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Sarkar, A orcid.org/0000-0003-1742-2122, Ademuyiwa, V, Stubley, S et al. (5 more authors) (2018) Pickering emulsions co-stabilized by composite protein/ polysaccharide particle-particle interfaces: Impact on in vitro gastric stability. Food Hydrocolloids, 84. C. pp. 282-291. ISSN 0268-005X

https://doi.org/10.1016/j.foodhyd.2018.06.019

Pickering emulsions co-stabilized by composite protein/ polysaccharide particle-particle interfaces: Impact on in vitro gastric stability

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Abstract

The objective of this study was to delay the rate and extent of gastric destabilization of emulsions using composite particle-particle layers at the O/W interface. Pickering emulsions (20 wt% oil) were prepared using lactoferrin nanogel particles (LFN, D_h=100 nm) (1 wt%) or a composite layer of LFN and inulin nanoparticles, latter was enzymatically synthetized by inulosucrase IslA from Leuconostoc citreum (INP) (D_h=116±1 nm) (1 wt% LFN 3 wt% INP). The hypothesis was that creating a secondary layer of biopolymeric particles might act as a barrier to pepsin to access the underlying proteinaceous particles. Droplet size, microscopy (optical and transmission electron microscopy (TEM)), ζ-potential and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were used to understand the colloidal fate of these Pickering emulsions in an in vitro gastric model (pH 3, 37 °C, pepsin). The ζ-potential measurements and TEM images confirmed that LFN and INP were at the O/W interface, owing to the electrostatic attraction between oppositely charged LFN (+29.3±0.7 mV) and INP (-10±1.8 mV) at both neutral and gastric pH. The SDS-PAGE results revealed that adsorbed LFN was less prone to pepsinolysis as compared to a typical protein monolayer at the interface. Presence of INP further decreased the rate and degree of hydrolysis of the LFN (>65% intact protein remaining after 60 min of digestion) by acting as a steric barrier to the diffusion of pepsin and inhibited droplet coalescence. Thus, composite particle-particle layers (LFN + INP) at droplet surface shows potential for rational designing of gastric-stable food and pharmaceutical applications.

Keywords

Lactoferrin nanogel particles, Pickering emulsion; particle-particle interface; inulin nanoparticles; pepsin digestion; layer-by-layer

1 Introduction

Recently, there has been growing research interests among food colloid scientists in designing Pickering emulsions i.e. emulsions stabilized by solid colloidal particles due to their ultrastability against coalescence (Dickinson, 2012, 2017). Pickering emulsions stabilized by inorganic or synthetic particles, such as silica, latex particles etc. are most common in literature (Binks & Lumsdon, 1999, 2001). However, these particles do often require chemical modifications to improve their partial wettability by the oil phase, which restrict their utilisation in food applications.

It is only recently that novel biocompatible particles have started to gain attention owing to their immediate suitability for use in food, pharmacetical and allied soft matter applications (Dickinson, 2012; Dickinson, 2015). Such particles range from laboratory synthsized protein microgel particles (Destribats, Rouvet, Gehin-Delval, Schmitt, & Binks, 2014; Liu & Tang, 2013; Matsumiya & Murray, 2016; Sarkar, et al., 2016b) to polysaccharide-based particles (Kalashnikova, Bizot, Bertoncini, Cathala, & Capron, 2013; Richter, Feitosa, Paula, Goycoolea, & de Paula, 2018; Tzoumaki, Moschakis, Kiosseoglou, & Biliaderis, 2011; Yusoff & Murray, 2011). Besides their exceptional physical stability, protein microgel particles (Sarkar, et al., 2016b) and chitin nanocrystals (Tzoumaki, Moschakis, Scholten, & Biliaderis, 2013) have also shown abilities to

reduce the rate of digestion of emulsified lipids in an in vitro duodenal model set up. As high desorption energies (order of several kBTs) are required to dislogde these particles from the oil-water interface, their competitive displacements by biosurfactant (bile salts) was prevented (Sarkar, Horne, & Singh, 2010; Sarkar, Ye, & Singh, 2016d). Thus, the presence of particles at interface slowed down the access of lipase to the emulsified lipid substrate. Such interesting property of altering lipid digestion offers promise for application of Pickering emulsions in satiety-enhancing foods, functional foods requiring sustained release of bioactive molecules (Araiza-Calahorra, Akhtar, & Sarkar, 2018).

However, it is worth recognizing that before the duodenal phase, harsh biochemical conditions occurring in the gastric tract might destabilize these emulsions and hinder such potential applications. Responsiveness of protein-based Pickering stabilizers to pepsin and their subsequent hydrolysis into peptide fragments is an important research challenge to tackle before such particles can be exploited for food applications (Sarkar, et al., 2016b; Shimoni, Shani Levi, Levi Tal, & Lesmes, 2013).

