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Araiza-Calahorra, A and Sarkar, A orcid.org/0000-0003-1742-2122 (2019) Designing biopolymer-coated Pickering emulsions to modulate in vitro gastric digestion: A static model study. Food and Function, 10 (9). pp. 5498-5509. ISSN 2042-6496

https://doi.org/10.1039/C9FO01080G

Designing biopolymer-coated Pickering emulsions to modulate in vitro gastric digestion: A static model study

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Abstract

The aim of this study was to restrict the degree of gastric destabilization of Pickering emulsions by using electrostatic deposition of a biopolymeric layer at the proteinaceous particle-laden oil-water interface. Pickering emulsions (20 wt% oil) were prepared using whey protein nanogel particles (WPN, $D_h \sim 91.5$ nm) (1 wt%) and the emulsions were coated by a layer of anionic polysaccharide, dextran sulphate (DxS) of molecular weight (MW) of 40 or 500 kDa, respectively. The hypothesis was that DxS coating on the protein nanogel particle-laden interface would act as a steric barrier against interfacial proteolysis of WPN by pepsin. During static in vitro gastric digestion, the droplet size, ζ-potential, microstructure (confocal microscopy with fluorescently labelled dextran) and protein hydrolysis were monitored. The ζ-potential measurements confirmed that 0.2 wt% DxS was sufficient to coat the WPN-stabilized emulsion droplets with clear charge reversal from +35.9 mV to -28.8 (40 kDa) and -46.2 mV (500 kDa). Protein hydrolysis results showed a significantly lower level of free amino groups upon addition of 0.2 wt% DxS of either 40 or 500 kDa MW to the WPN ($p \leq 0.05$). Emulsions coated with DxS-500 kDa presented stable droplets with lower degree of pepsin hydrolysis of the adsorbed layer as compared to those coated with DxS-40 kDa or uncoated protein nanogel-stabilized interface after 120 min of digestion, highlighting the importance of charge density and molecular weight of the polymer coating. Insights from this study could enable designing gastric-stable emulsions for encapsulation of lipophilic compounds that require delivery to the intestine.

Keywords

Pickering emulsion; nanogel; whey protein; dextran sulfate, electrostatic; in vitro gastric digestion

Abbreviations:

WPN: Whey protein nanogel particles; DxS: Dextran sulfate; E_{WPN}: Pickering emulsions stabilized by WPN, DxS-E_{WPN}-40: Pickering emulsions stabilized by WPN and coated with dextran sulfate of molecular weight (MW) 40 kDa; DxS-E_{WPN}-500: Pickering emulsions stabilized by WPN and coated with dextran sulfate of MW 500 kDa.

1. Introduction

The global prevalence of obesity has nearly tripled in the last 40 years [1]. Currently, one of the proposed strategies to attenuate this global epidemic is food structuring to increase satiety. Such strategies include designing food structures that delay the digestion of calorie-dense lipids to provoke generation of satiety hormones and enhance post-meal satiation via an 'ileal brake' mechanism [2-4]. Although significant attempts have been made to delay the digestion of lipid-based food structures i.e. via designing model oil-in-water emulsions through interfacial engineering, many if not most of these interfacial layers, are competitively displaced by bio-surfactants i.e. bile salts, consequently preventing such delay [5-7]. The key conclusion from these colloidal studies is that a more structurally complex and/or thicker interfacial structure is needed that is resistant to this competitive displacement and at the same time not readily digested by human proteolytic enzymes [8-11].

Recently, colloidal particles have been used for the interfacial design of either oil-in-water or water-in-oil interfaces and these are referred to as Pickering emulsions [12, 13]. An important reason why Pickering emulsions have gained significant interest is that as opposed to conventional emulsions stabilized by proteins or surfactants, Pickering emulsions are highly

resilient to coalescence and Oswald ripening [13]. Such ultra-stability is given by the high desorption energies (thousands of kT/particle), which makes it practically impossible to desorb these particles once they are adsorbed at the interface. Thus, Pickering stabilizers are promising candidates for delaying lipid digestion as there is negligible possibilities of these particles to be displaced by bile salts once they have been adsorbed at the oil-water interface [11, 14]. For example, protein microgel particles have been recently used as Pickering stabilizers for oil-in-water emulsions [15]. Such protein microgel particle-stabilized interfaces have been shown to delay lipolysis to a certain extent by providing a transient barrier to the access of bile salts and lipase [16]. However, such behaviour was only observed when the gastric phase of these Pickering emulsions was completely bypassed in a highly unrealistic condition and consequently the effect of digestion of the protein microgel particles by pepsin was not taken into account. In other words, the main drawback of these protein particle-stabilized emulsions is that there is hydrolysis of the particle-laden interface by pepsin [16] under simulated gastric digestion conditions [17]. Peptic hydrolysis of the adsorbed protein particle layer at the interface may lead to changes in emulsion microstructure and stability and thus might not be able to delay lipid digestion in the later phases. Thus, the unresolved challenge is to develop particle-stabilized emulsions that are protected against pepsin-induced hydrolysis in the gastric phase.

One approach to protect these emulsions from gastric destabilization might be biopolymer coatings through the layer-by-layer (LbL) deposition methodology. The LbL technique has been conventionally used to increase the stability of protein-stabilized emulsions against environmental stresses, such as changes in pH, ionic strength, temperature and spray-drying [18-23]. This technique consists of electrostatic deposition of an ionic polysaccharide onto the surface of a mutually oppositely charged protein-stabilized emulsion droplet [24]. In addition, there has also been significant progress into the use of biopolymer coatings to improve the chemical stability of encapsulated components, such as digestion, release and absorption rates

of lipid droplets within the gastrointestinal tract (GIT) [25-28]. In the domain of Pickering emulsions, one study reported a particle-particle interface, where negatively-charged inulin particles were electrostatically deposited on Pickering emulsions stabilized by positively-charged lactoferrin nanogel particles. In this study, it was shown that the electrostatic deposition of inulin particles decreased the rate and degree of hydrolysis of the lactoferrin nanogel particles at the oil-water interface during gastric digestion [29]. Electrostatic adsorption of lactoferrin-based nanoparticles or nanogel particles with polysaccharides such as pectin, carrageenan and inulin nanoparticles has been previously reported [29-31]. Nevertheless, use of biopolymer coating at particle-laden interfaces is a simple tool to delay the gastric destabilization in simulated gastric condition, which has not attracted much attention in literature [30].

