instability, during gastrointestinal transit, of the oil phases in which they are solubilized (Golding & Wooster, 2010; Parada & Aguilera, 2007). Oil-in-water (O/W) emulsions stabilized by protein or surfactant have been commonly used to encapsulate and stabilise lipophilic molecules (Araiza-Calahorra, Akhtar, & Sarkar, 2018; McClements, Decker, & Weiss, 2007). Nevertheless, their limited stability during gastric digestion, due to flocculation and coalescence of the oil droplets - largely attributed to pepsinolysis or harsh acidic/ ionic environments, leads to inadequate release of lipophilic molecules during subsequent intestinal digestion (Golding, et al., 2010; Hur, Decker, & McClements, 2009; Sarkar, Goh, & Singh, 2010; Sarkar, Goh, Singh, & Singh, 2009; Singh & Sarkar, 2011). Therefore, a strong emphasis has been placed on developing delivery systems that can protect the droplets in the gastric phase and then release the bio-actives molecules at specific locations during intestinal digestion (Matalanis & McClements, 2013; McClements, 2017; McClements, Decker, & Park, 2008).

In this direction of research, many authors have investigated manipulating the interface of droplets to restrict pepsinolysis of proteinaceous stabilizing layers by creating a more tortuous path for pepsin to reach the interface, for example by coating the adsorbed protein layer by layers of other material(s). In this fashion, gastric stability of emulsion droplets has been achieved by coating protein-stabilized droplets with a variety of non-digestible dietary fibres (Beysseriat, Decker, & McClements, 2006; Meshulam & Lesmes, 2014) and/or particles (Liu & Tang, 2016; Sarkar, Ademuyiwa, et al., 2018; Sarkar, Li, Cray, & Boxall, 2018; Sarkar, Zhang, Murray, Russell, & Boxal, 2017; Shao & Tang, 2016). The second strategy used in

literature involves encapsulating emulsion droplets within a gel. Emulsion gels have shown some success in providing gastric stability, attributed to the inhibition of diffusion of pepsin to the surface of emulsion droplets within the gel, largely controlled by the rheology/microstructure of the gel matrix (Guo, Bellissimo, & Rousseau, 2017; Guo, Ye, Lad, Dalgleish, & Singh, 2014; Sarkar, et al., 2015a). An alternative strategy is to embed the emulsion droplets into gelled particles: 'emulsion microgel particles'.

Emulsion microgel particles are a relatively new class of "smart" soft solid vehicles where several emulsion droplets are encapsulated within a biopolymer hydrogel particle (Torres, Murray, & Sarkar, 2016, 2017; Torres, Reyes, Murray, & Sarkar, 2018; Torres, Tena, Murray, & Sarkar, 2017). This structure offers several advantages over conventional O/W emulsions. The soft solid shell encapsulating the emulsion droplets can protect lipophilic bio-actives against oxidation and offers the opportunity to tune its physicochemical properties as a function of environmental conditions (e.g., swell or de-swell as a function of pH, ionic strength, temperature and enzymatic condition), allowing the protection or release of the lipophilic constituents (Beaulieu, Savoie, Paquin, & Subirade, 2002; Gunasekaran, Ko, & Xiao, 2007; Matalanis, Decker, & McClements, 2012; Torres, et al., 2016). Thus, emulsion microgel particles might enable targeted release of bio-active molecules at the different stages of digestion. Previous studies using different types of emulsifiers (e.g., protein) and gelling agents (e.g., alginate, κ-carrageenan, starch, gelatine, casein) to form emulsion-filled hydrogel particles have already started to examine the digestion and release mechanisms of the encapsulated emulsion droplets. (Corstens, et al., 2017; Mun, Kim, Shin, & McClements, 2015; Ozturk, Argin, Ozilgen, & McClements, 2015; Tangsrianugul, Suphantharika, & McClements, 2015; van Leusden, et al., 2018; Zhang, Zhang, & McClements, 2016). Surface erosion of the gel particles during gastric digestion was perceived as the main degradation mechanism for digestible gel matrices (e.g., casein and gelatine). Whilst, the stable gel matrices during gastric

environment should be good candidates for the formation of resistant emulsion microgel particles, the possible thermodynamic incompatibility between different hydrocolloids forming the particles might lead to uncontrolled swelling and diffusion of the lipophilic material (McClements, 2017). Therefore, engineering emulsion microgel particles from a single hydrocolloid (used as both the gelling agent and emulsifier), where the droplets are strongly linked by their adsorbed layer to the surrounding gel, is more likely to prevent any uncontrolled destabilisation due to possible thermodynamic incompatibility, etc. Of course, using a suspension of microgel particles as the carrier of the droplets will be far more versatile, in terms of technological usage, than macroscopic pieces of filled gel

Whey protein, primarily composed of β-lactoglobulin, has been demonstrated to limit pepsinolysis, due to its globular structure (Nacer S, Sanchez, Villaume, Mejean, & Mouecoucou, 2004). Additionally, whey protein has recently been analysed to form cold-set microgel particles and emulsion microgel particles, of around 30 μm size, via the addition of calcium (Ca^{2+}) ions to a preheated whey protein suspension and whey protein-stabilized O/W emulsion. Hence, whey protein can be used as emulsifier and gelling agent to produce emulsion microgel particles with actively bound emulsion droplets. Cold set gelation of whey protein with Ca^{2+} results from the formation of a network between Ca^{2+} and free carboxylic groups found on the acidic amino groups (i.e., aspartic acid and glutamic acid) of the main protein after their exposure on unfolding due to pre-heating (Egan, Jacquier, Rosenberg, & Rosenberg, 2013; Torres, Murray, et al., 2017).