Hence, it might be useful to create a relatively complex interface to protect the emulsions against gastric destabilization or at least slow down the rate of hydrolysis of the interfacial material by pepsin. In this regard, recently, cellulose nanocrystals have shown success on enhancing the stability of whey protein-stabilized oil-in-water (O/W) emulsions against enzymatic attacks (Sarkar, Li, Cray, & Boxall, 2018; Sarkar, Zhang, Murray, Russell, & Boxal, 2017). Binding of CNC to the protein film at the interface offered resistance to the protein film against pepsinolysis and inhibited

droplet coalescence in the gastric phase that occurs typically in case of emulsions stabilized by protein film alone (Sarkar, Goh, Singh, & Singh, 2009b; Sarkar, et al., 2016a; Sarkar & Singh, 2016c; Sarkar, et al., 2017; Singh & Sarkar, 2011). However, the safe human consumption of CNC can be debated due to its chemical processing technique, e.g. sulfuric acid treatment.

In this regard, inulin, a β-(2 $\rightarrow$ 1)-linked polysaccharide of D-fructose (Tadros, Vandamme, Levecke, Booten, & Stevens, 2004) can be an alternative candidate to create a steric barrier to a protein-based interfacial material against pepsin hydrolysis. Inulin is a polysaccharide comprised of fructose sugar units that grow linearly and are branched. Its physicochemical and functional properties depend on its degree of polymerization and percentage of branching. Inulin has been used by the food industry as a soluble dietary fibre and fat/sugar replacement, and in the pharmaceutical industry as a stabilizer and excipient. Hydrophobically modified inulin has shown ability to create stable emulsions under gastric conditions (Meshulam, Slavuter, & Lesmes, 2014b).

Inulin is not hydrolysed by human gastrointestinal enzymes and is delivered undigested in colon and behaves as a prebiotic (Glibowski, Kordowska-Wiater, & Glibowska, 2011; Rastall, 2010; Tuohy, 2007). Hence, use of inulin might not only help to provide a steric stabilization to protein particle-laden interface but can also act as a prebiotic in the colon. Since inulin is biocompatible, non-toxic and can form hydrogels, it has been used as a slow-release drug delivery system. Wolff, et al. (2000) documented the enzymatic formation of high molecular weight inulin

globular particles of nanometric size, using a recombinant inulosucrase from Streptococcus mutans and Aspergillus sydowi conidia. In the present study, we have used self-assembled high molecular weight inulin nanoparticles synthesized by inulosucrase from Leuconostoc citreum CW28.

Positively-charged protein-based nanoparticles derived from lactoferrin and their subsequent use as nano-scale Pickering stabilizers have been previously published (Meshulam & Lesmes, 2014a; Shimoni, et al., 2013). Authors have referred to these as 'lactoferrin nanoparticles' as they were prepared by the controlled heating and pH adjustment of dilute lactoferrin solutions. However, to our knowledge, there is no experimental evidence of the fabrication of colloidal 'nanogel particles' from lactoferrin using a top down approach (heat-set hydrogel preparation route followed by controlled shearing without any pH adjustment) and using them to create Pickering emulsion. Such nanogel particles are formed by a complex interplay of thermal denaturation, electrostatic repulsion, aggregation and formation of covalent disulfide bonds (Sarkar, et al., 2016b; Schmitt, et al., 2010). Hence, these lactoferrin nanogel particles might be hypothesized to be less susceptible to pepsin in the gastric phase as compared to the lactoferrin nanoparticles reported in literature, by virtue of the hierarchical structure of the former.

Formation of multilayered emulsions using proteins and polysaccharides is a well-established approach (Goh, Sarkar, & Singh, 2014; Guzey & McClements, 2006). For instance, thermal and gastrointestinal stability of lactoferrin-stabilized lipid droplets have been shown to be improved by adsorption of pectins or alginate, respectively (Tokle, Lesmes, Decker, &

McClements, 2012; Tokle, Lesmes, & McClements, 2010). However, to date, use of particle-particle interface as a physical tool to delay the rate of gastric destabilization in simulated gastric condition has not been elucidated.

Hence, in this study, we have used a two-fold approach. On the one hand, we created lactoferrin 'nanogel' particle-stabilized Pickering emulsions. On the other hand, we generated a novel composite particle-particle layer at the oil-water interface by coating the droplets with oppositely charged inulin nanoparticles aiming to delay the rate of gastric destabilization of emulsions. The hypothesis was that the presence of hydrophilic inulin nanoparticles at the protein nanogel particle-stabilized oil-water interface could enhance the kinetic stability of the corresponding emulsions in gastric regime by acting as a steric barrier to the pepsin from attacking the proteinaceous particles at the interface.

2 Materials and Methods

2.1 Materials

Bovine lactoferrin (LF) powder (Prodiet® lactoferrin), purchased from Ingredia Nutritionals (Arras, France) contained >95.0% lactoferrin protein as per supplier's specification. Inulin particles (INP) were from Leuconostoc citreum prepared at Departamento de Ingenieria Celular y Biocatálisis, Instituto de Biotecnología – UNAM (Cuernavaca, Mexico). Sunflower oil was purchased from a local supermarket (Tesco, UK). Pepsin enzyme (P7000-25G, activity: 536 U mg^{-1}) was purchased from Sigma-Aldrich Company Ltd, Dorset, UK. All other chemicals used were of analytical grade unless otherwise specified. Mini-Protean Precast TGX Gels (8–16%) and Precision Plus Protein All Blue

Standards were purchased from Bio-Rad Laboratories, Inc, USA. Milli-Q water with an ionic purity of 18.2 MΩ·cm at 25 °C (water purified by treatment with a Milli-Q apparatus) was used as a solvent for all the experiments.

