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Conjugate microgel-stabilized Pickering emulsions: Role in delaying gastric digestion

Andrea Araiza-Calahorra[a], Zachary J. Glover[a,b], Mahmood Akhtar[a] and Anwesha Sarkar[a]*

[a] *Food Colloids and Bioprocessing Group, School of Food Science and Nutrition, University of Leeds, Leeds, LS2 9JT, UK*

[b] *Department of Physics, University of Southern Denmark, Campusvej 55, 5230, Odense, Denmark*

*Corresponding author:

Dr. Anwesha Sarkar

Food Colloids and Bioprocessing Group,

School of Food Science and Nutrition, University of Leeds, Leeds LS2 9JT, UK.

E-mail address: A.Sarkar@leeds.ac.uk (A. Sarkar).

Abstract

In this study, a new class of microgels called 'conjugate microgels' was designed, where whey protein isolate (WPI) was conjugated with dextran (Dx, 500 kDa) (WPI-Dx) via Maillard reaction before fabricating the microgel particles. Such microgel particles were assessed for their abilities to act as Pickering stabilizers for oil-in-water emulsions and also checked if they offered gastric stability to the Pickering emulsions during *in vitro* digestion against interfacial pepsinolysis. WPI-Dx conjugates were obtained by controlled dry heating (60 ^{o}C, 79% RH, 24-48 h incubation). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and *ortho*-phthaldialdehyde (OPA) profile revealed that the degree of conjugation ranged from 11.6 to 28.1%. The WPI-Dx conjugates were re-dispersed and heat-treated to form heat-set gels with moduli ranging from ~45 to 250 kPa. Microgel particles (hydrodynamic diameters of 130–150 nm, ζ-potentials of –4.5 to –8.0 mV) were created by controlled shearing of these heat-set gels. Interfacial shear rheology measurements and microscopic examination confirmed that conjugated microgel particles with lower degree of conjugation ($WPDx_{10}M$) were effective as Pickering stabilizers. When present in an aqueous dispersion, $WPDx_{10}M$ had reduced the degree of gastric proteolysis (120–130 μM free NH_2) as compared to non-conjugated counterparts (187–205 μM free NH_2). When present at the droplet surface, cross-correlation image analysis revealed that $WPDx_{10}M$ was successful in delaying interfacial gastric proteolysis. Insights from this study suggest that conjugate microgel particles might be useful to design gastric-stable Pickering emulsions in the future for effective delivery of lipophilic compounds to the intestines.

Keywords

Maillard reaction; protein-polysaccharide conjugate; Pickering emulsions; microgel; gastric digestion; *in vitro* model

1. Introduction

Proteins are widely used as ingredients in complex colloidal systems to bring microstructural functionality to the food products such as the stabilization of emulsions and foams, thickening and gelation. Numerous attempts have been made in literature to further improve the functional properties of proteins to provide resilience to environmental stresses such as pH, ions and heat treatments for applications in food, pharmaceutical and cosmetic industries. Such modifications to proteins have been obtained through physical, chemical and/or enzymatic treatments (Akhtar & Ding, 2017; Rodríguez Patino & Pilosof, 2011). Chemical modifications have not been widely used due to associated safety issues or lack of acceptance by consumers, while enzymatic cross-linking is time consuming and often not affordable (Chevalier, Chobert, Popineau, Nicolas, & Haertlé, 2001; van Beilen & Li, 2002). Nevertheless, covalent linkage (or glycation) with polysaccharides *via* the Maillard reaction, has been widely used to improve the functional properties of proteins (Akhtar & Dickinson, 2003; Dickinson & Semenova, 1992; Goh, Sarkar, & Singh, 2014; Kato, Sato, & Kobayashi, 1989; Wong, Day, & Augustin, 2011).

Covalent conjugation between proteins and polysaccharides is formed through the condensation of the reducing sugar of the polysaccharide and the deprotonated ε-amino group of a lysine residue, which are the primary source of reactive amino groups in proteins (Kato, 2002). An important reason why Maillard conjugation between proteins and polysaccharides has gained significant interest is that, as opposed to other methods of conjugation such as acetylation, amidation, and succinylation, the Maillard reaction occurs naturally during thermal processing, which means it does not require additional chemical reactants other than the naturally present reducing sugar and the lysine residues in the proteins (Oliver, Melton, & Stanley, 2006). More importantly, through a well-controlled Maillard reaction, the protein functionality can be significantly improved for novel food applications. For example,

77 numerous studies have investigated the use of different proteins and polysaccharides, such as
78 β-casein-glucose (Darewicz & Dziuba, 2001), soy protein-dextran (Diftis & Kiosseoglou,
79 2004), β-lactoglobulin-dextran (Dickinson & Galazka, 1991), whey and egg white protein-
80 glucose 6-phosphate (Aoki, Fukumoto, Kimura, Kato, & Matsuda, 1994), casein-maltodextrins
81 (Shepherd, Robertson, & Ofman, 2000). Most of these studies were oriented towards
82 improving protein solubility, emulsifying and foaming capacity, or to improve the resilience
83 of the colloidal systems against environmental stresses (pH, ions etc.).

84 Most food proteins have a well-defined secondary and tertiary structure such that they
85 aggregate spontaneously and irreversibly depending on the degree and rate of heat-treatment
86 applied. It has been observed that Maillard conjugation of proteins with polysaccharides tends
87 to influence the final textural properties of heat, cold or acid-induced gels (Cabodevila, Hill,
88 Armstrong, Sousa, & Mitchell, 1994; Matsudomi, Nakano, Soma, & Ochi, 2002; Meydani,
89 Vahedifar, Askari, & Madadlou, 2019; Spotti, et al., 2019; Spotti, et al., 2013a, 2013b; Sun, et
90 al., 2011; Sun, Hayakawa, & Izumori, 2004). For example, studies on the gelation properties
91 of soy protein isolate-xylose of glucono-δ-lactone, dried egg white-galactomannan and
92 ovalbumin-ketohexose conjugated gels have shown that enhanced fracture properties and
93 reduced syneresis can be achieved by the Maillard reaction, as compared to their controls.
94 Nevertheless, there is paucity of studies focusing on the influence of the Maillard reaction on
95 the gelation of whey protein isolate-dextran conjugated systems (Spotti, et al., 2019; Spotti, et
96 al., 2013a, 2013b; Sun, et al., 2011). Dextrans are widely used to conjugate proteins since they
97 are reductive in nature and their neutral character inhibits the formation of any electrostatic
98 complex with proteins. In addition, they are suitable for protein gelation studies since they are
99 unable to form gel-like structures (Sun, et al., 2011). Nevertheless, the use of such conjugated
100 heat-set gels to create microgel particles and use them as Pickering stabilizer has not been
101 investigated so far.

In our previous study, we have developed and characterised whey protein nanogel particles (WPN) as Pickering stabilizers for oil-in-water emulsions (Araiza-Calahorra & Sarkar, 2019b). Furthermore, we have demonstrated that electrostatic deposition of dextran sulphate of a molecular weight of 500 kDa, to the cationic WPN can decrease the rate and extent of gastric proteolysis of the WPN-interfacial layer and prevent gastric coalescence of the Pickering emulsion droplets in simulated gastric conditions at pH 3.0 (Araiza-Calahorra & Sarkar, 2019a). However, it is well known that human physiology has a complex milieu of pH, ionic conditons and bio-surfactants and therefore, electrostatic complexation between proteinaceous particles and polysaccharide might not provide sufficient barrier to droplets against physiologically-driven coalescence (Sarkar, Zhang, Holmes, & Ettelaie, 2019; Singh & Sarkar, 2011).