In our previous study, we have comprehensively characterised the use of whey protein nanogel particle (WPN) as Pickering stabilizers for oil-in-water emulsions (E_{WPN}) [32]. In this study we aimed to understand the influence of biopolymer coating on the gastric fate of whey protein nanogel-stabilized Pickering oil-in-water emulsions using the INFOGEST in vitro static gastric model [33]. Whey protein isolate (WPI) was used to produce the soft solid particles i.e. whey protein nanogel particles (WPN), acting as the Pickering stabilizer. To coat the emulsion stabilized by WPN, dextran sulphate (DxS), which is a branched-chain polysaccharide with 1–6 and 1–4 glycosidic linkage with approximately 2.3 sulphate groups per glucosyl unit, of two molecular weights i.e. 40 and 500 kDa, was used. The DxS was selected due to the abundance of highly negatively charged sulphate groups that will allow electrostatic deposition to cationic WPN-stabilized droplets at gastric pH i.e. below the isoelectric point (pI ~5.2) of whey protein. We hypothesize that DxS coating on the protein nanogel particle-laden interface would act as a steric barrier against interfacial proteolysis by pepsin and the barrier properties will depend on the molecular weight of the DxS used. A

123 combination of complementary techniques, such as light scattering, zeta-potential, confocal
124 microscopy (with fluorescently-labelled DxS), protein hydrolysis using sodium dodecyl
125 sulphate polyacryl amide gel electrophoresis (SDS-PAGE) of the adsorbed layer and
126 standardized ortho-phthaldialdehyde (OPA) assay were used to test the above-stated
127 hypothesis.
128

129 ## 2. Materials and methods

130 ### 2.1. Materials

131 Whey protein isolate (WPI) with ≥ 90% protein content was gifted from Fonterra Co-operative
132 Group Limited (Auckland, New Zealand). Porcine pepsin (P7000, measured enzyme activity:
133 371 U mg^{-1} using haemoglobin as substrate), dextran sulphate sodium salt (DxS) of molecular
134 weight (MW) 40 and 500 kDa containing 15–19% and 16–19% of sulphur content,
135 respectively, and fluorescein isothiocyanate (FITC)-labelled dextran sulphate (MW 40 and 500
136 kDa) were purchased from Sigma-Aldrich Company Ltd, Dorset, UK. Sodium chloride,
137 sodium hydroxide, sodium phosphate monobasic monohydrate, sodium phosphate dibasic
138 anhydrous and hydrogen chloride were purchased from Thermo Fisher Scientific,
139 Loughborough, UK. The lipid phase consisted of medium-chain triglyceride (MCT-oil)
140 Miglyol® 812 with a density of 945 kg m^3 at 20 ^oC (Cremer Oleo GmbH & Co, Germany).
141 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) reagents including
142 Mini-Protean Precast TGX gels (8–16%) and Precision Plus Protein All Blue Standards were
143 purchased from Bio-Rad (Bio-Rad Laboratories Ltd., Richmond, CA, USA). All reagents were
144 of analytical grade and used without further purification unless otherwise reported. All
145 solutions were prepared with Milli-Q water with a resistivity of 18.2 MΩ cm at 25 °C (Milli-
146 Q apparatus, Millipore, Bedford, UK). Sodium azide (0.02 wt %) was added as a preservative.
147

2.2. Preparation of whey protein nanogel particles (WPN)

The whey protein nanogel particles (WPN) were produced based on modification of a previously developed top-down technique [16, 29]. The WPI powder (10 wt%) was dissolved in 10 mM phosphate buffer at pH 7.0 for 2 hours to ensure complete dispersion. The WPI solution was heated in a temperature-controlled water bath at 90 ^{o}C for 30 min to form a heat-set gel (quiescent), followed by cooling down for 15 min and storage at 4 ^{o}C overnight to form heat-set hydrogels. Obtained WPI gels were pre-homogenized with phosphate buffer (5 wt%) using a hand blender (HB724, Kenwood) for 1 minute and transferred to a vacuum box (John Fraser and Sons Ltd, London, UK) for degassing. The resulting 5 wt% whey protein gel was passed through a Leeds Jet homogenizer, a bespoke two-chamber homogenizer developed in the School of Food Science and Nutrition (University of Leeds, Leeds, UK) at 300 bars for two passes, respectively. Final whey protein nanogel particles (WPN) were diluted with buffer to the desired protein concentration for the emulsion preparation.

2.3. Preparation of Pickering oil-in-water emulsions

Whey protein nanogel-stabilized emulsions (E_{WPN}). Pickering oil-in-water emulsions were prepared using MCT-oil (20 wt%) and a protein content of 1 wt% in the final emulsion. Briefly, coarse E_{WPN} (20:80 w/w) droplets were prepared using Ultra Turrax T25 homogenizer (IKA-Werke GmbH & Co., Staufen Germany) at 13, 500 rpm for 1 min. Following this, the coarse emulsions were homogenized using Leeds Jet homogenizer (School of Food Science and Nutrition, University of Leeds, UK) at 300 bars using two passes to prepare fine E_{WPN} droplets.