2.2 Preparation of inulin nanoparticles

Inulin nanoparticles (INP) were synthetized enzymatically using Leuconostoc citreum whole cells with inulosucrase IslA enzyme as a catalyst (Ortiz-Soto, Olivares-Illana, & López-Munguía, 2004). The INP enzymatic synthesis was carried out in a Braun fermenter containing 50 mM phosphate buffer at pH 6.5 and 250 g L^{-1} sucrose at 30 °C and 250 rpm during 40 h with pH regulation by addition of 4 N NaOH. The cells were recovered by centrifugation at 14,000 rpm (Sharples AS-16) maintaining the polymer in the supernatant. The polymer was precipitated by addition of ethanol (1:3 v/v) and dried in a Labnet dryer (National Labnet Co., Woodbridge, NJ). The high molecular weight inulin nanoparticles was analyzed by gel permeation chromatography in a Waters 600E HPLC system controller (Waters Corp. Milford, MA) employing a refractive index detector (Waters 410), and a serial set of Ultrahydrogel (UG 500 and linear) columns at 358C with 0.1 M $NaNO_3$ as the mobile phase at 0.9 mL min-1 (Jiménez-Sánchez, et al., 2018).

2.2 Preparation of lactoferrin nanogel particles (LFN)

Lactoferrin nanogel particles (LFN) were prepared using heat-induced disulfide crosslinking of concentrated protein dispersion using a process previously described by Sarkar, et al. (2016a) with slight modification. Appropriate quantities of LF (12 wt%) were dispersed in Milli-Q water for 2 h

198 to ensure complete dissolution at pH 7. The LF solution was heated at 90 ℃
199 for 30 minutes and cooled at room temperature for 30 minutes followed by
200 storage at 4 ℃ overnight to form LF heat-set hydrogel. The hydrogels were
201 mixed with MilliQ water (3 wt% LF) at pH 7.0 and were pre-homogenized
202 using a blender (KM336, Kenwood, UK) for 15 minutes and degassed in a
203 vacuum box (John Fraser and Sons Ltd, London, UK). Following this, the gels
204 were homogenized using two passes through a two-stage valve homogenizer
205 (Panda Plus 2000, GEA Niro Soavi Homogeneizador Parma, Italy) operating
206 at first/second stage pressures of 350/50 bar, respectively to create LFN. The
207 LFN aqueous dispersion was centrifuged at 3000 rpm for 20 min and filtered
208 using 0.45 µm filters (Millipore Corp., Bedford, MA, USA) to remove any large
209 aggregates. The resulting 3 wt% LFN was diluted to 1.25 wt% before
210 emulsion preparation. Sodium azide (0.02 wt%) was added to the LFN to
211 prevent microbial growth.
212
213 2.3 Preparation of LFN-stabilized and LFN + INP-stabilized Pickering
214 emulsions
215 Pickering emulsions were prepared by mixing 20.0 wt% oil phase and 1 wt%
216 LFN particles in the final emulsion. The mixture of 20 g sunflower oil and 80 g
217 of LFN solution (1.25 wt% LFN) was sheared using a conventional rotor-stator
218 type mixer (L5M-A, Silverson machines, UK) operating at 10,000 rpm for 2
219 minutes to prepare the pre-emulsions. The pre-emulsions were then
220 homogenized using two passes through the Panda Plus 2000 homogenizer
221 operating as above to create LFN-stabilized emulsions (Figure 1a).

For the preparation of particle-particle-stabilized emulsions, primary emulsions were prepared using 40 wt% sunflower oil and 60 wt% aqueous phase (3.45 wt% LFN in aqueous phase). Appropriate quantities of hydrophilic unmodified INP (6 wt%) were dispersed in Milli-Q water for 2 h to ensure complete dissolution at pH 7. Primary emulsion (40 wt% oil, 2 wt% LFN) was combined with INP dispersion (6 wt%) in the 1:1 w/w ratio and stirred using a magnetic stirrer for 2 hours. The resulting secondary particle-stabilized emulsions (LFN + INP-stabilized emulsions, Figure 1b) contained 20 wt% oil, 1 wt% LFN and 3 wt% INP. The choice of 3 wt% INP for the preparation of secondary emulsions was based on complete coverage of the LFN-stabilized emulsions droplets by INP. Both the LFN and LFN + INP-stabilized emulsion samples were prepared in triplicates. Sodium azide (0.02 wt%) was added to the emulsions to prevent microbial growth during refrigerated storage at 4 ℃.

