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PAPER

Influence of electrostatic heteroaggregation of lipid droplets on their stability and digestibility under simulated gastrointestinal conditions

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There is emerging interest in the impact of food structure on lipid digestion and its relationship to human nutrition. The objective of this study was to investigate the influence of heteroaggregation of lipid droplets on their potential biological fate using a simulated gastrointestinal tract (GIT). At neutral pH, a highly viscous "mixed emulsion" was formed by mixing anionic β-lactogobulin (β-Lg) coated lipid droplets with cationic lactoferrin (LF) coated lipid droplets due to electrostatic attraction. We compared the behavior of β-Lg-emulsions, LF-emulsions and mixed emulsions under *in vitro* oral, gastric, and small intestinal conditions. In the oral stage, the β-Lg emulsion and mixed emulsion were stable but the LF emulsion aggregated, which was attributed to electrostatic interactions with mucin. In the gastric stage, extensive droplet aggregation occurred in all three emulsions, which was attributed to proteolysis of adsorbed proteins by pepsin, as well as the influence of high acidity and ionic strength on electrostatic interactions. Despite the differences in the initial compositions and microstructures of the three emulsions, we did not observe an appreciable difference in the rate or extent of their lipid digestion in the small intestine. Qualitatively similar results were obtained using a simple GIT model (small intestine only) and the full GIT model (oral, gastric, and small intestine). The knowledge gained from this study will be useful for the creation of functional foods to improve health and well-being.

1. Introduction

There is considerable interest in the utilization of structural design principles to create colloidal foods with improved nutritional profiles, by controlling lipid digestibility within the gastrointestinal tract,[1,2] by encapsulating, protecting and delivering lipophilic bioactive agents through the diet,[3,4] and by reducing the caloric density of high calorie foods.[5] Structural design principles have been utilized to create novel functional attributes in foods by controlling the spatial organization of their ingredients to form structures such as particles, fibers, tubes, sheets, and coatings.[5–7] Recent studies have shown that novel properties can be created using food-grade ingredients *via* the controlled heteroaggregation of oppositely charged lipid droplets.[8,9] The microstructure and rheological properties of these systems can be manipulated by controlling the total lipid content, the ratio of positive-to-negative particles, the particle size, and environmental conditions (*e.g.*, pH, ionic strength, and temperature). This approach may therefore have potential application in the food industry to create products with novel textural characteristics or reduced fat contents (since highly viscous products can be formed at lower fat contents than in the absence of heteroaggregation).

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Previously, we induced heteroaggregation by mixing an emulsion containing cationic protein-coated lipid droplets with one containing anionic protein-coated droplets.[9,10] This was achieved by using two globular proteins with different isoelectric points (pI) to stabilize the lipid droplets: β-lactogobulin (pI ≈ 5) and lactoferrin (pI ≈ 8). At pH values between the isoelectric points of the two proteins, the respective lipid droplets have opposite charges and therefore tend to aggregate through electrostatic attraction. The purpose of the current study was to examine the influence of heteroaggregation on the potential biological fate of ingested emulsions, since this might influence the rate of lipid digestibility and/or the release of any encapsulated bioactive components. Indeed, a number of studies have shown that droplet aggregation under simulated or actual gastrointestinal tract (GIT) conditions may influence the biological fate of ingested emulsions.[11–13] Previous research has examined the stability and digestion of lipid droplets coated with either β-lactoglobulin[14–17]or lactoferrin.[18–20] However, there have been no previous studies on the influence of heteroaggregation of protein-coated lipid droplets on their behavior under GIT conditions.

In this study, we focused on the influence of heteroaggregation on the stability and digestibility of lipid droplets within a simulated gastrointestinal tract. We hypothesized that the electrostatic attraction between oppositely charged protein-coated lipid droplets would affect the aggregation stability, rheological properties, and digestibility of emulsions under model GIT

conditions. We also compared the results obtained using a full GIT model that simulates the oral, gastric and small intestinal stages, with a simple GIT model that only simulates the small intestinal stage. This was done because simple GIT models are often used as an initial screening tool for identifying the major factors that influence the behavior of lipids under GIT conditions.[21] The knowledge gained from this study should be useful for creating emulsion-based functional food products designed to improve human health and well-being.

2. Experimental

2.1 Materials

Corn oil was purchased from a commercial food supplier (Mazola, ACH Food Companies, Inc., Memphis, TN) and stored at 4 °C until use. Lactoferrin powder (LOT #10404498) was supplied by FrieslandCampina (Delhi, NY), and the manufacturer reported that it contained 97.7% protein and 0.12% ash. Purified β-lactoglobulin powder (BioPURE, LOT #JE-001-0-415) was supplied by Davisco Foods International (Eden Prairie, MN). The manufacturer reported the composition of this powder to be 97.4% total protein, 92.5% β-lactoglobulin (β-Lg), and 2.4% ash. All other chemicals used in this research were purchased from Sigma-Aldrich (St Louis, MO). Double distilled water was used to make all solutions.