Hence, this study aims to design, for the first time, oil-in-water Pickering emulsions stabilized by whey protein isolate (WPI) − dextran (Dx) conjugated micrometric-sized gel particles as a Pickering stabilizer, and test its efficacy in delaying gastric proteolysis of the interfacial material. The mechanical properties of heat-induced WPI-Dx conjugate gels obtained via Maillard reaction was investigated and the gastric fate of the microgel particles designed by a top-down shearing approach of these conjugate heat-set gels and corresponding Pickering emulsions was studied. In addition, we introduced a proof-of-concept cross-correlation image analysis of the emulsion systems to quantify and analyse the protein hydrolysis caused by pepsin. We hypothesize that conjugation of proteins with polysaccharide creates a tortuous network in the microgel particles that is capable of delaying the digestibility of the proteinaceous microgel particles by pepsin during simulated gastric digestion.

2. Materials and methods

2.1. Materials

Whey protein isolate (WPI) with ≥ 90% protein content was gifted by Fonterra Co-operative Group Limited (Auckland, New Zealand). Dextran (Dx) of molecular weight (MW) 500 kDa and porcine pepsin (P7000, measured enzyme activity: 371 U mg^{-1} using haemoglobin as the substrate) were purchased from Sigma-Aldrich Company Ltd (Dorset, UK). Medium-chain triglyceride (MCT-oil) Miglyol® 812 with a density of 945 kg m^3 at 20 ºC was used as the lipid phase (Cremer Oleo GmbH & Co, Germany). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) reagents including Bolt™ 4-12% Bis-Tris Plus gels, 20x Bolt™ MES SDS Running Buffer, 4 x Bolt™ LDS Sample Buffer and PageRuler™ Plus Pre-stained Protein Ladder were purchased from Thermo Fisher Scientific (Loughborough, UK). All reagents were of analytical grade and used without further purification unless otherwise reported. All solutions were prepared with Milli-Q water with a resistivity of 18.2 MΩ cm at 25 °C (Milli-Q apparatus, Millipore, Bedford, UK). Sodium azide (0.02 wt %) was added as a preservative.

2.2. Preparation of whey protein isolate - dextran conjugate

Maillard conjugates of WPI and Dx (WPI-Dx) were prepared using the method described by Ding, Valicka, Akhtar, and Ettelaie (2017). Briefly, WPI and Dx were completely dissolved in a 1:2 w/w ratio in 100 mL Milli-Q water with gentle stirring under room temperature. The pH of the solution was adjusted to either pH 7.0 or 11.0 as shown in Table 1. The solutions were stored in the refrigerator at 4 ºC overnight and then frozen at -20 ºC for 6 h. These were then freeze dried for a period of 24 h. After freeze drying, Maillard reaction of the resulting WPI and Dx mixture was promoted by incubating the powder in a desiccator pre-heated at 60 ºC for 24 to 48 hours, with a relative humidity (79%) controlled by saturated potassium bromide (KBr) solution. The WPI-Dx conjugates of different degrees of conjugation (DC) were

151 stored in a dark and dry place for further characterization. An untreated WPI-Dx mixture *i.e.*
152 non-conjugated WPI-Dx, without any modification was similarly prepared as a control.
153
154 *2.3 Determination of free amino groups*
155 The DC of the conjugates and degree of hydrolysis during gastric digestion of samples were
156 quantified by detecting the content of free amino groups using a standardized *ortho*-
157 phthaldialdehyde (OPA) method, as described by Nielsen, Petersen, and Dambmann (2001)
158 with minor modifications. Briefly, the OPA reagent was prepared using 3.81 g sodium
159 tetraborate, 0.088 g dithiothreitol and 0.1 g sodium docecyl sulphate. Exactly 0.080 g OPA was
160 dissolved in 2 mL ethanol and added to the above-mentioned solution and made up to 100 mL
161 with Milli-Q water and the solution was kept in the dark. Each of the WPI-Dx conjugates
162 prepared with different DC was dissolved in Milli-Q water with gentle stirring at a
163 concentration corresponding to a WPI content of 1.0 mg/mL. For each prepared sample, 160
164 µL was added to 1200 µL OPA reagent in a PMMA cuvette, mixed for 5 s and the absorbance
165 was measured at 340 nm using a UV-VIS spectrophotometer (6715 UV/VIS
166 Spectrophotometer, Jenway, UK), using blank prepared with OPA reagent and Milli-Q water.
167 The baseline was established by using non-conjugated WPI-Dx solution. The degree of
168 conjugation can thus be calculated as follows:

169 $$Degree\ of\ conjugation\ (DC)\ \% = \frac{\left(C_{untreated} - C_{conjugate}\right)}{C_{untreated}} \times 100\%$$

170 where, $C_{untreated}$ is the concentration in the non-conjugated WPI-Dx mixture and $C_{conjugate}$ is the
171 concentration of the conjugated samples. The analysis of each sample was carried out in
172 triplicate.
173 The same OPA procedure was applied for quantification of protein hydrolysis. A
174 reference calibration curve of L-leucine solution (0 - 200 µM) was used and the protein
175 hydrolysis was expressed as a µM free amino groups per mass of the total protein in sample.

176 *2.4. Preparation of heat-induced gels and microgel particles*

177 The WPI-Dx Maillard conjugate and non-conjugated powders described in section 2.2
178 were dispersed in phosphate buffer for 2 h to ensure complete dissolution and to maintain the
179 final pH of the dispersion at pH 7.0. Protein concentration was 11.57 wt% for non-conjugated
180 and ~10 % DC samples, and the protein concentration was 8.02 wt% for ~20 and 30 % DC
181 samples. The aqueous solutions of the non-conjugated and three conjugate samples were heated
182 at 65 ^{o}C in a temperature-controlled water bath for 1 h to form a heat-set gel (quiescent),
183 followed by cooling down for 15 min and stored at 4 ^{o}C for 24 h before further analysis. The
184 gel formation was induced by heat-induced protein aggregation. When aqueous dispersions of
185 WPI are heated at ≥ 65 °C, heat treatment causes unfolding of the globular proteins causing the
186 exposure of the free sulfhydryl group and the inner hydrophobic amino acids. Protein
187 aggregation is caused initially by hydrophobic interactions followed by formation of
188 intramolecular disulphide-bonds. Large aggregates are formed by further sulfhydryl-catalyzed
189 disulphide-bond interchange and non-covalent interactions between the dimers (Croguennec,
190 O'Kennedy, & Mehra, 2004; Nicolai, et al., 2011).