However, to our knowledge no study has yet investigated the digestion mechanism of encapsulated emulsion droplets using only one biopolymer as both emulsifier and gelling agent, nor has the behaviour of whey protein based emulsion microgel particles during in vitro gastrointestinal digestion been investigated.

The hypothesis behind this study is that encapsulating whey protein stabilized O/W droplets into whey protein microgel particles will protect the fine emulsion droplets from gastric flocculation and coalescence. Such gastric stability will allow more efficient release of free fatty acids from the smaller droplets (higher interfacial area) during lipolysis. The first stage of this study was therefore to develop the encapsulation of the droplets into microgel particles and demonstrate their enhanced stability under in vitro gastric conditions. Secondly, the rate of lipolysis under subsequent in vitro intestinal conditions was measured, using the original O/W emulsion (9.6 wt% WPI – 20 wt% oil) as a control. We used a combination of particle size characterization, zeta-potential measurements, confocal microscopic imaging, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of the interfacial protein before and after in vitro gastric digestion as well as pH-STAT based free fatty acid release measurements during in -vitro intestinal digestion (pre- or post-gastric digestion).

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). Porcine pepsin (P7000, 526 U mg^{-1} using haemoglobin as a substrate), porcine pancreatin (P7545, 8 ×USP and trypsin activity of 6.48 U mg^{-1} using TAME, N-p-Tosyl-L-arginine methyl ester hydrochloride, as a substrate) and porcine bile extract B8631 (total bile salt content 49 wt% with 10 – 15% glycodeoxycholic acid, 3 – 9% taurodeoxycholic acid, 0.5 – 7% deoxcycholic acid, 5 wt% phospholipids) were purchased from Sigma-Aldrich Company Ltd, Dorset, UK. All solutions were prepared with Milli-Q water having resistivity of 18.2 MΩ cm at 25 °C (Milli-Q apparatus, Millipore, Bedford, UK).

Nile Red and Rhodamine B were purchased from Sigma-Aldrich (Steiheim, Germany). Dimethyl sulfoxide (DMSO) was purchased from Fluorochem (Hadfield, UK). All other chemicals were of analytical grade and purchased from Sigma-Aldrich unless otherwise specified.

2.2 Preparation of whey protein based emulsion microgel particles

Whey protein emulsion microgel particles were prepared using a bottom-up approach, as reported previously (Torres, Murray, et al., 2017). Briefly, 20 wt% sunflower oil was emulsified with 12 wt% WPI that has been previously heat-treated at 85 °C for 40 min at pH 7 (final concentration of WPI in the emulsion: 9.6 wt%). Secondly, the heat-treated WPI-stabilised emulsion was mixed with a solution of 0.1 M calcium chloride (at a ratio of 55 : 45) and passed once through the Leeds Jet Homogenizer at a pressure of 250 bar. The resulting particles were collected in a beaker and immediately diluted with Milli-Q water to 50 wt% and stirred for 30 min at low speed to limit particle aggregation. Sodium azide (0.02 wt%) was added as an antimicrobial agent to the samples stored for 24 h at 4 °C. For control purposes, whey protein microgel particles (without oil) were also prepared using the same procedure and final concentration of whey protein (9.6 wt%).

2.3 Static in vitro gastric and intestinal digestion

The different samples (WPI microgel particles, emulsion and emulsion microgel particles) were digested by subjecting them to simulated gastric fluid (SGF) mimicking fasted conditions of the stomach or simulated intestinal fluid (SIF) or sequential simulated gastric and intestinal fluids (SGF + SIF) using the slightly adapted digestion protocol of Minekus, et al. (2014) and Sarkar, Murray, et al. (2016) and Mat, Le Feunteun, Michon, and Souchon (2016). Ten