Whey protein nanogel-stabilized emulsion coated with dextran sulphate (DxS-E_{WPN}). Two types of emulsions of dextran sulphate (DxS)-coated Pickering emulsions (DxS-E_{WPN}) were produced by mixing E_{WPN} at pH 3.0 (40 wt% MCT, 2.5 wt% WPN in aqueous phase) produced

using afore-mentioned method, with aqueous dispersions of DxS of 40 kDa or 500 kDa, while maintaining the pH at pH 3.0. For confocal laser scanning microscopy, another set of samples were prepared using Fluorescein isothiocyanate (FITC)-labelled DxS of 40 kDa or 500 kDa. The biopolymer solutions were prepared by dissolving the powdered DxS of desired MW into Milli-Q water (pH 3.0), and stirring overnight at 21 ^{o}C to ensure dissolution. The E_{WPN} and aqueous dispersions of DxS were mixed in 1:1 w/w to produce DxS-E_{WPN} (20 wt% MCT, 1 wt% WPN) with different concentrations of DxS-40 kDa or DxS-500 kDa (0.05-1 wt%), pH was readjusted to pH 3.0 using 1.0 M HCl and samples were stirred for 2 hours at 25 ^{o}C to allow electrostatic deposition of DxS to the E_{WPN} droplets. Hereafter, the emulsions named as DxS-E_{WPN}-40 and DxS-E_{WPN}-500 represent the polymer-coated emulsions containing DxS of MW, 40 kDa or 500 kDa, respectively.

2.4 In vitro gastric digestion of particles and emulsions

The aqueous dispersions of WPN, WPN+DxS-40 kDa and WPN+DxS-500 kDa, respectively and the corresponding emulsions i.e. E_{WPN}**,** DxS-E_{WPN}-40 and DxS-E_{WPN}-500 were digested using slightly adapted digestion protocol[33], i.e. without the simulated oral phase considering that neither WPN nor DxS are susceptible to α-amylase. 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 $MgCl_2(H2O)_6$, 0.024 g L^{-1} of $(NH_4)2CO_3$ and 2000 U/mL pepsin at pH 3.0. The mixture was incubated for 2 h at 37 ^{o}C under agitation using a shaking water bath (Grant Instruments Ltd, Cambridge, UK). As control, samples were also subjected to SGF treatment without added pepsin i.e. SGF buffer. 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

198 by neutralizing to pH 7.0 using freshly prepared 1 M NH_4HCO_3 except for size and charge
199 measurements, in latter experiments, samples were characterized immediately after digestion.
200
201 2. 5 Particle size and droplet size distribution
202 The physicochemical properties and stability of aqueous dispersions of WPN+DxS-40 kDa and
203 WPN+DxS-500 kDa, respectively and their corresponding emulsions i.e. E_{WPN}**,** DxS-E_{WPN}-40
204 and DxS-E_{WPN}-500 before and after digestion were monitored using their particle size
205 distribution, ζ-potential and microstructural changes as a function of gastric digestion time.
206 Particle size of the aqueous dispersions of WPN, WPN+DxS-40 kDa and WPN+DxS-500 kDa,
207 respectively was determined using dynamic light scattering (DLS) at 25 ^oC using a Zetasizer
208 Nano-ZS (Malvern Instruments, Malvern UK) in a PMMA standard disposable cuvette.
209 Particle size of the samples before and after gastric digestion was measured after diluting the
210 samples in SGF buffer (pH 3.0). Droplet size distributions of the three Pickering emulsion
211 samples i.e. E_{WPN}**,** DxS-E_{WPN}-40 and DxS-E_{WPN}-500 were determined using static light
212 scattering at 25 ^oC using Malvern MasterSizer 3000 (Malvern Instruments Ltd, Malvern,
213 Worcestershire, UK). The refractive index of the MCT (Miglyol® 812 oil) and the dispersion
214 medium were set at 1.445 and 1.33, respectively. The absorbance value of the emulsion
215 droplets was 0.001. The mean particle size distribution of the emulsions was reported as
216 volume mean diameter (d_{43}) and surface mean diameter (d_{32}) based on five measurements on
217 triplicate samples.
218
219 2.9. ζ-potential
220 The ζ-potential of aqueous dispersions of WPN, WPN+DxS-40 kDa and WPN+DxS-500 kDa,
221 respectively and their corresponding emulsions i.e. E_{WPN}**,** DxS-E_{WPN}-40 and DxS-E_{WPN}-500
222 before and after digestion was determined using a particle electrophoresis instrument

(Zetasizer, Nano ZS series, Malvern Instruments, 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., 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.

2.6. Confocal scanning laser microscopy (CLSM)

The microstructure of the Pickering emulsions i.e. E_{WPN}**,** DxS-E_{WPN}-40 and DxS-E_{WPN}-500 was observed before and after the gastric digestion experiments using a Zeiss LSM 880 inverted confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany). A stock solution of Nile Red (1 mg/ mL in dimethyl sulfoxide, Sigma-Aldrich) was used to stain the MCT-oil to a final concentration of 0.02 mg mL^{-1} and a stock solution of Fast Green (1 mg mL^{-1} in Milli-Q water) was used to stain the WPN to a final concentration of 0.1 mg mL^{-1}. Fluorescein isothiocyanate (FITC)-labelled-DxS of 40 kDa or 500 kDa was used to image DxS-E_{WPN}-40 and DxS-E_{WPN}-500, respectively, where FITC-DxS was used during the emulsion preparation process. The emulsion samples were placed on a concave confocal microscope slide, secured with a glass coverslip and finally imaged using an oil immersion 40× lens. The pinhole diameter was maintained at 1 Airy Unit to filter out majority of the light scattering. Nile Red was excited at a wavelength of 488 nm, Fast Green at 633 nm and FITC-DxS at 495 nm. The emission filters were set at 555 - 620 nm for Nile Red, 660 - 710 nm for Fast Green and 450 – 520 nm for FITC-DxS.