191 To obtain microgel particles, the afore-mentioned non-conjugated and conjugate heat-set
192 gels were pre-homogenized with phosphate buffer at pH 7.0 (2 wt% protein) using a hand
193 blender (HB724, Kenwood) for 1 min and transferred to a vacuum box (John Fraser and Sons
194 Ltd, London, UK) for degassing. The resulting microgel particles were passed twice through a
195 Jet homogenizer (a bespoke two-chamber homogenizer developed in the School of Food
196 Science and Nutrition, University of Leeds, Leeds, UK) at 300 bar for two passes. Final non-
197 conjugated microgels particles and conjugate microgel particles are referred to as N-WPDxM
198 and WPDxM, respectively, and were diluted with phosphate buffer to the desired protein
199 concentration for the preparation of Pickering emulsions.

200

201 *2.5 Mechanical properties of heat-induced WPI-Dx gels*

202 Uniaxial single compression tests on the gel samples (10.10 mm diameter × 8.30 mm
203 height) were performed with a TA-TX2 Texture Analyser Micro Systems Ltd., (Surrey, UK)
204 using a cylindrical probe (diameter 59 mm), attached with a 50 kg load cell. The tests were
205 carried out at room temperature at a constant speed of 1 mm/s and the gels were compressed
206 until rupture (80% strain with respect to their initial height). The parameters calculated from
207 the uniaxial compression test were true fracture stress, which is the load at the point of the
208 fracture divided by the cross-section area during fracture and the Young's modulus, which is
209 calculated as the slope of the initial linear region of maximum stress versus the Henky strain.
210 Measurements were performed in triplicate and mean values and standard deviations were
211 calculated.

212

213 *2.6. Preparation of Pickering oil-in-water emulsions*

214 Pickering oil-in-water emulsions (E_{WPDxM}) were prepared using MCT-oil (20 wt%) and
215 WPDxM gel particles to give a final protein content of 1 wt% in the final emulsion. Briefly,
216 coarse E_{WPDxM} droplets were prepared using Ultra Turrax T25 homogenizer (IKA-Werke
217 GmbH & Co., Staufen Germany) at 13, 500 rpm for 1 min. Following this, the coarse emulsions
218 were homogenized twice using a Jet homogenizer (School of Food Science and Nutrition,
219 University of Leeds, UK) at 300 bar to prepare fine emulsion droplets.

220

221 *2.7 Interfacial shear viscosity*

222 The interfacial shear viscosity was measured as previously described by Murray and
223 Dickinson (1996) and Sarkar, Zhang, Murray, Russell, and Boxal (2017) using a two-
224 dimensional Couette-type viscometer. Briefly, a stainless steel biconical disc (radius 14.5 mm)
225 was suspended from a thin torsion wire with its edge in the plane of the oil-water interface of

the solution contained within a cylindrical glass dish (radius 72.5 mm). The deflection of the disc was measured by reflection of a laser off a mirror on the spindle of the disc onto a scale at a fixed distance from the axis of the spindle. The interfacial viscometer was operated in a constant shear-rate mode, as described in a recent study (Zembyla, Murray, & Sarkar, 2018). For the measurements, a layer of pure *n*-tetradecane was layered over an aqueous solution of microgel particles at a concentration of 0.5 wt% in phosphate buffer at pH 7.0. The constant shear rate apparent interfacial viscosity, η_i, is given by the following equation:

$$\eta_i = \frac{g_f}{\omega} K(\theta - \theta_0) \quad (1)$$

where, K is the torsion constant of the wire, θ is the equilibrium deflection of the disc in the presence of the film, θ_o is the equilibrium deflection in the absence of the film, *i.e.* due to the drag force of the sub-phase on the disc, g_f is the geometric factor, and ω is the angular velocity of the dish. A fixed value of $\omega = 1.27 \times 10{-}3$ rad s^{-1} was used.

2.8 In vitro gastric digestion of conjugate microgel particles

The aqueous dispersions of the non-conjugate and conjugate microgel particles and the corresponding selected emulsion *i.e.* E_{WPDxM} were digested using slightly adapted digestion protocol from Minekus, et al. (2014). Briefly, 10 mL of pre-incubated sample (37 °C, 1 h) at pH 3.0 was mixed with 10 mL of simulated gastric fluid (SGF), consisting of 0.257 g L^{-1} of KCl, 0.061 g L^{-1} of KH_2PO_4, 1.05 g L^{-1} of $NaHCO_3$, 1.38 g L^{-1} of NaCl, 0.0122 g L^{-1} of $MgCl_2(H_2O)_6$, 0.024 g L^{-1} of $(NH_4)2CO_3$ and 2000 U/mL pepsin at pH 3.0 without using any oral processing step. The mixture was incubated for 2.5 h at 37 ºC under agitation using a shaking water bath (Grant Instruments Ltd, Cambridge, UK). During the gastric phase, samples were periodically withdrawn from the sample-SGF mixture at 5, 30, 60, 90, 120 and 150 min for size, charge, microscopy and SDS-PAGE analysis. Proteolysis of the samples was terminated by neutralizing to pH 7.0 using freshly prepared 1 M NH_4HCO_3 except for size and

charge measurements, in latter experiments, samples were characterized immediately after digestion.

2.9 Particle size and droplet size distribution

The physicochemical properties and stability of aqueous dispersions of N-WPDxM and WPDxM prepared using the non-conjugated and conjugated gels and their corresponding emulsions *i.e.* E_{WPDxM} before and after digestion were monitored using their particle size distribution, ζ-potential and microstructural changes as a function of gastric digestion time as previously described (Araiza-Calahorra, et al., 2019a). The particle size of the conjugate microgel particles was also investigated as a function of pH (pH 2.0 – 7.0) and in presence of ions (50 mM NaCl, 10 mM $CaCl_2$) to understand their behaviour in simulated physiological fluids in the absence of any physiological enzymes (Araiza-Calahorra, et al., 2019b). Briefly, the particle size of the aqueous dispersions of WPDxM was determined using dynamic light scattering (DLS) at 25 ^{o}C using a Zetasizer Ultra (Malvern Instruments Ltd., Malvern, Worcestershire, UK) in a PMMA standard disposable cuvette. Particle size of the samples before and after gastric digestion was measured after diluting the samples in SGF buffer (pH 3.0). Droplet size distributions of the emulsion samples (were determined using static light scattering at 25 ^{o}C using Malvern MasterSizer 3000 (E_{WPDxM}) Malvern Instruments Ltd., Malvern, Worcestershire, UK). The mean particle size distribution of the emulsions was reported as volume mean diameter (d_{43}) and surface mean diameter (d_{32}) based on five measurements on triplicate samples.

2.10. ζ-potential

The ζ-potential of aqueous dispersions of the conjugate microgel particles (WPDxM) and emulsion samples (E_{WPDxM}) before and after digestion was determined using a particle

electrophoresis instrument (Zetasizer Ultra, Malvern Instruments Ltd, Malvern, Worcestershire, UK). Samples were diluted in SGF buffer (pH 3.0) (0.1 wt% particle or 0.002 wt% emulsion droplet concentration) and added to a folded capillary cell (Model DTS 1070, Malvern Instruments Ltd., Malvern, Worcestershire, UK). Samples were equilibrated for 1 min and the data was processed using the Smoluchowski model. The ζ-potential results were reported as mean result of at least five reported readings made on triplicate samples.

