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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.

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, pharmaceutical and allied soft matter applications (Dickinson, 2012, 2015). Such particles range from laboratory synthesized protein microgel particles (Destribats, Rouvet, Gehin-Delval, Schmitt, & Binks, 2014; Liu & Tang, 2013; Matsumiya & Murray, 2016; Sarkar, Ye, & Singh, 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 dislodge these particles from the oil-water interface, their competitive displacements by biosurfactant (bile salts) was prevented (Sarkar, Horne, & Singh, 2010b; Sarkar et al., 2016b). 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).

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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, 2016; 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 → 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, 2014).

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, 2014; 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, & McClements, 2010; Tokle, Lesmes, Decker, & McClements, 2012). 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).

2.3. 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 to ensure complete dissolution at pH 7. The LF solution was heated at 90 °C for 30 min and cooled at room temperature for 30 min followed by storage at 4 °C overnight to form LF heat-set hydrogel. The hydrogels were mixed with MilliQ water (3 wt% LF) at pH