2.7 Quantification of protein hydrolysis

Protein hydrolysis was quantified using the standardized ortho-phthaldialdehyde (OPA) method, as described by [34] with minor modifications. Briefly, OPA reagent consisted of 3.81 g sodium tetraborate, 0.088 g dithiothreitol and 0.1 g sodium docecyl sulphate. Exactly, 0.080 g OPA was dissolved in 2 mL ethanol and added to the above-mentioned solution and made up to 100 mL with Milli-Q water. The solution was kept in the dark. Absorbance at 340 nm was measured, using a UV-VIS spectrophotometer (6715 UV/VIS Spectrophotometer, Jenway, UK) blanked with OPA reagent and Milli-Q water. Quantification of protein hydrolysis was performed by using a reference calibration curve of L-leucine solution (0 - 200 μM). Exactly, 160 μL of standard solutions were added to 1200 μL OPA reagent in a PMMA cuvette, mixed for 5 seconds and absorbance was measured after standing for 2 min. The same procedure was applied to the samples. The protein hydrolysis was expressed as a μM free amino groups per mass of the total protein in sample.

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

The protein composition of the aqueous dispersions of WPN, WPN+DxS-40 and WPN+DxS-500, respectively and corresponding absorbed phases of the emulsion droplets (E_{WPN}**,** DxS-E_{WPN}-40 and DxS-E_{WPN}-500) after gastric hydrolysis by pepsin was examined using SDS-PAGE under reducing conditions. The WPN-SGF, WPN+DxS-40 kDa-SGF or WPN+DxS-500 kDa-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 precast gels placed on a Mini-PROTEAN II system (Bio-Rad Laboratories, Richmond, CA, USA). 5 μL of protein molecular weight marker was added in the first lane. After running the gel at 100 V for an hour, the gel was stained for 120 min 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).

For measuring the composition of adsorbed phase in case of E_{WPN}, DxS-E_{WPN}-40 or DxS-E_{WPN}-500, the emulsion–SGF mixtures (1.5 mL) after digestion and termination of the pepsin-induced hydrolysis were collected at specific time points and centrifuged for 40 min at 4,000 g at 20 ^{o}C using an Eppendorf centrifuge (Thermo Scientific, Waltham, MA). The cream layer was collected carefully and a certain amount of cream was then mixed with SDS buffer at a sample : SDS buffer ratio of 1:4, heated at 95 °C for 5 min with 10 μL of sample loaded and the SDS-PAGE experiment was conducted. 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. 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.

2.8. Statistical analysis

Mean and standard deviation 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 Optimization of the biopolymer-coated Pickering emulsions

295 Firstly, the influence of pH on the ζ-potential of WPN, DxS-40 kDa and DxS-500 kDa was
296 examined (Supplementary Fig. S1). The ζ-potential of the WPN went from being highly
297 positive to highly negative as the pH was increased from 2.0 to 7.0 due to the protonation of
298 the ionisable groups as they move from above to below the isoelectric point, respectively
299 (Supplementary Fig. S1). On the other hand, the ζ-potential of the DxS was negative at all pH
300 values examined within the experimental window (supplementary Fig. S1). The magnitude of
301 the negative charge of the low MW DxS-40 Da was smaller as compared to that of the high
302 MW DxS-500 kDa ($p < 0.05$), which is expected owing to the charge densities of the sulphate
303 groups per unit of the dextran molecule being proportional to the MW of the biopolymer.
304 Secondly, we examined the influence of MW and concentration of DxS on the droplet size
305 and charge of emulsions stabilized by WPN (Fig. 1). At pH 3.0, the ζ-potential of the E_{WPN} was
306 highly positive since WPN is below its isoelectric point (supplementary Fig. S1). As the
307 biopolymers were added, there was a significant reduction in net ζ-potential values. Upon
308 adding 0.1 wt% DxS-40 kDa or DxS-500 kDa, the net charge almost reduced to zero
309 particularly in case of DxS-E_{WPN}-40 (Fig. 1a1) whilst some positive charge remained in case
310 of DxS-E_{WPN}-500 (Fig. 1a2). This suggests that the coverage by DxS was incomplete at such
311 low concentration. When the DxS concentration was increased to 0.2 wt%, the ζ-potential
312 values of the DxS-E_{WPN}-40 or DxS-E_{WPN}-500 droplets showed a charge reversal from positive
313 to negative values, highlighting that electrostatic interaction was sufficient between WPN-
314 laden interface and DxS, irrespective of the MW. This result was attributed to the electrostatic
315 deposition of anionic groups of the biopolymers onto the cationic surface of the protein-
316 stabilized droplets [35-38]. The net charge reached a plateau at ≥ 0.2 wt% DxS, which suggests
317 that the anionic DxS had fully saturated the surface of cationic E_{WPN} droplets, with DxS-40
318 having lower magnitude of ζ-potential as compared to DxS-500, as discussed before.

Droplet size measurements indicated that addition of DxS increased the volume-average mean diameter (d_{43}) of the DxS-E_{WPN}-40 droplets, which ranged from 6.84 to 98.4 μm (Fig. 1b1), whereas for DxS-E_{WPN}-500 droplets, the d_{43} ranged from 6.84 to 58.9 μm (Fig. 1b2). These observations suggests droplet flocculation due to the formation of polymeric bridges between anionic DxS adsorbed to WPN-laden interface and some cationic patches in the surface of the neighbouring uncoated E_{WPN} droplets [31]. In addition, these flocculated droplets were not easily disrupted by the dilution or shear effects within the static light scattering (SLS) equipment. Interestingly, at an optimum concentration of 0.2 wt% DxS (Fig. 1c1 and 1c2), the droplet size distribution became smaller with shrinkage in the more prominent peak in the size range of 10-1000 μm to 1-100 μm. This suggests that on adding higher amounts of DxS, a reduction in the droplet flocculation and electrostatic stabilization of the droplets was observed, as corroborated by the charges on the electrical charge observed in Fig. 1a1 and 1a2. Since 0.2 wt% of anionic DxS was the minimum concentration needed to completely coat the cationic E_{WPN} droplets at pH 3.0, irrespective of the MW of DxS, resulting in negatively charged DxS-E_{WPN}-40 or DxS-E_{WPN}-500 emulsion droplets (Fig. 1a1 and 1a2), this concentration was selected for the preparation of the DxS-E_{WPN}-40 or DxS-E_{WPN}-500 droplets.