2.11Cryogenic-Scanning Electron Microscopy

Cryogenic scanning electron microscopy (cryo-SEM) of the emulsion was conducted using heptane as the dispersed phase. Preliminary analysis on heptane or MCT-oil emulsions reveled that both systems presented the same overall microstructural behavior. Nevertheless, heptane was used as the dispersed phase, to avoid interference by crystallization of oil during the freezing step as described by Destribats, et al. (2013) and Araiza-Calahorra, et al. (2019b). The emulsion sample was mounted on rivets attached to the sample stub. The samples were plunge-frozen in liquid nitrogen "slush" at −180 °C, then transferred to the cryo-preparation chamber on the SEM. The frozen emulsion droplets were cleaved and then etched at −95 °C for 4 min. Next, the samples were coated with 5 nm of platinum (Pt). Finally, the Pt-coated samples were transferred to the SEM for imaging at −135 °C. The heptane emulsion sample was imaged in a FEI Quanta 200F ESEM with a Quorum Polar Prep 2000 cryo system.

2.12. Confocal scanning laser microscopy (CLSM) and cross-correlation analysis

Microstructural observations were made using a Zeiss LSM 880 inverted confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany) using an oil immersion 63× lens and the pinhole diameter maintained at 1 Airy Unit to filter out the majority of the scattered light. A stock solution of Nile Red (1 mg/ mL in dimethyl sulfoxide) was used to stain the

MCT-oil to a final concentration of 0.02 mg/ mL and a stock solution of Fast Green (1 mg/mL in Milli-Q water) was used to stain the protein particles to a final concentration of 0.1 mg/ mL. Nile Red and Fast Green were excited at wavelengths of 488 and 633 nm, respectively. The emission filters were set at 555 – 620 nm for Nile Red and at 660 – 710 nm for Fast Green. Samples were placed on a concave confocal microscope slide and secured with a glass coverslip before imaging.

In addition, a combination of confocal microscopy and cross-correlation image analysis was applied to two channel microscopy images of emulsion samples stabilized by conjugate microgel particles (E_{WPDxM}) and emulsion samples stabilized by whey protein-based nanogel particles (E_{WPN}) before and after digestion with SGF containing pepsin. Briefly, fresh emulsion samples were stained and mixed with SGF containing pepsin. Samples were imaged after 5 – 10 min of incubation and z-stacks images were obtained using a scan rate of 400 Hz in sequential scan mode to avoid cross-talk between fluorophores. Images were accepted for analysis if they were part of 3 or more image planes within an image stack and of sufficient technical quality to discern discrete particles at the droplet interface. Image analysis was conducted using MATLAB R2018b (Mathworks, US), details have been previously described (Glover, et al., 2019b). Briefly, a region of interest around an oil droplet channel was selected and the largest circle in that image crop was found using the function *'imfindcircle'* in MATLAB. The centre point and the radius of the circle was determined and a cropped image was created from the original image with the droplet at the centre. The cropped images were 3.5× the diameter of the droplet in width and height to ensure no overlap with other droplets and protein structures occurred.

For the cross-correlation analysis, each pixel in the image was given a polar co-ordinate and the image was split into 20 radial segments. A threshold was applied to the red channel using the function '*graythresh'*, based on Otsu's method. For every radial segment, the

intensity of the fat and protein was radially averaged using the MATLAB function *'accumarray'* and a 1D cross-correlation was performed between the fat and the protein using the function *'xcorr'*. The cross-correlation intensity was integrated for every radial segment using the function '*trapz'* and the integrated values were scaled to the radius of the droplet in the selected region of interest to avoid artifacts caused by minor changes in z-position over time. The cross-correlation analysis was performed for pairs of images at different time points where the same droplet was selected each time. Microscopy images were optimized for publication using Fiji, ImageJ.

2.13 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The protein composition of the aqueous dispersions of WPI and Dx conjugate solutions and the peptides generated in the N-WPDxM and WPDxM particles after gastric hydrolysis by pepsin was examined using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The WPI-Dx sample and N-WPDxM and WPDxM-SGF, mixtures (1.5 mL) after gastric digestion with pepsin were mixed with SDS buffer (0.5 M Tris, 2.0% SDS, 0.05% β-mercaptoethanol, pH 6.8), at a 1:2 ratio (sample : SDS buffer), heated at 95 °C for 5 min and 10 μL was loaded into the precast gels placed on an Invitrogen™ Mini Gel Tank system (Thermo Fisher Scientific, Loughborough, UK). Exactly, 5 μL of the protein molecular weight marker was added in the first lane. After running the gel at 200 V for 22 min, the gel was fixed in a Milli-Q: Methanol: Acetic acid (50:40:10 vol%) solution for 1 h and stained for 2 hrs with a Coomassie Brilliant Blue R-250 solution in 20% isopropanol. The gels were destained overnight in Milli-Q water and scanned using a ChemiDoc™ XRS + System with image LabTM Software (Bio-Rad Laboratories, Richmond, CA, USA). The intensities of the protein bands were quantified using Image Lab Software Version 6.0. Bands within the lanes was selected automatically by the software to cover the whole band. Background intensity

was subtracted after scanning an empty lane, which served as the blank. The percentage composition of each sample was determined by scanning the gradual reduction in peak volume intensity for each intact protein bands of WPI (β-lactoglobulin (β-lg), α-lactalbumin (α-la) and bovine serum albumin (BSA)). The SDS PAGE experiments were carried out in triplicates and band intensities was reported as an average of three reported readings.

2.14. Statistical analysis

Means and standard deviations were calculated from three individual measurements performed on triplicate samples and analysed using the one-way analysis of variance (ANOVA) and Student's t-test where significance was accepted at $p < 0.05$.

3. Results and discussion

3.1 Identifying the degree of conjugation (% DC)

To confirm the covalent conjugation of the carbonyl group of the reducing sugar with the free amino groups in the proteins, many studies have focused on the quantification of free amino groups (Wooster & Augustin, 2006). Thus, to estimate the extent of the Maillard reaction in the conjugate samples designed in this study, the loss of free amino groups of WPI was estimated using the OPA method taking the non-conjugated WPI-Dx mixture as the reference and the DC was calculated and shown in Table 1. Additionally, covalent coupling of WPI and Dx after dry heating was qualitatively established using SDS-PAGE patterns (Figure 1). Based on the DC obtained (see Table 1), the following nomenclature was followed henceforth, WPI-Dx_{10} denotes the sample that presented a DC of 11.57%, WPI-Dx_{20} refers to the sample with a DC of 19.32%, and WPI-Dx_{30} refers to the sample that presented a DC of 28.14%. Accordingly, the same subscripts will be used henceforth to refer to the corresponding microgel particles ($WPDx_{10}M$, $WPDx_{20}M$ and $WPDx_{30}M$).