millilitres of each sample were incubated for 2 hours at pH 3 with 7.5 mL of SGF composed of 6.9 mM KCl, 0.9 KH_2PO_4, 72.2 mM NaCl, 0.1 mM $MgCl_2(H_2O)_6$, 0.5 mM $(NH_4)_2CO_3$, 5 µL $CaCl_2$ at 0.3 M, 1.6 mL pepsin (at 2000 U mL^{-1} in the final chyme) and 0.695 µL water. After 2 hours of incubation the pH of the sample + SGF (20 mL) was adjusted to pH 6.8 with 1 M NaOH and mixed with 11 mL of SIF. The SIF after gastric digestion (SGF + SIF) at pH 6.8 contained 6.8 mM KCl, 0.8 mM KH_2PO_4, 123.4 mM NaCl, 0.33 mM $MgCl_2(H_2O)_6$, 40 µL $CaCl_2.H_2O$ at 0.3 mM, 2.5 mL bile salts at 160 mM, 1.31 mL water and 5 mL pancreatin solution (at 800 U mL^{-1} based on trypsin activity). In a separate experiment, the different samples were mixed in SIF in the absence of any pre-gastric digestion. Samples (2 mL) were mixed with 15 mL of SIF (4.7 mM KCl, 0.6 mM KH_2PO_4, 85.7 mM NaCl, 0.8 mM $MgCl_2(H_2O)_6$), 20 mL bile salts at 25 mM, 10 mL $CaCl_2$ at 1.5 mM, 2 mL water and 1 mL pancreatin solution at 498 U mL^{-1} based on trypsin activity. The in vitro intestinal digestion was carried out over 3 hours at pH 6.8 and 37 °C.

Aliquots were collected at different time points throughout the course of in vitro digestions (SGF, SIF and SGF + SIF) and were characterized. To stop pepsin activity at specific time points, 0.2 M sodium bicarbonate at pH 7 was added to the samples. The pancreatin activity was stopped by adding 1 mM of 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (Pefabloc©) at appropriate time points.

2.4 Particle size measurements

Static light scattering was used to measure the size distribution of the emulsion droplets and emulsion microgel particles undergoing in vitro digestion (at 0, 5, 60, 120 min during gastric digestion; at 0, 30, 180 min during intestinal digestion; and at 0, 30, 180 min after gastric and during intestinal digestion) using a Malvern Mastersizer 3000E hydro, (Malvern

Instruments, Worcestershire, UK). Samples were diluted in distilled water until the instrument gave an obscuration of 4 to 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 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. 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.

2.5 ζ-potential measurements

The ζ-potential of the emulsion droplets and emulsion microgel particles undergoing in vitro digestion was determined using a particle electrophoresis instrument (Zetasizer, Nano ZS series, Malvern Instruments, Worcestershire, UK). The emulsion and emulsion microgel particles were diluted to 0.005 wt% droplet concentration. The diluted sample 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.

2.6 Analysis of peptic hydrolysis of interfacial proteins

The protein composition at the interface of the emulsion droplets or encapsulated within whey protein microgel particles before and after in vitro gastric hydrolysis by pepsin was determined by analysing the cream phase using sodium dodecyl sulphate polyacrylamide gel

electrophoresis (SDS-PAGE) under reducing conditions (Sarkar, Ademuyiwa, et al., 2018; Sarkar, Murray, et al., 2016). For control purposes the protein compositions of 9.6 wt% WPI solution and whey protein microgel particles without any oil droplets were also determined. The different samples before and after in vitro gastric digestion were centrifuged for 40 min at 14,500 g and 20 °C. The cream layer of the emulsion and emulsion microgel particles was carefully removed, dispersed in Milli-Q water (to obtain a final concentration of WPI of 0.192 wt%) and centrifuged again for 40 min at 14500 g and 20 °C. Approximately, 65 μL of cream layer was carefully collected and mixed with 25 μL of SDS sample buffer (62.5 mM Tris-HCl, pH6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue) and 10 μL of diothiothreitol (DTT, of a final concentration of 50 mM) and heat treated at 95 °C for 5 min. The SDS-PAGE was carried out by loading 5 μL of standard protein marker and 10 μL of sample into gels previously prepared on a Mini-PROTEAN II system (Bio-Rad Laboratories, Richmond, CA, USA). The resolving gel contained 16% acrylamide and the stacking gel was made up of 4% acrylamide. The SDS-PAGE ran for 60 min at 100 V. After running, the gel was rinsed in Milli-Q water and stained for 2 h with 90% Proto-Blue Safe Colloidal Coomassie G-250 stain and 10% ethanol solution. The gels were destained in Milli-Q water overnight and scanned and analysed using a Gel Doc^{TM} XR+ System (Bio-Rad Laboratories, Richmond, CA, USA). Each band within the lanes was selected automatically by the software to cover the whole band. Background intensity was subtracted after scanning an empty lane. The SDS PAGE experiments were carried out in triplicates and band intensities was reported as an average and standard deviation of three reported readings.

2.7 Analysis of free fatty acid release after in vitro intestinal digestion

The free fatty acid release from the emulsion and emulsion microgel particles was analysed during the in vitro intestinal digestion without or with in vitro gastric digestion, the latter

subsequently described as sequential digestion. The in vitro intestinal digestion was carried out over 1 hour whilst maintaining the pH at 6.8 by the addition of 0.05 M NaOH using a pH-STAT (TIM 854, Radiometer). The volume of 0.05 M NaOH added to the samples was used to calculate the concentration of free fatty acid (FFA) generated in the reaction vessel during digestion of the emulsified or encapsulated lipids. The percentage of FFA released was calculated, taking into account the auto-digestion of pancreatic juice (assuming the generation of 2 FFAs per triacylglycerol molecule by the action of lipase action) using eq (1) (Sarkar, Murray, et al., 2016):

$$\%FFA = 100 \times \left(\frac{V_{NaOH} M_{NaOH} Mw_{Lipid}}{2 \times W_{Lipid}}\right) \quad (1)$$

where V_{NaOH} is the volume (mL) of sodium hydroxide, M_{NaOH} is the molarity of sodium hydroxide (0.05 M), Mw_{Lipid} is the average molecular weight of sunflower oil (0.880 kg mol^{-1}) and W_{Lipid} is the weight of lipid initially present in the reaction vessel.