3.2 In vitro gastric digestion of aqueous dispersions of WPN and DxS+WPN

Firstly, we determined the behaviour of the aqueous dispersions of WPN in absence and presence of 0.2 wt% DxS-40 kDa or DxS-500 kDa, respectively (Fig. 2, 3 and supplementary Table S1 and supplementary Fig. S2 and S3). This sets the scene to understand how the particles might behave when present in the continuous phase of the respective emulsions and compare it when they are present at the adsorbed phase. To determine the behaviour of the aqueous dispersion of WPN in presence or absence of DxS in the in vitro gastric digestion model, changes in their physiochemical properties and protein composition were examined as a

function of digestion time. As controls, an aqueous dispersion of 1 wt% WPN and 1wt% WPN in presence of 0.2 wt% DxS-40 kDa or DxS-500 kDa in SGF without pepsin were also analysed (supplementary Table S2).

WPN. At pH 3.0, WPN had a hydrodynamic diameter of 91.5 nm and a ζ-potential of +30.2 mV (supplementary Table S1). Formation of WPN during the heat treatment is the result of the association of small aggregates via hydrophobic, electrostatic and disulphide bonds [39]. After addition of SGF buffer (pH 3.0) without pepsin, changes in ζ-potential were not significant ($p > 0.05$) (see time zero in Supplementary Fig. S2). Upon gastric incubation in SGF containing pepsin, the size distribution of the particles became multi-modal and it was no longer possible to measure the hydrodynamic diameter using DLS as the particle size was too polydisperse to be considered for Rayleigh fitting (data not shown). This indicates that WPN was hydrolysed by pepsin and created different sizes of particle aggregates. As can be expected, a significant decrease in the ζ-potential value was also observed within the first 15 min of digestion, which remained fairly constant at ~+20 mV even after 2 h (supplementary Fig. S2).

The results are in agreement with the protein hydrolysis monitored using SDS-PAGE (Fig. 2a1 and b1). It can be observed that, after just 5 min of incubation, the intensity of the bands corresponding to the major whey proteins i.e. β-lactoglobulin (β-lg), α-lactalbumin (α-la) as well as bovine serum albumin (BSA) bands was reduced considerably (remaining intact proteins were 24%, 19.27% and 0%, respectively), with simultaneous appearance of a mixture of peptides with molecular weights < 10 kDa after 2 h incubation (Fig. 2a1). These results can be explained in terms of temperature-induced conformational changes in the whey protein structure. In native state, β-lg (the major protein in whey protein isolate) has most of the hydrophobic amino acids buried inside the β-barrel structure making them not easily accessible to pepsin, which makes native β-lg resistant to pepsin hydrolysis [40]. Upon heating, unfolding

of the protein molecule exposes the hydrophobic amino acids, making it highly susceptible to hydrolysis by pepsin [41]. From our results it is evident that the thermal treatment during WPN preparation exposed the hydrophobic amino acids, which were not refolded during the particle formation making them more accessible to pepsin hydrolysis during gastric incubation as compared to native WPI.

WPN+DxS. At pH 3.0, aqueous dispersions of WPN containing either DxS-40 kDa or DxS-500 kDa had highly negative ζ-potential values of -21.6 mV and -37.4 mV, respectively, which suggest that the DxS saturated the surface of cationic WPN (Supplementary Table S1). Addition of DxS caused particle aggregation irrespective of the MW, and it was not possible to measure the hydrodynamic diameter using DLS (supplementary Fig. S3b and S3c).

Presence of SGF without pepsin did not showed significant ($p < 0.05$) changes in the ζ–potential for both 40 and 500 kDa DxS (-37.7 and -40.5 mV, respectively) (see time zero in supplementary Fig. S2). During gastric incubation with pepsin, the magnitude of ζ-potential of the particles was reduced from -37.7 mV to a constant value ranging between -4.4 and +2.2 mV for 40 kDa DxS samples (Supplementary Fig. S2). The ζ–potential of the WPN+DxS-500 kDa particles was more negative than that of the WPN+DxS-40 kDa and was reduced from -40.5 mV to a constant value ranging between -15.7 and -22.3 mV, during the 120 min of gastric incubation (Supplementary Fig. S2). The most likely explanation for this effect is that there was electrostatic screening of charge of the biopolymer-coated particles by the SGF buffer, as can be corroborated by the control samples subjected to the SGF without pepsin (supplementary Table S2).

The SDS-PAGE electrograms of WPN+DxS for both biopolymers are shown in Figs. 2a2 and 2a3. It can be seen that for both biopolymers i.e. DxS-40 kDa or DxS-500 kDa, the β-lg bands remained relatively resistant to pepsin hydrolysis as compared to that of WPN alone (Fig. 2a1). Particularly, it can be observed that the band corresponding to α-la protein remained

resistant to pepsin hydrolysis for DxS-500 kDa, with a clear band still present after up to 2.5 h incubation in SGF (Fig. 2a3). Quantification of the SDS-PAGE gel bands (Figs. 2b2 and 2b3) suggests that in presence of DxS-40 kDa, the proportion of β-lg and α-la decreased to 11.47% and 0.25%, respectively after 2 h of incubation (Fig. 2b2). As for DxS-500 kDa, the proportion of intact protein bands were much higher, with 27.70% of β-lg and 14.88% of α-la remaining after 2 h of incubation (Fig. 2b3).