2.4 Particle sizing of LFN and INP

The mean hydrodynamic diameter (D_h) of LFN or INP was measured in a disposable cuvette (ZEN0040) using a dynamic light scattering with non-invasive back scattering (DLS-NIBS) instrument, Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) at 25 ± 0.5 ℃ with a 633 nm laser. The LFN or INP dispersions were measured using refractive index of 1.53 and the absorbance was taken as 0.001. The Stokes-Einstein equation was used to calculate the D_h using the Stokes Einstein equation (1).

$$D_h = (K_B T)/(6\pi\eta D) \quad (1)$$

where, D_h is the particle hydrodynamic diameter, K_B is the Boltzmann's constant, T is the absolute temperature, D is the translational diffusion coefficient, η is the viscosity of the aqueous phase (Pa-s). The particle size distribution by number was also determined for the INP using nano-tracking analysis (NTA) using a NanoSightTM LM10 system equipped with a LM14 green (535 nm) laser module and a cooled Andor camera (Andor-DL-658-OEM). The particles were diluted 1:100000 in water before analysis.

2.5 Droplet sizing of the Pickering emulsions

A Malvern MasterSizer 3000 (Malvern Instruments Ltd, Malvern, Worcestershire, UK) was used to measure the droplet size distribution of both the emulsions before and after in vitro gastric digestion. The relative refractive index, i.e., the ratio of sunflower oil (1.456) to that of dispersion medium (1.33) was 1.095. Droplet size measurements were reported as Sauter-average diameter (d_{32}) and volume-average diameter (d_{43}) from the particle size distributions, using equations 2 and 3, respectively:

$$d_{32} = \frac{n_i d_i^3}{n_i d_i^2} \qquad (2)$$

$$d_{43} = \frac{n_i d_i^4}{n_i d_i^3} \qquad (3)$$

where, n_i is the number of particles with diameter d_i. Mean and standard deviations were calculated on five measurements on triplicate samples.

2.6 ζ-potential

The ζ-potential values of the particle dispersions (LFN and INP) and the corresponding emulsions before and after gastric digestion (0, 120 minutes) were measured using Malvern Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, Worcestershire, UK). Emulsions were diluted to 0.005 wt% droplet concentration and the samples were transferred into DTS1070 folded capillary cells and after 120 s of equilibration, the collected electrophoretic mobility data was converted to ζ-potential using classical Smoluchowski equation. Each individual ζ-potential data point was reported as an average and standard deviation of at least five reported readings made on triplicate samples.

2.7 Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) was used to observe the structure of the INP and the original emulsions stabilized by LFN and LFN + INP. Samples (10 µL) were fixed with 2.5% (v/v) glutaraldehyde and post fixed in 0.1% (w/v) osmium tetroxide. Then, the samples were subjected to serial dehydration in ethanol (20-100%) before being embedded in araldite. Ultra-thin sections (silver-gold 80-100 nm) were deposited on 3.05 mm grids and stained with 8% (v/v) uranyl acetate and lead citrate. The sections were cut on an “Ultra-cut” microtome. Images were recorded using a CM10 TEM microscope (Philips, Surrey, UK).

297 2.8 Optical microscopy

298 The microstructural characteristics of the emulsions before and after in vitro
299 gastric digestion were imaged using a Leica optical light microscope,
300 equipped with a Canon Power Shot and TASV43 program. A small quantity of
301 emulsion before and immediately after gastric digestion (0, 120 min) was
302 placed on a concave microscope slide, covered with a cover slip and imaged
303 using a 40× magnification objective lens.

304

305 2.9 In vitro gastric digestion

306 Emulsions were digested by mixing them with simulated gastric fluid (SGF)
307 with pepsin using the harmonized digestion protocol at 37 ℃ (Minekus, et al.,
308 2014). Briefly, 20 mL of the emulsions (20 wt% oil) were incubated for 2 hours
309 in 20 mL of SGF, latter contained 0.514 g L^{-1} KCl, 0.123 g L^{-1} KH_2PO_4, 0.042
310 g L^{-1} $NaHCO_3$, 0.06 g L^{-1} NaCl, 0.0004 g L^{-1} $MgCl_2(H_2O)_6$, 0.0009 g L^{-1}
311 $(NH_4)_2CO_3$ and 2000 U/ mL pepsin. The pH value of SGF was adjusted to pH
312 3 using 0.1 M HCl to simulate after meal ingestion conditions. To observe the
313 change of emulsions during digestion, sample aliquots were collected during
314 gastric digestion at 0, 5, 10, 30, 60, 90, 120 and 150 min. These gastric
315 digesta samples were neutralized to pH 7 using freshly prepared 1 M
316 NH_4HCO_3 to inactivate pepsin and samples were stored at −20 °C until further
317 analysis.