376 Table 1 presents the Maillard reaction conditions used for the conjugation of Dx to WPI as
377 a function of pH and reaction times. The pH of the solutions and the heating conditions are
378 based on those commonly used for preparing Maillard conjugates using dextran (Akhtar, et al.,
379 2003; Fechner, Knoth, Scherze, & Muschiolik, 2007; Ho, Ishizaki, & Tanaka, 2000; Liu, Ma,
380 McClements, & Gao, 2016; Liu, Wang, Sun, & Gao, 2016). As shown in Table 1, the DC
381 increased with increasing the pH of the protein-polysaccharide solution dispersion from pH 7.0
382 to pH 11.0. This behaviour might be expected considering that browning reaction is known to
383 be promoted at higher pH levels (Kato, 1956). For example, it has been observed that a greater
384 Maillard reaction was produced at pH above 7.0, as compared to pH 5.0 and 6.0 in solutions
385 containing glucose and amino acids (Willits, Underwood, Lento Jr., & Riccuti, 1958).
386 Additionally, it has been observed that during the Maillard reaction, pH often decreases leading
387 to a slowing down of the reaction (Mikami & Murata, 2015; Wolfrom, Kolb, & Langer, 1953).
388 It should be noted that no buffer was used to control the pH of the solutions, as it has been
389 suggested to influence the nature and quantity of the Maillard reaction products by catalysing
390 the conversion of glycosylamine into the Amadori product during the first stage of the reaction
391 (Potman & van Wijk, 1989). Thus, at pH 7.0, it is likely that a decrease of the pH, naturally
392 caused during the Maillard reaction, may have led to the slowing down of the reaction, as
393 compared to pH 11.0. In previous studies, it has been observed that, in the absence of buffer,
394 changes in the pH of 3 – 4 units may occur (Madruga & Mottram, 1995).
395 The degree of conjugation was increased by increasing the reaction time from 24 to 48
396 h (Table 1) in line with a previous study (Wooster, et al., 2006). The levels of conjugation are
397 similar to those reported in other studies using WPI and Dx (Spotti, et al., 2013a, 2013b).
398 However, slight discrepancies might arise from the fact that a bigger molecular weights of the
399 Dx used in our study may have been less reactive with the free amino groups of the proteins

400 due to a less open chain as compared to a biopolymer containing a shorter carbonic chain length
401 (Chevalier, et al., 2001).

402 In order to assess the level of reacted materials in WPI-Dx solutions, SDS-PAGE was used.
403 As shown in Figure 1, for native WPI solution (lane 2), the intensities of bands at about 66 kDa,
404 18 kDa and 14 kDa that correspond to bovine serum albumin (BSA), β-lactoglobulin (β-lg)
405 and α-lactalbumin (α-la), respectively, are clearly identified. For WPI-Dx_{10}, WPI-Dx_{20} and
406 WPI-Dx_{30} (lanes 3 to 5), the intensities of the two major bands corresponding to β-lg and α-la
407 were reduced as compared to that of the WPI solution (lane 2) (Figure 1). For β-lg, the
408 remaining intact protein was 82.19%, 59.99% and 54.03%, and for α-la was 66.73%, 47.52%
409 and 18.24%, respectively. In addition, in WPI-Dx_{30} solution, an intense drag at the loading end
410 of the gel was observed (shown by arrow in Figure 1). This indicates that WPI and Dx are
411 conjugated into larger oligomers that could not migrate into the resolving gel. Similar results
412 have been reported previously in WPI-Dx conjugate systems, where smearing of bands and
413 reduced intensity of the characteristics proteins bands in the resolving gel have been observed
414 (Akhtar, et al., 2003; Sun, et al., 2011; Zhu, Damodaran, & Lucey, 2010). Although it is not
415 possible to determine the exact molecular weights of the oligomers generated in the conjugate,
416 it can be concluded that there were significant changes in the WPI-Dx systems upon
417 conjugation and that the Maillard reaction was carried out successfully. The optical images of
418 the freeze-dried non-conjugated and conjugate powders can be seen in Supplementary Figure
419 1, where the conjugated powder clearly showed increased levels of visual brown coloration
420 with increased DC in line with the SDS-PAGE results (Figure 1) and the DC reported in Table
421 1.

422

423 *3.2 Mechanical properties of the conjugate WPI-Dx heat-set gels*

In order to prepare the microgel particles, a heating step was required at first to convert the protein solution into heat-set hydrogel before shearing these gels into microscopic gel particles. The fracture properties of the parent gels were characterized to give an indication of the large deformation properties of the resultant microgel particles. To study the influence of glycosylation on the mechanical properties of the heat-set protein gels, the WPI-Dx conjugate gels with different DC were compared with WPI systems (*i.e.* no dextran added). The mechanical properties of WPI and WPI-Dx conjugate gels incubated for different reaction times are shown in Figure 2.

The appearance of the 10 wt% WPI gel was translucent and brittle, which indicates that a fine-stranded microstructure was formed (Figure 2a) (Clark, Judge, Richards, Stubbs, & Suggett, 1981). The gels formed by WPI presented an average fracture stress of 175.40 kPa (Figure 2), which is slightly higher than that previously reported for WPI gels of 8 – 20 wt% protein concentration (11.2 – 171.2 kPa) formed at pH 7.0 (Foegeding, 1992). This could be explained by the differences in heat-treatment conditions and presence of ions. Upon addition of Dx (non-conjugated WPI-Dx gels), the fracture stress increased to ~950 kPa (Supplementary Figure 2), which indicates that addition of Dx had a pronounced effect on the gelation behaviour of proteins. This is in agreement with previous studies where presence of dextran, a neutral polysaccharide, has been shown to increase the modulus of whey protein isolate gels (Spotti, et al., 2013a; Spotti, Santiago, Rubiolo, & Carrara, 2012). It was proposed that other molecular interactions such as hydrogen bonds can be promoted by the addition of dextran, which caused the increased strength of the heat-set induced gel (Spotti, et al., 2013b).

For the heat-set conjugate gels with different DCs (WPI-Dx_{10}, WPI-Dx_{20}, WPI-Dx_{30}), a significant contribution of the Maillard conjugation to the fracture mechanics of the gels was observed ($p < 0.05$) (Figure 2), though all the DC levels resulted in self-supporting gels (Figure 2b-d). It is noteworthy that even under carefully replicated experiments there was incorporation

449 of air into the WPI-Dx_{30} samples subjecting the fracture measurements to some error (Figure
450 2d). Fracture stress was significantly lower for sample WPI-Dx_{30} with values of 272.56 kPa
451 (Figure 2) as compared to 770.48 and 905.39 for WPI-Dx_{10} and WPI-Dx_{20}, respectively. It is
452 possible that due to the loss of native structure of the protein due to conjugation, groups that
453 are primarily responsible for the gel network such as sulfhydryl groups were not freely
454 available for covalent interaction (Spotti, et al., 2013b). Although the Young's modulus of
455 WPI-Dx_{10} increased as compared to that of WPI counterparts ($p < 0.05$), it was lower as
456 compared to that of the non-conjugated WPI-Dx gels (see Supplementary Figure 2) and it
457 decreased by four-fold with increasing DC from 448.55 kPa for non-conjugated WPI-Dx gels
458 to 92.15 kPa for the WPI-Dx_{30} gels (Figure 2). Similar trends were observed by Spotti, et al.
459 (2013a) who reported that increased Maillard reaction time decreased the Young's modulus
460 values obtained for WPI-Dx conjugate gels irrespective of the dextran molecular weight
461 studied (6 -70 kDa). However, the discrepancies in Young's modulus values between our study
462 and that of Spotti, et al. (2013a) could be due to differences in molecular weight of dextran
463 used in our study (500 kDa), which is an order of magnitude higher as compared to the ones
464 used by Spotti, et al. (2013a).