The kinetics of the FFA released from the emulsion or emulsion microgel particle was analysed using a nonlinear regression model eq (2):

$$\Phi(t) = \Phi_{max} + (\Phi_0 - \Phi_{max})\exp(-kt) \quad (2)$$

where Φ (t) is the amount of FFA released at time in the in-vitro intestinal digestion, Φ_{max} is the maximum FFA released that can be obtained during the simulated intestinal digestion, Φ_0 is the amount of FFA released at time 0 min during the in vitro intestinal digestion, k is the digestion rate constant and t is the time in during the simulated intestinal digestion. At the start of the simulated intestinal digestion (t = 0 min), it was assumed that no FFA were released, resulting in $\Phi_0 = 0$. Therefor eq 2 can be simplified into eq 3 (Sarkar, Ye, & Singh, 2016):

$$\Phi(t) = \Phi_{max}(1 - \exp(-kt)) \quad (3)$$

The FFA versus digestion time resulting from the lipolysis reaction can then be characterised

using quantitative terms, such as Φ_{max} and k, by fitting eq 3 to the experimental data and finding the values that minimize the difference between the experimental data and the model.

Origin 2015 was used to fit the nonlinear regression model to the experimental data and solve Φ_{max} and k.

2.8 Confocal scanning laser microscopy

Emulsion microgel particles undergoing in vitro digestion were imaged using a confocal laser scanning microscope (CLSM). A Zeiss LSM 700 CLSM (Carl Zeiss MicroImaging GmbH, Jena, Germany) with a 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 emulsion and emulsion 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.

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 In vitro gastric digestion of emulsion microgel particles

285 Figures 1A and B highlight the particle size distribution of both the whey protein
286 stabilised-emulsion and emulsion microgel particles undergoing either a change of pH (i.e.,
287 from pH 7 to pH 3) or undergoing simulated gastric digestion over time in presence of pepsin
288 (i.e., 5, 60, 120 min). At pH 7 and in absence of SGF, the majority of the emulsion droplets
289 were in the range of 0.01 – 5 μm, with d_{32} = 0.13 μm and d_{43} = 0.76 μm. The emulsion droplets
290 were negatively charged with a ζ-potential of –47.5 mV (Figure 2A) and the emulsion appeared
291 to consist of uniformly dispersed droplets as observed via CLSM (Figure 1C). The decrease of
292 pH to pH 3 in the presence of SGF without pepsin led to the expected charge reversal of
293 emulsion droplets due to the protonation of the ionisable groups. The ζ-potential did not reach
294 high magnitudes at pH 3 (+32.2 mV) owing to some degree of electrostatic screening of WPI
295 by SGF ions (Sarkar, Goh, et al., 2010; Zhang, Zhang, & McClements, 2017) (Figure 2A),
296 meanwhile such charge screening effects did not influence the droplet size distribution (Figure
297 1A). In the presence of SGF containing pepsin, emulsions underwent a drastic increase in
298 droplet size, ranging from 3 to 800 μm, suggesting flocculation or even coalescence of droplets,
299 possibly due to the hydrolysis of the interfacial protein by pepsin (Singh, Ye, & Horne, 2009).
300 CLSM images of the emulsion + SGF after 120 min provided further evidence of the
301 flocculation of the droplets (Figure 1D), where large flocs of emulsion droplets of >10 μm can
302 be observed. A few much larger droplets are also noticeable, suggestive of coalescence during
303 the simulated gastric digestion, as observed in previous studies (Golding, et al., 2010;
304 Macierzanka, Sancho, Mills, Rigby, & Mackie, 2009; Sarkar, Goh, et al., 2010; Sarkar, et al.,
305 2009; Singh, et al., 2011).

306 Shifting the focus to emulsion microgel particles at pH 7 in the absence of SGF, the
307 particles ranged in size from 3 to 1000 μm with d_{32} = 57 μm and d_{43} = 206 μm (Figure 1B) and
308 a ζ-potential of -21.2 mV (Figure 2B). This suggests that the fine emulsion droplets were
309 clustered into discrete emulsion microgel particles (Figure 1B), in accordance with previous