318

319 2.10 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

320 To determine the influence of INP on digestion of the adsorbed LFN at the
321 O/W interface, the cream phase of both the LFN and LFN + INP-stabilized

emulsions sampled at various time intervals during in vitro gastric digestion was analysed using SDS-PAGE. Samples (1 mL) were heated at 95 ℃ for 5-10 min to stop digestion. The aliquots were centrifuged for 40 min at 14500g and 20 ℃ using a table-top micro-centrifuge (Eppendorf MiniSpin plus, Scientific Laboratory Supplies. Ltd. UK). A certain amount of cream layer was carefully collected, mixed with 50 µL SDS buffer (1 M Tris, pH 6.8) and 10 μL of Dithiothreitol (DTT) (500 mM) and again heated at 95 ˚C for 5-10 min. The SDS-PAGE was carried out by loading 5 µL of protein marker and 20 µL of digested adsorbed phase samples + loading buffer mixtures into precast gels, and then placed in Mini-PROTEAN II system (Bio-Rad Laboratories, Inc, USA). The running process had two stages: 100 V for 10 min followed by 200 V for 40 min. The gels were then stained for an hour with ProtoBlue Safe Colloidal Coomassie G-250 stain in ethanol (90:10 v/v). The gels were destained overnight using MilliQ water and then scanned using a ChemiDoc™ XRS+ system with image LabTM Software (Bio- Rad Laboratories, Inc, USA). Each band within the lanes was selected automatically by the software to cover the whole band. Background intensity was subtracted after scanning an empty lane. The SDS PAGE experiments were carried out in triplicates and band intensities was reported as an average and standard deviation of three reported readings.

2.11 Statistical analysis

All experiments were carried out in triplicates, with each repetition being measured three times. Results are presented as the mean and standard deviation of these nine measurements unless mentioned otherwise. The

results were statistically analyzed by analysis of variance (ANOVA) using Graphpad 5 Prism software and differences were considered significant when $p<0.05$ were obtained.

3 Results and discussion

3.1 Characteristics of LFN and INP

The properties of LFN and INP were evaluated before analysing the Pickering emulsions stabilised by these particles. This serves to understand the behaviour of the particles in bulk phase first, which sets the scene to gain insights on the behaviour of the particles at the oil-water (O/W) interface. The size reduction of the LF hydrogel owing to the homogenization step led to the formation of "nanogel particles" characterized by a D_h of 100 ± 0.8 nm (Table 1). During heating, the globular LF molecules were denatured causing unfolding of the polypeptide chains subsequently exposing the hydrophobic amino acid residues (Torres, Murray, & Sarkar, 2017). Individual protein molecules began to aggregate through hydrophobic interactions followed by the formation of inter-molecular covalent bonds of disulphide origin. These covalent bonds were responsible for the structural integrity of the derived nanogel particles (Sarkar, et al., 2016a; Schmitt, et al., 2010).

Interestingly, the size of LFN were three-fold smaller than that of typical "microgel particles" prepared using a top down approach, which is most likely due to the harsher shearing conditions used in the former as compared to that of the latter (Sarkar, et al., 2016a). In the case of enzymatically synthetized INPs, the particle size distribution curves by intensity and number were derived from DLS-NIBS and NTA, respectively. Of notice, both techniques

372 revealed a monomodal and narrow size particle distribution (Supplementary
373 Data Figure S1), thus confirming the absence of more than one population of
374 particles. The average diameter calculated by both techniques was similar
375 ($p>0.05$) (Table 1). The DLS-NIBS provides the particle distribution by
376 intensity, and it is known to be sensitive to the presence of large particles and
377 to polydispersity. Hence, it tends to overestimate the width of the particle size
378 distribution. By contrast, NTA, shows greater accuracy for both monodisperse
379 and polydisperse samples as well as higher peak resolution than DLS (<0.5
380 fold and >3 fold difference in diameter, respectively) (Filipe, Hawe, & Jiskoot,
381 2010). In agreement with this, close inspection of the particle size distribution
382 curves shown in Figure 1S do confirm that the width of the distribution of INP
383 sample was broader than that registered by NTA. Also, a slight shoulder was
384 observed on the greater side of the size distribution measured by NTA, which
385 is not discernible in the DLS Gaussian monomodial peak. The use of both
386 techniques to characterize the INP particle size distribution, thus offers, for the
387 first time, a complementary robust characterization of this sample. Moreover,
388 the size of the INP were of the same order as LFN (Table 1). The TEM image
389 of INP (Figure 1) also suggests that the nanoparticles were spherical
390 particles. Both the particles (LFN and INP) had a relatively low polydispersity
391 index (PDI < 0.15) as determined by DLS-NIBS (Table 1).

392 As expected, LFN particles were highly positively charged, which is in
393 line with the previous report of high isoelectric point (pI≈8.5) of LF (Adal, et al.,
394 2017; Sarkar, Goh, & Singh, 2009a). The ζ-potential of the LFN at pH 7.0
395 (Table 1) was in good agreement with heated LF (Peinado, Lesmes, Andrés,
396 & McClements, 2010) but higher in magnitude than a native LF dispersion of