465 Differences on the observed mechanical properties of conjugated gels could be
466 caused by changes in the structure of the protein due to either the heat treatment or the coupling
467 of dextran during Maillard reaction (Spotti, et al., 2013b). Such changes to the structure could
468 affect the protein unfolding and further thermal denaturation and aggregation reactions altering
469 the mechanism of gelation of the WPI-Dx conjugate gels. In addition, conjugation of Dx may
470 also suppress the intermolecular association between neighbouring proteins in aqueous
471 solutions due to possible steric hindrance effect caused by Dx (Sun, et al., 2011).

472

473 *3.3 Characteristics of conjugate microgel particles*

Conjugate microgel particles (WPDxM) were prepared by controlled shearing of the afore-mentioned WPI-Dx_{10}, WPI-Dx_{20} and WPI-Dx_{30} gels using a top down approach developed previously (Araiza-Calahorra, et al., 2019a, 2019b). For control purposes, an aqueous dispersion of mixed particles (*i.e.* non-conjugated N-WPDxM) was analysed (Supplementary Figure 3). Aqueous dispersions of $WPDx_{10}M$, $WPDx_{20}M$, and $WPDx_{30}M$ particles at pH 7.0 presented monomodal particle size distributions (Figure 3) with polydispersity index ranging from 0.2 – 0.3 and hydrodynamic diameters ranging from 136 to 146 nm (Table 2). The microgel particles were slightly negatively-charged at pH 7.0 (Table 2). The negative charge of the particle dispersions might be attributed to the fact that WPI was above its isoelectric point.

When compared to whey protein nanogel and microgel particles as previously described by Araiza-Calahorra, et al. (2019b) and Sarkar, et al. (2016), respectively, a decrease in the magnitude of the negative charge was observed in these conjugate microgel particles. This may be attributed to the addition of the neutral dextran molecule, which might have saturated the surface of these conjugated microgel particles. In addition, if we compare the ζ-potential of conjugate microgel particles, such as $WPDx_{10}M$ and $WPDx_{20}M$ (Table 2) with that of non-conjugated N-WPDxM systems (Supplementary Figure 3), a similar ζ-potential values ($p > 0.05$) can be observed, suggesting that conjugation did not directly affect the charge groups of the proteins.

The colloidal stability of 1 wt% of aqueous dispersions of conjugate microgel particles ($WPDx_{10}M$, $WPDx_{20}M$, and $WPDx_{30}M$) at various pH and three different salinities (50 mM NaCl and 10 mM $CaCl_2$) was investigated (Supplementary Figures 4 and 5). Changes in hydrodynamic diameter were measured since the stability of the microgel particles largely depends on the attractive and repulsive interactions between the constituent proteins, which dictates the swelling behaviour of the microgel particles. From Supplementary Figure 5, it can be observed that in the absence of added electrolytes (NaCl or $CaCl_2$), dispersions of $WPDx_{10}M$ conjugate particles were found to aggregate within the pH range of $4.0 < pH < 5.5$ as revealed

by the turbidity (Supplementary Figure 4) and size evolution of the microgel particles that increased drastically to several micrometres (Supplementary Figure 5). The size evolution of $WPDx_{10}M$ was found to corroborate with the near zero zeta-potential (data not shown), suggesting that a low degree of conjugation did not significantly affected the isoelectric point (*pI*) of WPI (Sarkar, et al., 2016). Interestingly, on increasing DC to 20 and 30% (*i.e.* $WPDx_{20}M$, and $WPDx_{30}M$ particles), the particles formed a slightly more stable dispersion within the same pH range of $4.0 < pH < 5.5$. Especially for higher degree of conjugated particles *i.e.* $WPDx_{30}M$, the increase on the hydrodynamic diameter was not as large as compared to $WPDx_{10}M$ samples in the same pH range (Supplementary Figure 5).

In the presence of 50 mM NaCl, $WPDx_{10}M$ particles presented a slight shift of the *pI* towards pH 5.0. Furthermore, in the presence of 10 mM $CaCl_2$, an observable shift of the instability domain towards neutral pH was also observed (Supplementary Figure 5). With increased DC, the $WPDx_{20}M$, and $WPDx_{30}M$ particles showed less responsiveness to pH and ions as opposed to $WPDx_{10}M$. Reduction in responsiveness to pH and ions upon increased degree of dextran attachment may be attributed to major conformational modification induced by the glycation (Chevalier, et al., 2001) or electrostatic screening of the protein structure by the neutral dextran molecule (Wooster, et al., 2006). In summary, physicochemical characterization of the conjugated microgel particles suggests that increased DC reduces the responsiveness of the particle to physiologically relevant ionic conditions and thus, might be more stable as opposed to non-conjugated WPI-based microgel particles.

3.4 Characteristics of conjugated microgel particles during in vitro *gastric digestion*

Besides pH and electrolytes, proteolysis by pepsin in the gastric regime is a major determining factor for breakdown of protein-based particles (Araiza-Calahorra, et al., 2019a; David-Birman, Mackie, & Lesmes, 2013; Sarkar, et al., 2019). Hence, changes in the

524 physiochemical properties and protein composition of aqueous dispersions of conjugate
525 particles were examined as a function of gastric digestion time. This sets the scene to
526 understand the influence of polysaccharide conjugation on the gastric behaviour of aqueous
527 dispersions of microgel particles and their behaviour when present at the oil-in-water interface.
528 Upon addition of SGF buffer without pepsin at pH 3.0, the particles presented a monomodal
529 size distribution (Figure 3) with no significant difference in polydispersity index (0.21– 0.28) and
530 the hydrodynamic diameter ranging from 125 – 133 nm (Table 2). As might be expected, the ζ-
531 potential became slightly positive since the WPI is now below its isoelectric point. After 5 min
532 of incubation in SGF containing pepsin at pH 3.0, different sizes of particle aggregates were
533 generated and the polydispersity was too high to be measured using dynamic light scattering.
534 Figure 4 describes the protein composition of the microgel particles as a function of gastric
535 incubation time as determined by SDS-PAGE. As controls, SDS-PAGE patterns of whey
536 protein nanogel particles (Araiza-Calahorra, et al., 2019a) and an aqueous dispersion of mixed
537 whey protein-dextran microgel particles (*i.e.* non-conjugated N-WPDxM, particles without any
538 Maillard reaction) are shown in Supplementary Figure 6. It can be observed that for $WPDx_{10}M$
539 after 30, 60 and 120 min in SGF containing pepsin, 17.09 ± 4.34, 13.34 ± 4.62 and 1.47 ±
540 0.08% of β-lg and 6.45 ± 2.49, 2.81 ± 0.61 and 0.17 ± 0.12% of α-la remained, respectively
541 (Figure 4a). However, the control sample of mixed microgel particles without conjugation or
542 WPN did not protect β-lg or α-la from immediate gastric proteolysis, as the intact bands were
543 not detectable even after 5 min of digestion (Supplementary Figure 6). This result indicate that
544 conjugation with Dx (10% DC) increased the resistance of the microgel particles to gastric
545 proteolysis. In contrast, digestion of microgels particles with higher DC *i.e.* both $WPDx_{20}M$
546 and $WPDx_{30}M$ did not even show traces of intact β-lg or α-la bands within the first 5 min of
547 gastric proteolysis (Figures 4b and 4c). It is possible that a greater degree of conjugation was
548 caused by the exposure of the hydrophobic amino acids naturally generated by increased

549 conjugation of a high molecular weight polysaccharide making the particles highly susceptible
550 to hydrolysis by pepsin (Nooshkam & Varidi, 2019). However, this needs to be further
551 investigated in a future study.