studies (Torres, Murray, et al., 2017). From CLSM images it is noticeable that all the emulsion droplets (stained in red) are encapsulated within the WPI matrix (stained in green) with no significant free or coalesced oil droplets (Figure 1E). The change in pH from pH 7 to pH 3 did not affect the emulsion microgel particle size distribution, although the ζ-potential became positive (+22.2 mV) due to the protonation of the WPI (Figure 2B) (Zhang, et al., 2017). Interestingly, even in presence of pepsin in the SGF, the particle size distribution of the emulsion microgel particles decreased only slightly, in contrast to the emulsion droplets. After 120 min of residence in SGF + pepsin, the emulsion microgel particle size decreased to d_{32} = 22 μm and d_{43} = 96 μm, reflecting the changes observed in the CLSM images (Figure 1F). Note that there was no evidence of large emulsion droplets (i.e., no coalescence) or significant release of droplets from the microgel particles, based on the particle size distribution data or the CLSM images. The ζ-potential data also remained fairly constant (no statistical difference was obtained over time, $p > 0.05$ (Figure 2B)). Therefore, at this early stage, it can be speculated that the decrease in size of the emulsion microgel particles (Figure 1B) is probably due to pepsin rupturing the protein network on the outside of the microgel particles, eroding them slightly, rather than degrading the interfacial protein of individual emulsion droplets, which would be expected to produce coalescence. The surface erosion of the microgel particles, rather than their degradation, was further confirmed by the analysis of microgel particles (for information on particles without any emulsion droplet, see Supplementary Figures S1 and S2).

In order to better understand the gastric stability of WPI stabilised-emulsion and emulsion microgel particles towards pepsinolysis, the hydrolysis patterns of the adsorbed protein phase (i.e., the cream layer) from the emulsion and the emulsion microgel particles are presented in Figure 3. As controls, an aqueous dispersion of 9.6 wt% whey protein and whey protein microgel particles (without any encapsulated emulsion droplets) were also analysed. In agreement with findings elsewhere, whey protein, composed primarily of β-lactoglobulin (18.4

kDa) and α-lactoglobulin (14 kDa), is resistant to pepsin-induced digestion owing to their globular conformation (Guo, Fox, Flynn, & Kindstedt, 1995; Macierzanka, et al., 2009). From Figure 3A and B (lines a and a'), after 120 min in SGF 51% of α-lactoglobulin and 92% of β-lactoglobulin remained as compared to the non-digested whey protein solution. Due to the globular nature of β-lactoglobulin, pepsin has very limited access to the carboxyl side of the aromatic amino acid buried inside the β-lactoglobulin dimers (Guo, et al., 1995; Luo, Borst, Westphal, Boom, & Janssen, 2017; Nacer S, et al., 2004).

The formation of whey protein microgel particles via a process of heat treatment and Ca^{2+} - induced gelation under turbulent flow conditions (Torres, Murray, et al., 2017) - led to WPI particles slightly less resilient towards digestion (Figure 3A and B, lines b and b'). After 120 min in SGF, α-lactoglobulin and β-lactoglobulin were broken down into peptides with Mw < 10 kD, although considerable quantities of intact α-lactoglobulin (45%) and β-lactoglobulin (70%) remained. This is most obviously explained by the heat treatment, causing some unfolding of β-lactoglobulin, enhancing the digestibility of WPI (Beaulieu, et al., 2002; Mackie & Macierzanka, 2010). From a previous study, heat treating whey protein at 85 °C for 40 min has been estimated to lead to protein denaturation by over 85% (Torres, Murray, & Sarkar, 2017). However, the cross-linking of whey protein with Ca^{2+} might have created a network around the aromatic amino acids of β-lactoglobulin, limiting the access to pepsin and slowing down pepsinolysis.

In the case of whey protein stabilised-emulsion (previously heat treated), α-lactoglobulin and β-lactoglobulin in the adsorbed phase also appeared to break down into peptides although 42% and 75% of the intact protein remained, respectively, (Figure 3A and B, line c and c'). Previous studies have shown that alongside heat treatment, emulsification also unfolds the secondary structure of β-lactoglobulin at the oil droplet interface which would increase the accessibility of pepsin towards the polypeptide chain (Macierzanka, et al., 2009; Mackie, et al.,

2010; Sarkar, et al., 2009). However, due to the acidic conditions in the gastric phase, increased internal hydrogen bonding can occur between two carboxyl groups or one carboxyl group and one amine group limiting the accessibility to pepsin (Nacer S, et al., 2004; Reddy, Kella, & Kinsella, 1988). The large flocs observed during simulated gastric digestion (Figure 1D) also suggested the formation of a coarse network which might further protect the protein from pepsinolysis.

In the case of the whey protein emulsion microgel particles, a considerable amount of interfacial material remained in the stacking gel. The emulsion microgel particle aggregates were possibly too large (> 250 kDa) to enter the resolving gel (Figure 3A, line d). The hydrolysis pattern of the emulsion microgel particles was similar to that of the whey protein microgel particles alone and the emulsion alone (Figure 3B, lines b', c' and d'), although slightly more intact *β*-lactoglobulin remained (85%) after 120 min of in vitro gastric digestion. A potential cause of this lower pepsinolysis of the emulsion microgel particles might be related to the mesh size of the emulsion microgel particles (Beaulieu, et al., 2002; Gunasekaran, et al., 2007; Sarkar, et al., 2015b). From previous studies, it was suggested that the theoretical mesh size of WPI microgel particles and WPI emulsion microgel particles is related to the elastic modulus of the gel network as well as the interfacial tension and emulsion droplet size of the encapsulated emulsion droplets. From the rubber elasticity theory modified by Flory the mesh size of the model whey protein gel can be calculated, via Equation 1:

$$\xi^3 = \frac{\kappa_B T}{G'_m} \quad (1)$$

where κ_B is the Boltzmann constant, T is the temperature and G'_m the storage modulus of the starch gel.