397 the same concentration (ζ = +14.2 mV, data not shown). The recorded
398 increase in ζ-potential for nanogel dispersions is expected as particulate
399 material is likely to have a more compact structure and consequently a higher
400 charge density than a native protein molecule. The charge on INP was
401 negative (ζ = −10 mV) (Table 1), which would allow the deposition of these
402 nanoparticles at the LNP-stabilized oil-in-water interface via electrostatic
403 attractive forces, as hypothesized. The slightly negative charge of the INP
404 could be attributed to the presence of a low quantity of residual free enzyme
405 on the INP's surface, a common phenomenon in these kind of enzymes; this
406 free enzyme could be released by proteolysis (Ortiz-Soto, et al., 2004).
407
408 3.2 Properties and microstructure of Pickering emulsions stabilized by LFN
409 or LFN + INP
410 The visual images of both the emulsions did not reveal any oiling off in the
411 particle-stabilized emulsions without or with INP (Figure 1). The LFN-
412 stabilized emulsions showed a multimodal size distribution with majority of
413 droplets (~75%) within the size range of 1-100 µm (Figure 2a) and a d_{43} of ~
414 25 µm. The morphology of the adsorbed particles at the droplet surface was
415 examined using negative staining and TEM observations of the emulsions.
416 The TEM images (Figure 2a) clearly reveals the droplets with adsorbed
417 spherical LFN at the interface. The arrangement of clearly distinguishable
418 LFN at the interface did not show a complete monolayer or multilayer
419 coverage of particles. The emulsions rather showed a sub-monolayer of
420 particles assembled at the interface, as often reported for Pickering emulsions
421 (Destribats, et al., 2014; Sarkar, et al., 2016a). The size ratio of the emulsion

droplet-to-LFN was 250:1, which was within the typical size ratio limits for Pickering emulsions (Sarkar, et al., 2016a).

A small fraction of droplets were also observed in the size range of 0.1-1 µm, which might be attributed to the free nanogel particles that were not adsorbed to the droplet interface, as also indicated in the TEM micrographs. Another peak area with droplet size between 100-1000 µm was observed (Figure 2a), which most likely represents the bridged LFN-stabilized droplets as observed in the TEM images. Such bridged droplets have previously been reported when emulsions are made with low volume fraction of particles as in our case with 1 wt% LFN (French, Taylor, Fowler, & Clegg, 2015).

In case of LFN + INP co-stabilized droplets, the emulsions showed a bimodal distribution with a large peak centred in the size range of 1-20 µm and a small peak in the size range of 0.1-1 µm, the area of latter was slightly larger than that observed in LFN-stabilized droplets. The small peak may be associated with the free (unbound) fraction of either LFN, INP or LFN-INP electrostatic complex. The main peak comprising the larger proportion of droplets can be attributed to the droplets co-stabilized by a composite LFN + INP layer. No peak in the 100-1000 µm size range was observed in contrast to the bridged LFN-stabilized droplets as discussed before (Figure 2a). This suggests that the presence of higher concentration of INP might have created a particle-particle interface. This was supported by TEM images (both lower and higher magnification images, Figure 2b) with a significant degree of droplet coverage by discernible particles achieving almost a saturation (Figure 2b). This might be attributed to the electrostatic complexation between anionic

446 INP and cationic LFN at pH 7 at the O/W interface (Table 1), which is further
447 discussed in the section dealing with the surface charge results.
448
449 3.3 Changes in microstructure during in vitro gastric digestion
450 The droplet size distribution of LFN and LFN + INP co-stabilised emulsions
451 before and after in vitro gastric digestion with corresponding changes in their
452 optical microstructures are presented in Figures 3 and 4. As can be observed
453 from Figure 3, the droplet size distribution remained the same when the pH
454 was shifted from pH 7 to gastric pH (pH 3) ($p>0.05$). After treatment with SGF
455 without pepsin, the peak at 100-1000 µm size range increased markedly
456 ($p<0.05$) owing to the gastric salt-induced charge screening and ion binding
457 effects, resulting in large aggregates, as can be observed in the optical
458 micrographs.
459 It is only after treatment with SGF containing pepsin (120 min), that this
460 is peak diminished with a subsequent appearance of a new one in the 1000-
461 10,000 µm size range ($p<0.05$) (Figure 3). Droplet aggregation was more
462 prominent in the optical micrographs in presence of pepsin, with appearance
463 of very few coalesced droplets, congruent with the d_{43} value of 196 µm. It is
464 worth recognizing that although LFN adsorbed at the interface appeared to be
465 digested by pepsin, the LFN peptide fragments still offered some degree of
466 protection to the droplets against coalescence as compared to that of a typical
467 protein monolayer-stabilized interface (Sarkar, Goh, & Singh, 2010; Sarkar, et
468 al., 2009b; Sarkar, et al., 2017; Singh, et al., 2011). This suggests that either
469 the aromatic groups were somehow buried inside the particles making them
470 less accessible by the pepsin or the particle fragments generated were still

viscoelastic enough to offer some resistance to complete droplet destabilization.

In case of LFN + INP co-stabilized emulsion (Figure 4), the peak from 1–100 μm remained relatively constant ($p>0.05$) when pH was shifted as well as when SGF was added without containing pepsin. This suggests that the steric-stabilized droplets were rather stable to gastric stage-induced change in pH and ions in contrast to the LFN-stabilized droplets (Figure 3). On addition of SGF containing pepsin, a small peak appeared in the 1000–10,000 μm size range at 120 min ($p<0.05$) suggesting proteolysis of LFN did occur even in the presence of INP (Figure 4). In agreement with laser diffraction results, a gradual appearance of well-connected networks of agglomerates was observed in the optical micrograph of the LFN + INP co-stabilized emulsion, without presence of any discernible coalesced droplets (Figure 4). Comparing the size and microstructural results of LFN- and LFN + INP-stabilized droplets after gastric digestion in presence of pepsin (Figures 3 and 4), it can be suggested that INP provided protection to the structural integrity of the LFN-stabilized emulsion droplets inhibiting droplet coalescence.