In addition, the free amino group (NH_2) content was determined by OPA method for the WPN and WPN after addition of 0.2 wt% DxS of 40 and 500 kDa before and after gastric digestion with added pepsin and shown in Fig. 3. The concentration of free NH_2 increased generally with digestion time for all samples. Results indicated that for WPN, there was an increase in the proteolysis profile in the first 30 min gastric digestion from 989.97 mM NH_2/g to 3,747 mM NH_2/g, after which it levelled off to values between 3,747–4088.66 mM NH_2/g during the 120 min gastric digestion. These values are in agreement with previously reported hydrolysis of different whey proteins [42, 43]. Levels of proteolysis were significantly ($p < 0.05$) lower after addition of DxS. For lower molecular weight DxS (40 kDa), after 30 min gastric digestion, the free NH_2/g concentration was 3,105.88 mM NH_2/g (as compared to 3,747 mM NH_2/g in WPN) with a relatively constant value between 3,105.88 – 3,808.97 mM NH_2/g during the 120 min (Fig. 3). The proteolysis profile for DxS of higher molecular weight of 500 kDa was 771.01 mM NH_2/g in the first 30 min with values between 771.01 – 2,752.21 mM NH_2/g after the 120 min gastric digestion, clearly indicating the barrier effect of DxS-500 kDa on proteolysis of WPN.

A possible explanation from the increased protection to WPN upon DxS addition might be related to the aggregation state of the particles, i.e. DxS was aggregating the WPN particles limiting the access of pepsin to the peptic cleave sites of WPN. In addition, the higher extent of protection to WPN against proteolysis by DxS-500 kDa as compared to that of DxS-40 kDa

419 may arise from a combination of high ζ-potential values, and/or the MW (degree of branching)
420 that somehow physically inhibited the enzyme to reach the hydrophobic moieties of the protein
421 nanogel particles. These results are in agreement with a previous study that have reported
422 increased resistance to gastric proteolysis during first 10 min of simulated gastric conditions of
423 lactoferrin nanoparticles, when coating these particles with low methoxyl pectins as compared
424 to that of high methoxyl pectins. The increased gastric resistance was largely associated with
425 the increased electro-kinetic charge of the former [30]. Overall, these results suggest that the
426 extent of the protective effect of DxS-500 kDa was markedly higher than that of DxS-40 kDa
427 in the bulk phase and it would be interesting to see whether such effects exist when DxS coats
428 the WPN-stabilized droplets at the surface.
429
430 3.3 Physicochemical and microstructural characterization of E_{WPN}, DxS-E_{WPN}-40 and DxS-
431 E_{WPN}-500 during in vitro gastric digestion
432 Pickering emulsion samples with or without the biopolymer coating of 0.2 wt% DxS were
433 prepared at pH 3.0, subjected to in vitro gastric model (SGF, pH 3.0) at 37 ^{o}C and then the
434 droplet size, charge, and microstructure were measured as a function of digestion time (Figs. 4
435 and 5). In addition, as controls, E_{WPN}, DxS-E_{WPN}-40 and DxS-E_{WPN}-500 in SGF without pepsin
436 were also analysed (Supplementary Fig. S3).
437 **E_{WPN}**. Freshly prepared E_{WPN} at pH 7.0 presented a bimodal droplet size distribution
438 with the droplet population in the peak area of 0.1-1.0 μm corresponding to the unadsorbed
439 WPN and the population in the peak area of 10-100 μm corresponding to the emulsion droplets
440 (Fig. 4a1). The mean droplet diameter (d_{43}) was 9.61 μm and the droplets presented a highly
441 negative charge of -26.70 mV, because the adsorbed layer of WPN was above its pI (data not
442 shown). Confocal images of the E_{WPN} shows the WPN particles (stained green by Fast Green)
443 clearly adsorbed on the surface of the oil droplets (stained by Nile Red) (Fig. 4a1). Upon

444 decreasing the pH to gastric pH 3.0, the droplet size distribution and the volume average mean
445 diameter (d_{43}) were not significantly changed ($p > 0.05$) (Fig. 4a1 and Table 1), but the droplets
446 presented a charge reversal to a highly positive charge of +35.9 mV, as the pH was below the
447 pI as previously discussed with respect to Fig. 1a1 and 1a2.

448 Incubation of E_{WPN} with SGF without pepsin did not significantly influence the droplet
449 size distribution (Fig. 4a1), mean droplet size (d_{43} 16.93 μm) and ζ-potential of the droplets
450 (see time zero in Fig. 4b1), indicating that E_{WPN} was stable to any aggregation under the ionic
451 environment of the gastric conditions. From the confocal images, it can be seen that the
452 emulsion droplets did not show significant aggregation (Fig. 5b1) as compared to initial
453 emulsions (Fig. 5a1). When E_{WPN} was incubated in SGF with pepsin, the mean droplet size
454 decreased from 16.93 μm to 9.72 μm within the first 5 min of gastric digestion and remained
455 fairly constant obtained over time (Fig. 4b1). The droplet size distribution, shows the
456 appearance of a population of smaller droplet size (Fig. 4a1), which was not observed in the
457 control samples (Supplementary Fig. S3). Interestingly, the ζ-potential of the droplets remained
458 fairly constant at +37.6 mV by the end of the gastric incubation (Fig. 4b1) indicating the
459 presence of enough WPN at the interface. Therefore, it can be suggested that the decrease in
460 size of the E_{WPN} with smaller sized population might have been caused by pepsin hydrolysis
461 either hydrolysing the protein at the surface of the emulsion droplets, or the particles bridging
462 different droplets. This result is in agreement with previous studies that have reported decrease
463 of d_{43} values after gastric digestion of whey protein microgel-stabilized Pickering emulsions
464 [16].