The estimation of the mesh size of an emulsion gel can be achieved using the Palierne model (Bousmina, 1999; Palierne, 1990, 1991), which takes into account the interfacial tension, the oil droplet size and the oil content in the emulsion (Equation 2):

$$G_b^*(\omega) = G_m^*(\omega)\frac{1+3\phi H(\omega)}{1-2\phi H(\omega)} \quad (2)$$

where $H(\omega) = \frac{4(\alpha/R)\left[2G_m^*(\omega)+5G_d^*(\omega)\right]+\left[G_d^*(\omega)-G_m^*(\omega)\right]\left[16G_m^*(\omega)+19G_d^*(\omega)\right]}{40(\alpha/R)\left[G_m^*(\omega)+G_d^*(\omega)\right]+\left[2G_d^*(\omega)+3G_m^*(\omega)\right]\left[16G_m^*(\omega)+19G_d^*(\omega)\right]}$

with, ϕ the concentration of oil, R the average radius of the emulsion droplets, α the interfacial tension of the OSA starch, ω the frequency, G^*_m, G^*_d and G^*_b the complex shear moduli of the matrix, the emulsion droplets and the emulsion gel, respectively.

Therefore, from equation 1 the mesh size of the whey protein gel was estimated to be 24.5 nm, whilst from equation 2 the mesh size of the emulsion gel was estimated to be 6.6 nm (Torres, Murray, et al., 2017). However, it should be recognized that the mesh size for the filled microgel particles could be misleading. The concentration of protein is the same for both microgel particles the overall modulus of the filled gelled phase does not necessarily translate to the equivalent modulus of an unfilled gel. However, the local structure of the protein gel in the vicinity of the surface of the droplets is likely to be affected, since the protein on the surface of the droplets is apparently actively bound to the bulk gel matrix (Dickinson, 2012; Dickinson & Chen, 1999). More importantly, the model used assumes the droplets are randomly distributed throughout the protein gel phase. We have little evidence that is not the case, but some droplet aggregates are seen and if some aggregates extend to form strands within some microgel particles, possibly even a secondary network, then this could have even larger effects on their overall modulus, making the calculation increasingly invalid. Unfortunately, no such

models seem to exist for dealing with such complexity and so we believe it is still worth stating the values calculated, recognizing the system may be considerably more complex than the calculation implies.

The radius of gyration of pepsin is ca. 2.3 nm (Amsden, 1998), so pepsin would more easily diffuse into the larger WPI microgel particle pores, resulting in a higher digestibility. In the case of the emulsion microgel particles, pepsin might only be able to digest a thin layer of WPI at the surface of the particles (Luo, Boom, & Janssen, 2015; Luo, et al., 2017). The tortuous network of the droplets within the emulsion microgel particles might also hinder pepsin reaching the interfacial whey protein of all the droplets. This would explain the decrease in size of the emulsion microgel particles rather than the release and coalescence of free oil droplets.

Therefore, we propose that the protection of the emulsion microgel particles from complete pepsinolysis was possibly caused by the chemical and/or physical mechanisms depicted in Figure 4. The key chemical mechanisms might be 1) the binding of calcium ions to whey protein limiting the access to the active sites of pepsin, 2) the increased internal hydrogen bonding at acidic pH, both of which would restrict the diffusion of pepsin to the aromatic amino acid. On the other hand, the physical mechanism might be that 3) the small mesh size of the particles would inhibit or slow down the diffusion of pepsin inside the emulsion microgel particles.

3.2 In vitro intestinal digestion of emulsion microgel particles

Simulated intestinal digestion of the emulsion and emulsion microgel particles were carried out using two conditions, one without simulated gastric pre-digestion and another post

simulated gastric digestion, i.e., sequential gastric-to-intestinal digestion, to understand the distinctive influence of the simulated gastric and intestinal regimes.