3.4 Changes in ζ-potential during in vitro gastric digestion

To provide indirect quantitative insights into the droplet behaviour, ζ-potential values are reported at pH 7 (freshly prepared emulsions), pH 3 (pH of SGF) and in presence of SGF without/with added pepsin (Figure 5). Freshly prepared LFN emulsions were positively charged (~+45 mV), which is expected as the LFN at the interface was below its isoelectric point (pI) (Adal, et al., 2017; Sarkar, et al., 2009a). The ζ-potential values of LFN emulsion

droplets were slightly higher in magnitude as compared to that of the nanogel particles themselves (−29.3 mV) at pH 7.0 (Table 1). This is expected due to the presence of higher local concentration of LFN at the droplet surface as compared to that when present in the bulk phase.

With the addition of anionic INP (3 wt%), the ζ-potential of the LFN-coated emulsion droplets decreased from +42 to −3.63 mV ($p<0.05$). This confirms the electrostatic binding of INP to the complementarily charged LFN adsorbed at the O/W interface almost achieving a complete coverage and steric stabilization as evidenced by near zero-charge (Figure 5). Electrostatic complexation of LF particles with aqueous polysaccharides, such as carrageenan and alginate, has been reported previously (David-Birman, Mackie, & Lesmes, 2013; Peinado, et al., 2010), but to our knowledge, this is the first study that highlights particle-particle electrostatic complex formation at the interface.

At gastric pH (Figure 5), there was no appreciable change in the magnitude of ζ-potential in both the primary and secondary Pickering emulsions ($p>0.05$). Presence of SGF without pepsin showed a significant reduction of ζ-potential values ($p<0.05$) in the primary LFN-stabilized emulsion confirming some degree of charge screening effects as indicated in the laser diffraction and optical microscopy results (Figure 3). However, such ion-induced aggregation was not evident in the LFN + INP-stabilized interfaces (Figure 5), which is highly consistent with the d_{43} values reported in Figure 4.

Interestingly, when pepsin was added, the proteolysis of the intact LFN at the interface resulted in substantial loss of surface charge (ζ = +29 mV) within 30 min with subsequent decrease in magnitude by 30% after 120 min

521 ($p<0.05$) (Figure 5). It is worth noting that although there was reduction in ζ-
522 potential, LFN-stabilized droplets still had sufficiently high magnitude of
523 positive charge as compared to a typical protein-coated droplets under the
524 same conditions (Sarkar, et al., 2009b). Alterations in surface charges due to
525 gastric pepsinolysis was not significant when LFN-stabilized droplets were
526 coated by INP at 30 or even after 120 min of digestion time ($p>0.05$) (Figure
527 5). This suggests that a relatively rigid layer of negatively charged INP formed
528 by intermolecular hydrogen bonding between INP-INP (Kim, Faqih, & Wang,
529 2001) remained intact as it was not attacked by human physiological enzymes
530 restricted or delayed the access of pepsin to the inner-adsorbed protein
531 nanogel particulate layer. Furthermore, the electrostatic complexation
532 between INP and LFN created a rather complex interface for diffusion of
533 pepsin to the substrate binding sites of LFN.
534
535 3.5 Response of the particle at interface to pepsin
536 To gain direct quantitative insight into the gastric stability of these Pickering
537 emulsions, the patterns of proteolysis of the interfacial layer of the emulsions
538 were obtained via SDS-PAGE analyses of adsorbed phase of the chyme
539 collected at designated time intervals during gastric digestion (Figure 6).
540 Interestingly, LFN showed a marked degree of proteolysis of the LF band (85
541 kDa) i.e. 65% of the intact LF band remaining within first 5 min (Figures 6a
542 and 6c), which became subsequently faint and 20% of intact protein remained
543 after first 30 min of digestion. The intact LF band in the LFN emulsions
544 disappeared only after around 90 minutes (Figures 6a). This suggests that
545 pepsin hydrolysed the interfacial layer of nanogel particles, giving rise to

546 droplet aggregation (Figure 3) as a consequence of loss of surface charge
547 (Figure 5).

548 Of note, the LFN at the interface was gradually hydrolysed into smaller
549 peptides (<15 kDa), which might not have been captured by the resolving
550 SDS-PAGE gel. However, appearance of smearing of bands in the lanes from
551 5-120 min (Figures 6a), possibly represent the peptides of higher molecular
552 weight (> 15 kDa). It is highly likely that these high molecular weight LFN
553 nanogel particle fragments generated by pepsin hydrolysis were anchored to
554 the droplet surface, thus conferring them some degree of protection against
555 accretion (Figure 3).