465 Looking at the confocal images, after 30 minutes incubation, the microstructure of the
466 emulsion (Fig. 4c1) changed dramatically showing very limited population of droplets. One
467 might argue it is due to the dilution effect with SGF, however that can be negated by looking
468 at the image at 0 min which had equivalent droplet concentration due to dilution with SGF

buffer without any added pepsin. This suggests that pepsin was hydrolysing the bridges between the WPN-coated droplets as well as was rupturing the particle-layer at the interface to a certain extent resulting in some degree of droplet coalescence. Such coalesced droplets were most likely rising to the top and thus were not visulaized by the confocal microscopy resulting in reduction in droplet volume (Figs. 5c1). Confocal micrographs of the emulsion after 120 min of gastric digestion provided further evidence of the appearance of individual droplets that appeared to be less aggregated emulsion droplets (Fig. 5d1) than the ones in 0 min (Fig. 4b1). A thin interfacial layer around the oil droplets even after 120 min was observed in the microscopic image (Fig. 5d1) suggesting that either WPN or peptide network of WPN were still present in the emulsion droplets after the gastric digestion that were somehow protecting the droplets against coalescence.

Table 1. Droplet size and ζ-potential values for E_{WPN}, DxS- E_{WPN}-40 and DxS- E_{WPN}- at pH 3.0, respectively.

	E_{WPN}		**DxS- E_{WPN}-40**		**DxS- E_{WPN}-500**	
	d_{43} / μm	ζ-potential / mV	d_{43} / μm	ζ-potential / mV	d_{43} / μm	ζ-potential / mV
pH 3.0	11.50 ± 1.20	+35.9 ± 0.80	22.2 ± 0.98	-28.8 ± 1.30	38.8 ± 0.07	-46.2 ± 2.42

DxS-E_{WPN}-40 and DxS-E_{WPN}-500. Before digestion, biopolymer-coated emulsions containing either of the two anionic DxS (40 or 500 kDa MW) had high negative charges (-28.8 and -46.2 mV for 40 kDa and 500 kDa, respectively) at pH 3.0 (Table 1), suggesting that the biopolymer had substantially adsorbed onto the cationic WPN-stabilized oil droplets at pH 3.0. Both 40 and 500 kDa DxS-E_{WPN} presented a bimodal particle size distributions (Fig. 4a2 and 4a3) with a mean droplet size (d_{43}) of 22.2 and 38.8 μm, respectively (Table 1). The increase of the droplet size might result from the aggregation of the E_{WPN} droplets after addition of DxS, as reflected by the confocal images (Figs. 5 a2 and 5a3). In addition, it can also be

observed in Figs. 5a2 and 5a3 that FITC-labelled DxS (stained in blue) is electrostatically adsorbed to the WPN particles (stained in green). At this point, it is worth mentioning that, Nile Red was not used to stain the oil droplets, since both Nile Red and FITC-labelled DxS possessed similar excitation wavelengths. For this reason, images where only acquired using Fast green (for WPN) and FITC-DxS for visualizing the biopolymer coating. Supplementary Fig. S4 shows a control image indicating that the presence of FITC-labelled DxS only records the DxS and not the WPN.

Incubation in SGF without pepsin showed no significant ($p < 0.05$) changes in the ζ-potential values and d_{43} (see time zero for Figs. 4b2 and 4b3) for DxS of both MW, confirming there was no SGF-induced effects on aggregation of the droplets. The confocal images (Figs. 5b2 and 5b3) further confirms that the FITC-labelled DxS remained adsorbed to the WPN and that the emulsion droplets sizes are in agreement with the light scattering data. After addition of pepsin, there was a pronounced decrease in the magnitude of their negative charge within the first 5 min of digestion, reaching a fairly constant value at longer digestion time scales (Figs. 4b2 and 4b3). This negative charge is in line with the anionic DxS being bound to the DxS-E_{WPN} droplets under SGF, as evidenced by the bright blue ring surrounding the emulsion droplets and indicating the presence of the FITC-labelled DxS (Figs. 5c2-c3 and 5d2-d3). Emulsions were stable to the gastric conditions, since there was no increase in the mean particle diameter (d_{43}) (Figs. 4b2 and 4b3), and there was no evidence of coalescence in the microscopy images (Fig. 5c2-c3 and 5d2- d3). Irrespective of digestion times, DxS appeared to be in the same region as the WPN highlighting that the electrostatic complexation at the interface remained almost unaffected in presence of pepsin. These results clearly indicate that DxS-E_{WPN}-40 and DxS-E_{WPN}-500 emulsion droplets at gastric conditions exhibited resistance to any pepsin-induced microstructural changes.