552 To investigate more quantitatively, the levels of free amino group (NH_2) were
553 determined using OPA method for the different microgel particles during the gastric digestion.
554 Figure 5 shows that at 0 min, all the three conjugate microgel particles ($WPDx_{10}M$, $WPDx_{20}M$,
555 and $WPDx_{30}M$) had 29.3 - 33.1 μM of free NH_2 per g of protein, which is lower than that found
556 was found previously in heat-induced whey protein nanogel particles (WPN) (~50 μM of free
557 NH_2 per g of protein) (Araiza-Calahorra, et al., 2019a) before any digestion had begun. This
558 can be expected as the free NH_2 acids from the protein was used in the conjugation process in
559 case of the conjugate microgel particles. Of more importance here is the behaviour of these
560 conjugate microgel particles as the gastric digestion commenced (Figure 5). The free pepsin
561 hydrolysis presented relatively constant values ranging from 120.1 ± 7.0 to 130.7 ± 9.9 μM
562 NH_2 per g of protein after 120 min of gastric digestion with no significant difference between
563 the microgel particles with different DC ($p > 0.05$). This is 36% lower than that of the
564 previously studied WPN (Araiza-Calahorra, et al., 2019a), that showed an increase in the
565 proteolysis in the first 30 min gastric digestion to 187.4 – 204.4 μM NH_2 per g when subjected
566 to the same 120 min of simulated *in vitro* gastric digestion. This proteolysis profile in WPN in
567 the previous study (Araiza-Calahorra, et al., 2019a) was directly correlated with heat-induced
568 conformational changes in the whey protein structure that exposed the hydrophobic amino
569 acids making it highly susceptible to hydrolysis by pepsin. Comparing with this previous study,
570 it can be inferred that covalent conjugation with dextran (500 kDa) in the present study would
571 be the only reason why the free amino group profile of all conjugated microgel particles was
572 much lower than the WPN profile during 5-120 minutes of digestion ($p < 0.05$). In summary,
573 OPA and SDS-PAGE patterns suggests that a low degree of conjugation was sufficient to delay

574 pepsinolysis of the microgel particles during gastric digestion. Hence, $WPDx_{10}M$ particles were
575 selected for the preparation of the whey protein microgel particle-stabilized Pickering emulsion
576 droplets, which is referred to as $E_{WPDx10M}$ henceforth.
577
578 *3.5 Characteristics of Pickering emulsions stabilized by WPM_{10} (E_{WPN10})*
579 Oil-in-water emulsions were prepared using high-pressure homogenization to determine if
580 the $WPDx_{10}M$ could successfully be used as Pickering stabilizers. Figure 6a shows the droplet
581 size distribution of the 20 wt% MCT oil emulsion stabilised by $WPDx_{10}M$ (1 wt% protein
582 concentration). Droplet size distribution showed a bimodal distribution with oil droplet ranging
583 from 1 to 50 μm (which does not change over one week), and the peak in the area of 0.1-1 μm
584 most likely corresponding to unabsorbed $WPDx_{10}M$. Similar results have been reported
585 previously where unadsorbed particles tend to form a smaller peak in static light scattering
586 results (Araiza-Calahorra, et al., 2019b; Du Le, Loveday, Singh, & Sarkar, 2020). As can be
587 expected from the charge of the aqueous dispersion of microgel particles (Table 2), the
588 emulsion droplets were slightly negatively-charged with a ζ-potential value of −5.39 mV
589 (Figure 6a). Interestingly, no notable emulsion instability was observed over several months
590 (data not shown), which indicates that only high adsorption energies via particle-stabilization
591 by the microgel particles was governing the stability of the conjugate microgel-stabilized
592 emulsions as opposed to any electrostatic contribution, the latter has been important contributor
593 to droplet stability in cases of previously reported Pickering emulsions stabilized by whey
594 protein-based microgel or nanogel particles (Araiza-Calahorra, et al., 2019b; Destribats, et al.,
595 2013; Sarkar, et al., 2016). The cryo-SEM image (Figure 6b) clearly demonstrates the presence
596 of a monolayer of spherical microgel particles at the interface of the $WPDx_{10}M$-stabilized
597 emulsions further confirming the Pickering stabilization.

598 In order to quantitatively determine the mechanical performance of the adsorbed microgel
599 particles at the O/W interface, interfacial shear viscosity (η_i) measurements were undertaken.
600 Interfacial shear viscosity experiments can give powerful insights into structural characteristics
601 of the adsorbed layer, and relate the interfacial properties to aspects of the formation and
602 stability of the emulsion (Murray, Durga, Yusoff, & Stoyanov, 2011; Zembyla, Lazidis,
603 Murray, & Sarkar, 2019; Zembyla, Murray, Radford, & Sarkar, 2019). Figure 6c shows the
604 time-dependent shear viscosity data of adsorbed films of WPN (used as control) (Araiza-
605 Calahorra, et al., 2019b) and WPDx$_{10}$M particles at the *n*-tetradecane-water interface (0.5 wt%,
606 pH 7.0, 25°C). For the first 3 h, η_i increased by approximately two folds for both particles
607 reaching 970 mN s m^{-1} for WPN, and 540 mN s m^{-1} for WPDx$_{10}$M. After 24 h, the highest
608 value (ca. 800 mN s m^{-1}) was obtained by WPN, whereas the lowest value obtained by WPM$_{10}$
609 was ca. 400 mN s m^{-1}. This decrease in η_i in WPDx$_{10}$M versus WPN can be surprising
610 considering that the fracture stress and Young's modulus of the parent conjugate gels with 10%
611 DC being four-five folds higher than that of the whey protein counterparts (Figure 2). It is
612 possible that the increased shear viscosity values in WPN may be due to stronger interactions
613 between closely packed WPN particles absorbed at the interface. On the contrary, because of
614 the altered structural conformation of the WPDx$_{10}$M particles caused by the conjugation and/
615 or the presence of a high molecular weight polysaccharide, the interactions among WPDx$_{10}$M
616 particles adsorbed at the interface were probably weakened, which decreased the interfacial
617 shear viscosity values of the resultant conjugate particle. This can be further corroborated by
618 the control samples (mixed non-conjugated N-WPDxM particles) subjected to interfacial shear
619 viscosity measurements (Supplementary Figure 7), where it can be observed that an increase
620 in η_i values was obtained in mixed non-conjugated N-WPDxM microgel particles as compared
621 to WPDx$_{10}$M but lower than WPN. This suggest that addition of a high molecular weight
622 polysaccharide might have prevent protein-protein interactions between particles, or that the