556 Also, it is noteworthy that the digestion kinetics of LFN was rather slow
557 when compared to a native LF-stabilized emulsion. In case of adsorbed phase
558 from LF-stabilized emulsion (Supplementary Data Figure S2), no intact LF
559 bands were discernible within first five min of digestion, consistent with
560 previous reports on native LF/ heat-treated LF nanoparticles (David-Birman,
561 et al., 2013) or adsorbed whey protein (Sarkar, et al., 2009b; Sarkar, et al.,
562 2016a; Sarkar, et al., 2017; Singh, et al., 2011). This suggests that formation
563 of these compact nanogel particles offered some degree of transient barrier to
564 the easy diffusion of pepsin by virtue of their hierarchal structure within the
565 nanogel, as opposed to that of a system with LF monolayer or LF
566 nanoparticles (David-Birman, et al., 2013; Tokle, et al., 2012).

567 Presence of INP showed a clear delaying effect on digestion of LFN at
568 the O/W interface (Figures 6b and 6c) with > 65% and ~ 25% of the intact LF
569 bands remaining after 60 and 120 min of gastric digestion, respectively.
570 Presence of 75% intact LFN particles (Figure 6b) in the adsorbed phase

supports the absence of coalescence in LFN + INP-stabilized droplets (Figure 4) and no change in ζ-potential values (Figure 5). This suggests that the delaying was driven by a barrier-dominant mechanism i.e. structure and thickness of the adsorbed LFN+INP layers. Such delaying of digestion of the intact protein bands have been previously reported in presence of polysaccharides, such as, carrageenan or alginate (David-Birman, et al., 2013) or other non-proteinaceous particles, such as, cellulose nanocrystals (Sarkar, et al., 2017). Interestingly, hydrophobic inulin has been also reported to provide improved gastric stability to emulsions when it is present at the interface, former being non-digestible by physiological enzymes (Meshulam, et al., 2014b). This suggests that electrostatic binding of INP to LFN at the interface had a prominent effect in providing a kinetic barrier to the diffusion of the pepsin to the LFN and subsequently diminishing the rate and final extent of interfacial proteolysis.

Despite the steric barrier effect, pepsin had access to the LFN-laden interface owing to the porosity of the INP layer (Sarkar, et al., 2016a), which supports that presence of INP did not completely limit but rather delayed digestion. Besides the formation of a composite particle-particle layer, electrostatic repulsion between pepsin and INP layer might have also been at play in delaying gastric digestion. As the net charge of both pepsin (Davies, 1990) and the LNP + INP co-stabilized droplets were negative at pH 3 (Figure 5), the mutual electrostatic repulsion might have also contributed to not allow pepsin in the close vicinity of the underlying positively charged binding points of the protein nanogel particulate layer.

595 It is also worth noting that there was unadsorbed LFN and LFN+INP in
596 the continuous phase (Figures 2a and 2b), respectively, which might have
597 been more readily accessible to pepsin, thus reducing the pepsin's overall
598 activity for the LFN present at the interface. Further research is needed to
599 uncover the interactions of pepsin with these unadsorbed particles and
600 particle-particle complexes.
601

602 ## Conclusions

603 In this study, we have investigated the influence of composite particle-
604 particle interfaces on the gastric stability of emulsions using complimentary
605 physicochemical and microstructural analysis. Primary Pickering emulsions
606 (20 wt% oil) co-stabilized by LFN particles (1 wt%) as well secondary
607 emulsions (1 w% LFN, 3 wt% INP) demonstrated good stability against
608 droplet coalescence at pH 7. Findings from this study report, for the first time,
609 that the rate of pepsinolysis of LFN particles at interface is significantly less as
610 compared to the protein monolayer counterpart. The presence of the
611 secondary interfacial layer of polysaccharide particles (INP) could provide a
612 protective coating to this protein nanogel particle-stabilized emulsion and
613 further delay gastric digestion. Presence of INP decreased the extent of in
614 vitro gastric digestion of the proteinaceous particles (LFN) by pepsin, which
615 was confirmed by SDS-PAGE of the adsorbed phase. This was mainly
616 attributed to the formation of strong particle-particle composite layers at pH 3
617 and to INP exhibiting effective steric barrier that slows down the access of
618 pepsin to the LFN. The gastric digestion was not completely inhibited owing to
619 the diffusion of the pepsin through the gaps in between the INP particles.

Thus, the present study has demonstrated an interesting link between the interfacial architecture at varying length scales using composite particle-particle layers and enhanced gastric stability, which could be useful in the rational design of physiologically relevant emulsions. Further studies are ongoing to understand the effect of polydispersity of these Pickering stabilizers and the porosity of these composite layers to tailor the kinetics of gastric stability of emulsions for optimized delivery of gastric-stable lipid droplets to the duodenum. Ongoing in vitro studies in our laboratories are consistent that INP themselves do not show prebiotic activity. Whether the developed Pickering systems would offer a route to deliver prebiotic formulations to the colon in vivo, is yet to be investigated.

Acknowledgements

The authors would like to gratefully acknowledge the contributions of Martin Fuller for his technical support in electron microscopy at the Electron Microscopy Facility, Faculty of Biological Sciences, University of Leeds, UK. The work of the Mexican co-authors was supported by National Autonomous University of Mexico (grant UNAM-PAPIIT IN213616). We acknowledge Prof. Dr. Martin Wiemann from IBE R&D gGmbH, Institute for Lung Health (Münster), for the generous access to the NTA instrument.

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