517 3.4 Protein composition of adsorbed phase of EWPN, DxS-EWPN-40 and DxS-EWPN-500
518 during in vitro gastric digestion
519 The SDS-PAGE of the adsorbed phases of all emulsion systems, i.e. E_{WPN}, DxS-E_{WPN}-40
520 and DxS-E_{WPN}-500 were compared (Fig. 6). The SDS-PAGE electrograms revealed that in
521 E_{WPN}, the major protein constituents, β-lg and α-la, as well as BSA were identified in the initial
522 undigested emulsion (Fig. 6a1). However, upon incubation in SGF containing pepsin, within
523 the first 5 min the BSA fraction almost completely disappeared, and the proportion of the β-lg
524 and α-la fractions decreased to 55.39% and 36.19%, respectively (Fig. 6b1). These results
525 suggest that the emulsification of the WPN slightly decreased the accessibility of pepsin as
526 compared to that when WPN was freely available in aqueous dispersion (Fig. 2a1). These
527 results can be explained in terms of wetting of WPN by the oil phase that consequently reduced
528 its accessibility to pepsin. However, after 120 min of gastric incubation, both β-lg and α-la
529 bands completely disappeared, indicating complete hydrolysis of these proteins. At the end of
530 the gastric phase (2.5 h), a fuzzy thick band representing a mixture of peptides with molecular
531 weights < 10 kDa was observed in the gels (Fig. 6a1). This supports the thin particle-laden
532 interface observed in the micrographs even after 2 hours of digestion, which might be the
533 peptide remnants of nanogel particles or peptides (Fig. 5d1).
534 In contrast, the digestion pattern of the DxS-E_{WPN} emulsion during gastric proteolysis
535 was very different from that E_{WPN} (Figs. 6a1 and 6a3). In addition, it should be noted that some
536 interfacial material remained in the stacking gel. It is possible that the protein aggregates of the
537 absorbed phase were too large (>250 kDa) to enter the resolving gel. The hydrolysis pattern for
538 DxS-E_{WPN}-40 emulsions after 5 minutes of gastric incubation revealed that the proportion of
539 BSA decreased to 17.4%, whereas significant proportion of β-lg and α-la fractions remained
540 with intact fractions of about 48.4% and 40.3%, respectively (Fig. 6b2). After 120 min, the
541 quantity of BSA, β-lg and α-la remaining undigested was 0.12%, 11.62% and 11.88%,

542 respectively (Fig. 6b2). Confocal fluorescent microscopy images of the emulsions after 120
543 min digestion indicated the aggregated microstructure arrangement of the droplets, which may
544 also contribute to the slowed pepsin hydrolysis, since pepsin would have to diffuse through the
545 outer parts of these aggregates before it could reach the protein sites underneath these DxS
546 coating (Figs. 5c2-d2). These results suggested that the polysaccharide coating decreased the
547 digestibility of the WPN located at the interface of E_{WPN}.

548 In the case of 500 kDa DxS-$E_{WPN,}$ a considerable amount of interfacial material was
549 also observed to remain in the stacking gel (Fig. 6a3). However, within the first 5 min of gastric
550 incubation, the BSA fraction was reduced to 95.27%, whereas the proportion of the β-lg and
551 α-lg fractions decreased to 89.70% and 94.518%, respectively (Figs. 6b3). At the end of the
552 2.5 h gastric digestion, around 83.42%, 67.50% and 73.75% of the BSA, β-lg and α-la fractions
553 remained still intact. These result are in agreement with previous studies that have reported that
554 the presence of a polysaccharide coating reduced the activity of pepsin for protein-stabilized
555 emulsions after 2 h gastric incubation [30 44] .

556 A possible explanation for this effect could be that the formation of a more complex
557 structure increased the barrier properties of DxS+WPN and reduced the pepsin diffusion to the
558 underneath WPN-stabilized interface. Previous proteolytic studies on casein-stabilized
559 emulsions with an absorbed dextran sulphate layer have suggested that there may be an
560 associative interaction of the enzyme (trypsin) with the absorbed DxS layer, as a large increase
561 in the interfacial shear viscosity was observed [45]. Thus, it seems reasonable to suppose that,
562 pepsin may be trapped or co-adsorbed within the DxS coatings, reducing the availability of
563 pepsin in the close vicinity of hydrophobic groups of WPN for potential proteolysis.
564 Consequently, we can reasonably infer that, a bigger molecular structure may impart a greater
565 barrier effects to WPN substrate against pepsin attak, which is consistent with the differences
566 in extent of hydrolysis between the two different MW of DxS used. A schematic diagram to

illustrate the physical state of the DxS-E_{WPN} when it is exposed to in vitro gastric conditions is presented in Fig. 7. The E_{WPN} is stable in presence of SGF in absence of enzymes, but when pepsin is added to the system, hydrolysis of the interfacial protein layer occurs producing remnants of nanogel particle or peptides. On the contrary, hydrolysis of the interfacial protein layer on the DxS-E_{WPN} is delayed by the presence of a physical barrier provided by a biopolymer either by providing an electrostatically repelling layer or simply providing a steric hindrance to pepsin, thus preventing gastric destabilization of the emulsion droplets.

4. Conclusions

In this study, we have studied the influence of coating of anionic dextran sulphate on the physicochemical properties and digestibility of whey protein nanogel particle-coated droplets under in vitro gastric conditions. Findings from this study report that the stability and degree of pepsin hydrolysis of the whey protein nanogel particles at the interface is restricted in presence of a secondary coating of dextran sulphate, latter could provide a steric and electrostatic barrier. In addition, the molecular weight of the coating had an appreciable effect on the degree of pepsin hydrolysis, with dextran sulphate of a molecular weight of 500 kDa presenting a better barrier to pepsin-hydrosis of the underlying protein nanogel particle-laden interface as compared to that of the 40 kDa molecular weight within the simulated gastric phase. Such results are mainly attributed to the higher negative charge density associated with higher degree of sulphate groups in the higher molecular weight system or the higher order molecular structure that trapped pepsin protecting the protein-laden interface against complete enzymatic hydrolysis. Insights from this study could enable creating future gastric-stable emulsions for food and pharmaceutical applications targeting altered lipid digestion profile in the intestinal phase or encapsulation of lipophilic bioactive compounds that need to be delivered to intestines without any gastric destabilization.

5. Conflict of interest

The authors declare no conflicts of interests.

6. Acknowledgments

Author AAC acknowledges financial support from the Mexican National Council of Science and Technology (CONACyT) for the award of an Academic Scholarship for her PhD. Authors wish to thank Professor Alan Mackie at University of Leeds, UK for useful discussion on gastric digestion assays.

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