623 changes on the molecular structure caused by the conjugation altered the structure weakening
624 the strength of the particle-laden film at the interface.
625
626 *3.6 Characteristics of E_{WPM10} during* in vitro *gastric digestion*
627 The Pickering emulsion samples stabilized by $WPDx_{10}M$ was exposed to an *in vitro*
628 gastric digestion model to analyse whether the conjugate microgel particles had any slowing
629 down effect on proteolysis of the particle-laden interface and protected the Pickering emulsions
630 against coalescence during simulated gastric conditions. The droplet size, charge, and
631 microstructure as a function of time were recorded and a combination of confocal laser
632 scanning microscopy and cross-correlation image analysis (Glover, et al., 2019a; Glover, et al.,
633 2019b) was performed to quantify the amount of proteinaceous microgel particles located at
634 the droplet interface (Figures 7-9).
635 *3.6.1 Stability under simulated gastric conditions*
636 As can be observed in Figure 7, the droplet size distribution of $E_{WPDx10M}$ and the
637 volume-average mean diameter (d_{43} = 7.61 μm) were not significantly influenced by incubation
638 in SGF without pepsin (Figure 7a, see Figure 6a for sample at pH 7.0), although the ζ-potential
639 became positive (+8.54 mV) due to the protonation of the WPI (see time 0 min in Figure 7b).
640 From the confocal images, it is noticeable that $WPDx_{10}M$ (stained in green) are stabilizing the
641 emulsion droplets (stained in red) with no discernible coalesced oil droplets either before
642 (Figure 8a) or after incubation in SGF without pepsin (Figure 8b). This indicates that E_{WPM10}
643 was stable to any aggregation under the ionic environment of the gastric conditions.
644 Interestingly, after addition of pepsin in the SGF, the emulsion exhibited a decrease in
645 the magnitude of surface charge and the d_{43} values increased to a certain extent but remained
646 bimodal after the 120 min (Figure 7a). It is worth noting that the first peak height diminished
647 owing to digestion of the unadsorbed microgel particles, which can be expected from the

discussion in the previous sections on digestion of aqueous dispersion of particles after 120 min (see Figure 4a). But the second peak showed very similar peak height but shifted slightly towards larger droplet size indicating aggregation of droplets. The decrease in ζ-potential might suggest that the increase in droplet size arose from the aggregation of the droplets during gastric digestion. From the confocal images, emulsion droplets did not seem to have coalesced and no large oil droplets were noticeable even after 120 min (Figure 8c). Results indicate that a steric barrier was provided by the neutral conjugate microgel particles which was retained at the interface and played an important role in stabilizing the emulsions in simulated gastric conditions.

3.6.2 Cross-correlation image analysis of confocal microscopy images

To assess the impact of conjugation on the gastric destabilization, two channel microscopy images (protein and fat droplets) of two emulsion samples, control Pickering emulsion stabilized by whey protein nanogel particles (WPN) (Araiza-Calahorra, et al., 2019b) and whey protein-dextran conjugated microgel particles ($WPDx_{10}M$) were obtained during simulated gastric conditions. Each image contained a distribution of Pickering emulsion droplets from which smaller regions containing individual emulsion droplets were analysed to extract local information. To analyse the amount of protein around the oil droplet, the droplet of interest was segmented into 20 radial segments ($\pi/10$) and the cross-correlation analysis was performed as a function of angle. Figure 9 shows the overlaying radial plot of the treated fat droplets and protein particles and the mean cross-correlation intensity distributed around the fat droplet within each segment of E_{WPN} (a) and $E_{WPDx10M}$ (b) after 0 min (t1) to 5 - 10 min (t2) of simulated gastric digestion. By overlaying the radial plots of the two different digestion time points (see Figure 9), it is clear that the changes in the maximal protein distribution from the oil droplet vary between samples. For E_{WPN}, it can be showed that the radial distribution of the maximal protein intensity at time t2 was on average smaller compared to t1 (Figure 9a). These

673 results corroborate with previous results by Sarkar, et al. (2016) and Araiza-Calahorra, et al.
674 (2019a) where whey protein-based microgel- as well as nanogel-stabilized Pickering emulsions
675 show interfacial proteolysis by pepsin during *in vitro* gastric step. Nevertheless, the emulsion
676 droplets did not seem to coalesce possibly due to fragments of peptides still present at the
677 interface. As for $E_{WPDx10M}$, a subtle change on the average distribution between t1 and t2 was
678 observed (Figure 9b). Whilst there might be some protein interference in the same pixel from
679 particles localized in a nearby emulsion droplet, which was due to the imaging procedure, the
680 observed subtle changes in the protein concentration around the oil droplet is still a good
681 indication of a reduced pepsin hydrolysis of the conjugate microgel particles located at the
682 interface in line with the delayed behaviour observed in the aqueous dispersion (Figure 4a).
683 Recent studies have shown that glycation alters the susceptibility of food proteins to
684 gastrointestinal digestion. For example, Corzo-Martínez, Soria, Belloque, Villamiel, and
685 Moreno (2010) reported a decreased in the proteolytic susceptibility after simulated
686 gastrointestinal digestion of β-lactoglobulin after conjugation with dextran (10 kDa).
687 According to this study, steric hindrance caused by the molecules of dextran attached to β-lg
688 contributed to the lower susceptibility of digestive enzymes toward the protein. In addition,
689 Lesmes and McClements (2012) and Xu, et al. (2014) have also shown an increased stability
690 under gastric conditions of β-Lg-dextran and whey protein-beet pectin conjugate emulsions,
691 respectively. In the former, it was also reported that increasing molecular weight of the
692 conjugated dextran from 10 kDa to 400 kDa rendered emulsion more stable to pepsin-induced
693 instability. Therefore, as depicted schematically in Figure 10, we propose that a restricted
694 access of digestive enzyme to potential cleavage sites of the protein in case of the conjugate
695 microgel particles was possibly due to the tortuosity of network structure created during
696 conjugation and parent thermo-set gel formation process. Hence, the conjugate microgel

protected the emulsion droplets from destabilization by a physical mechanism caused by steric hindrance effects due to the high molecular weight of the dextran.

4. Conclusions

This study shows that whey protein was covalently linked to dextran via Maillard reaction as determined by OPA and SDS-PAGE, later shown by a gradual disappearance in the intrinsic characteristic band pattern of the WPI fractions. Changes in pH and reaction time increased the degree of conjugation. It was found that the degree of conjugation during Maillard reaction greatly influenced the large deformation properties of the heat-set gels fabricated using these conjugates, such heat-set gels were used to create conjugate microgel particles for the first time by controlled shearing process. These protein-polysaccharide conjugate microgel particles had different responsiveness to pH, ions (NaCl / $CaCl_2$) and pepsin depending on the degree of conjugation. The conjugation of whey protein isolate with dextran delayed the gastric digestion of conjugate microgel particles, which might be attributed to the steric hindrance effect that limits pepsin access to the proteinaceous group with the particles. Conjugate microgel particles with a low degree of conjugation of ~10% effectively acted as Pickering stabilizers for oil-in-water emulsions and by using a proof-of-concept cross-correlation analysis of confocal images it was demonstrated that such conjugate microgel particle-stabilized Pickering emulsions exhibited decreased pepsin digestibility kinetics as compared to droplets stabilized by non-conjugated whey protein-based nanogel particles. The novel insights generated in this study may be applied to rationally design Maillard-based conjugate microgel particle-stabilized emulsions to improve emulsion stability in the human gastric regime for effective delivery of lipophilic ingredients in the human intestines.

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