Figure 5 shows the particle size distribution and representative CLSM images of the emulsion and emulsion microgel particles undergoing in vitro intestinal digestion with or without the in vitro gastric pre-digestion step. Without the pre-gastric digestion, 30 min was enough to destabilise the emulsion. The emulsion droplets became polydisperse, with prominent peaks at about 100 – 1000 μm, suggesting coalescence and confirmed by the CLSM images. The substantial decrease of the ζ-potential from −47.5 ± 0.9 mV before in vitro intestinal digestion, to −78.2 ± 1.1 and −91.3 ± 4.4 mV at 30 min and 180 min after in vitro intestinal digestion, respectively, corroborates the emulsion droplet destabilization (Figure 6A). During the in vitro intestinal digestion, bile salts will displace the WPI from the interface allowing access of lipase. The lipolysis of the oil droplets will produce free fatty acids, as well as mono- and diglycerides, which are negatively charged surface active digestion products, decreasing the ζ-potential of the emulsion (Mackie, et al., 2010; Sarkar, Horne, & Singh, 2010a, 2010b; Sarkar, Ye, et al., 2016; Torcello-Gomez, Maldonado-Valderrama, Martin-Rodriguez, & McClements, 2011). The emulsion undergoing in vitro intestinal digestion post-gastric digestion behaved differently. The emulsion droplet size distribution at both 30 and 180 min showed polydispersity with a prominent peak ranging between 1 and 50 μm (Figure 5C and D). Additionally, the ζ-potential of the emulsion was stable over time, at around −40 mV (Figure 6A). The flocculation of the emulsion droplets in the SGF might have delayed the displacement of whey protein from the interface by bile salts. Additionally, the flocculation and coalescence of the oil droplets in the gastric regime decreases the interfacial area, that would reduce the lipolysis kinetics (Torcello-Gomez, et al., 2011).

For the emulsion microgel particles, the in vitro intestinal digestion without any pre-gastric digestion led to some release of the emulsion droplets, as observed by the peak ranging

from 0.01 to 1 μm after both 30 and 180 min in Figure 5E and F. From the CLSM images, the released emulsion droplets after 30 min did not seem to have coalesced, no large oil droplets are noticeable (Figure 5E). Interestingly, after 180 min the particle size distribution of the emulsion microgel particles did not appear to have changed significantly, although due to the polydispersity of the sample, a few large droplets were evident from the CLSM micrographs (Figure 5F).The ζ-potential measurements showed an initial net decrease in the ζ-potential from -21.2 ± 2.2 to -52.9 ± 2.4 mV at time 0 and 30 min, respectively (Figure 6B). Over the next 180 min, the ζ-potential stabilized at an average value of -47.8 ± 2.1 mV. At intestinal pH (pH 6.8) and ionic strength, the deprotonation of the carboxyl groups of β-lactoglobulin drastically increased ($p < 0.05$) the net negative charge of the emulsion microgel particles, hence contributing to higher repulsive forces. This electrostatic repulsion might have led to the swelling of the particles via water absorption, which might allow the release of emulsion droplets (Beaulieu, et al., 2002; van Leusden, et al., 2018). The swelling of the microgel particles was particularly noticeable by the particle size change of the whey protein microgel particles (containing no oil) over the intestinal digestion time (see Supplementary Figure S2). The increase of the particle size might also result from the aggregation of fragmented particles during intestinal digestion. Additionally, the large decrease in ζ-potential might also suggest surface erosion and destabilisation of the whey protein microgel particles by the trypsin in the pancreatin, allowing the release of the emulsion droplets, which had a ζ-potential of -47.5 mV (Figure 2A) (see Supplementary Figure S4 for particles without oil droplets showing no noticeable change). The stability of the ζ-potential over the next 180 min suggested that pancreatin did not significantly hydrolyse further the interfacial protein on the emulsion droplets. In contrast, the behaviour of emulsion microgel particles during in vitro intestinal digestion post-gastric digestion differed. It is noticeable from Figure 5G that, after 30 min, all the microgel particles were hydrolysed, releasing their emulsion droplets, which subsequently

coalesced. After 180 min, fewer and smaller oil droplets can be observed in the CLSM micrographs suggesting almost complete lipolysis of the oil droplets. However, it should be noted that large coalesced oil droplets might still be present, as depicted by the light scattering results (Figure 5H), which might have migrated to the top of the microscopic slide due to density gradient and were not captured during imaging. The ζ-potential after 180 min slightly decreased to −26.0 ± 1.3 mV.

To assess the impact of emulsion droplet encapsulation in microgel particles, the free fatty acid (FFA) release during intestinal digestion was monitored via a pH-STAT technique (at 37 °C) (Figure 7). The experimental data was fitted with a nonlinear regression mathematical model (eq 3) and the corresponding fitting parameters (the rate constant, k and the maximum FFA release, Φ_{max}) are also reported in Figure 7. It should be noted that this may not be the best model for the initial part of the digestion, since this model assumes individual emulsion droplets rather than clustered droplets. However, the fitting still gives some indication of the effects on the rate constant as well as the half time of the digestion.

For control purposes, the FFA release of both emulsion and emulsion microgel particles pre-gastric digestion was also assessed and reported in the supplementary information (Figure S5). As a general trend, both pre-gastric digested emulsions and emulsion microgel particles showed a steep increase in the percentage of FFA release but this stabilised after 30 min. The total amount of FFA released as well as the rate constant and half time of both emulsion and emulsion microgel particles were not significantly different ($p > 0.05$). After 60 min of in vitro intestinal digestion, the emulsion generated a FFA release of 54.9 ± 3.7%, whilst the emulsion microgel particles generated a release of 60.5 ± 3.2% FFA (Figure S5). Therefore, it can be assumed that after the full 3 hours of intestinal digestion all the FFA (66.66%) would have been released from both the emulsion and emulsion microgel particles. During the intestinal digestion, lipolysis of the emulsion droplets only occurs after bile salts displace the emulsifier

506 from the oil-in-water interface. Previous studies have demonstrated that bile salts displace
507 protein via an orogenic displacement process involving the nucleation of bile salt domains at
508 weak points within the protein network film (Mackie, Gunning, Wilde, & Morris, 2000).
509 Subsequently, lipase-colipase complexes are able to adsorb to the oil interface to initiate the
510 hydrolysis of the emulsion droplets.

511 In O/W emulsions, bile salts generally rapidly displace WPI from the interface permitting
512 lipase to adsorb and release FFAs (Maldonado-Valderrama, et al., 2008; Sarkar, Horne, et al.,
513 2010a; Singh, et al., 2011). Such results are in accordance with the sharp decrease in ζ-potential
514 data (< –75 mV) obtained in the first 30 min of the pre-gastric in vitro intestinal digestion. In
515 the emulsion microgel particles a limited or delayed lipolysis might have been expected.
516 However, the swelling capacity of the whey protein microgel particles at pH 6.8 might have
517 allowed relatively easy diffusion of trypsin and chymotrypsin into the particles, which would
518 then hydrolyse the whey protein and break up the protein gel network that previously
519 immobilized the droplets. Free emulsion droplets could then diffuse out of the swollen and
520 fragmenting microgel particles into the continuous phase. Lipase and bile salts might also be
521 able to diffuse into the microgel particles to reach the oil-water interface initiating the lipolysis
522 of the emulsion droplets. The bile salts, as well as displacing the interfacial protein, have also
523 been demonstrated to destabilise the tertiary structure of β-lactoglobulin, accelerating its
524 proteolysis by both trypsin and chymotrypsin (Gass, Vora, Hofmann, Gray, & Khosla, 2007;
525 Reddy, et al., 1988). This whey protein breakdown might further aid dissolution of the WPI
526 microgel particles and allow access of lipase to the emulsion droplet interface, as well as
527 releasing emulsion droplets into the aqueous phase for lipase to hydrolyse.

528 From Figure 7, it is noticeable that the emulsion post-gastric digestion had a
529 significantly lower ($p < 0.05$) release of FFA after 60 min with $\Phi_{max} = 44.1 \pm 3.1\%$, as compared
530 to the FFA release from emulsion pre-gastric digestion ($\Phi_{max} = 54.9 \pm 3.7\%$, Figure S5). The

oil droplet coalescence during the in vitro gastric digestion (Figure 5) might have affected the FFA release. Without pre-gastric digestion, the emulsion droplets coming into contact with the simulated intestinal phase had a size ranging from 0.1 to 5 μm, whereas post-gastric digestion these were nearly 10 times larger (compare Figure 1A to Figure 5). As mentioned above, the rate of lipolysis is inversely proportional to the oil droplet size, since with larger oil droplets a lower number of triacylglycerol molecules are exposed to lipase (Golding, et al., 2010; Mackie, et al., 2000; Singh, et al., 2009). Additionally, flocculation of WPI during the in vitro gastric digestion (as seen in Figure 1A and D), which appeared to form a network of aggregated WPI around the emulsion droplets, might have slightly restrained the diffusion of the bile salts and lipase to the oil droplet interface.

In comparison, encapsulating the emulsion droplets into whey protein microgel particles allowed a similar FFA release to the emulsion and emulsion microgel particle pre-gastric digestion (compare Figure 7 and Figure S5). As observed during the simulated gastric phase, the emulsion droplets seemed to have been protected from any flocculation and/or coalescence. Thus, during the simulated intestinal phase, the swelling and breakdown of the microgel particles through the action of pH, bile salts and proteolysis allows the release of the fine encapsulated emulsion droplets which can be readily hydrolysed. These results suggest that encapsulating the emulsion droplets into whey protein microgel particles protects them from degradation, uncontrolled flocculation and coalescence in the gastric regime, enabling a more complete release of FFA during subsequent intestinal digestion.

4. Conclusion

This study has shown that whey protein based emulsion microgel particles have the ability to protect and target the release of emulsion droplets at a desired physiological site. Under in vitro gastric conditions (i.e., acidic pH and in the presence of endoproteinase (pepsin)), non-

encapsulated emulsion droplets were destabilised and coalesced in an uncontrolled manner. In comparison, encapsulating the emulsion droplets into whey protein microgel particles protected the emulsion droplets from flocculation and coalescence. The formation of a network between calcium ions and the carboxylic groups of whey protein possibly protected the aromatic amino acids of the protein from the cleavage by pepsin, hindering the proteolysis of the emulsion microgel particles. Also the tighter network density and henceforth the smaller mesh size of the microgel particles also possibly prevented the enzyme from diffusing to the surface of the emulsion droplets, limiting pepsinolysis of the interfacial protein. Under in vitro intestinal conditions the whey protein microgel particles swelled and disintegrated due to the combined action of pH, bile salts and proteolysis allowing the full release of the free fatty acids from the emulsion droplets. Thus, emulsion microgel particles might have applications for encapsulation of lipophilic bioactive material that needs stability in the gastric phase but complete release in the intestinal phase.

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