2.2 Formation of single-protein emulsions

Aqueous emulsifier solutions were prepared by dispersing either β-lactoglobulin (β-Lg) powder or lactoferrin (LF) powder into distilled water, and then stirring for at least 3 h at room temperature to ensure complete dispersion. After this time the protein powders were seen to be fully dissolved (a transparent solution was formed). The pH of the protein solutions was then adjusted to 7.0 using 1 M NaOH or HCl. Oil-in-water emulsions containing a single protein type were prepared by blending 10 g of corn oil and 90 g of aqueous protein solution for 2 min using a hand blender (M133/1281-0, 2 speed, Biospec Products Inc., ESGC, Switzerland) and then recirculating them four-times through a high pressure homogenizer (Microfluidizer M-110 L processor, Microfluidics Inc., Newton, MA) operating at 90 MPa. The β-Lg emulsion was then heated to 90 °C for 30 min to cross-link the adsorbed proteins, so as to prevent any competitive adsorption effects. All emulsions were then stored for 24 hours prior to utilization. Preliminary experiments reported elsewhere[10]established that 1% β-Lg and 3% LF were suitable levels to form single-protein emulsions with relatively small droplet diameters ($d_{43} \approx 0.35$ μm), and so these levels were used to form the mixed-protein emulsions.

2.3 Formation of mixed-protein emulsions

Initially, two 10 wt% oil-in-water emulsions stabilized by either 1% β-Lg or 3% LF were prepared in distilled water, and then adjusted to pH 7.0. These two single-protein emulsions had similar initial droplet diameters ($d_{43} \approx 0.35$ μm). Mixed emulsions were then prepared by mixing 40 wt% of the β-Lg emulsion with 60 wt% of the LF emulsion, stirring for 10 min, then allowing them to stand for 24 h prior to analysis. This particle ratio was selected based on our previous results, which showed that the maximum amount of droplet aggregation occurred under these conditions.[9,10]

2.4 Simulated gastrointestinal tract

Each emulsion sample was passed through a full simulated GIT model that consisted of a mouth phase, gastric phase and small intestine phase, or a simple simulated GIT model that only consisted of the small intestine phase. Measurements of the microstructure, particle size distribution, and particle charge were measured at each stage (Section 2.5).

Oral stage. Artificial saliva (pH 6.8) containing 3% mucin was prepared according to the composition shown in Table 1. This saliva composition was based on those reported in previous studies.[19,22] The *in vitro* oral model consisted of a conical flask (125 mL) containing artificial saliva maintained at 37 °C with continuous shaking at 100 rpm for 15 min in a temperature controlled air incubator (Innova Incubator Shaker, Model 4080, New Brunswick Scientific, New Jersey, USA) to mimic the conditions in the mouth. Each emulsion was mixed with artificial saliva (ratio 1 : 1 w/w). The resulting mixture contained 5% (w/w) oil and was taken for characterization at the end of the incubation period. In reality, a food is likely to spend considerably less time in the oral cavity than the incubation time used here, and the ratio of saliva-to-food will depend on the type of food consumed and the individual. However, it has been reported that using a longer incubation time with relatively mild shaking of a food sample in the laboratory can help to simulate the shorter incubation time a food spends in the human mouth, where the mechanical agitation is more intense.[19]

Gastric stage. Simulated gastric fluid (SGF) was prepared by adding 2 g NaCl, 7 mL HCl, and 3.2 g of pepsin (from porcine gastric mucosa) to a flask and then diluting with distilled water to a volume of 1 L, and finally adjusting to pH 1.2 using 1.0 M HCl (United States Pharmacopeial Convention, 2000). Samples taken from the oral stage were mixed with SGF (ratio 1 : 1 w/w) so that the final mixture contained 2.5% (w/w) oil. This mixture was then adjusted to pH 2.5 using 1 M NaOH and incubated at 37 °C for 2 h. Samples were taken for characterization at the end of the incubation period.

Small intestinal stage. Samples obtained from the simulated gastric model were incubated for 2 h at 37 °C in a simulated small intestinal fluid (SIF) containing 2.5 mL pancreatic lipase (4.8 mg mL^{-1}), 4 mL bile extract solution (5 mg mL^{-1}) and 1 mL calcium chloride solution (750 mM), and the free fatty acids (FFA) released were monitored by determining the amount of 0.25 M NaOH needed to maintain a constant pH of 7.0 within the reaction chamber using an automatic titration unit. The pH-stat used (Metrohm USA, Inc.) was controlled by dedicated software (Tiamo 1.2.1 software, Metrohm GA, Switzerland). All additives were dissolved in double distilled water (pH 7.0) before use. Lipase addition and initialization of the titration program were carried out only after the addition of all other pre-dissolved ingredients and careful balancing of the pH to 7.0. Samples were

Table 1 Chemical composition of artificial saliva used to simulate oral conditions[19,22]

Chemical name	Chemical formula	Concentration ($g\ L^{-1}$)
Sodium chloride	NaCl	1.594
Ammonium nitrate	NH_4NO_3	0.328
Potassium phosphate	KH_2PO_4	0.636
Potassium chloride	KCl	0.202
Potassium citrate	$K_3C_6H_5O_7 \cdot H_2O$	0.308
Uric acid sodium salt	$C_5H_3N_4O_3 \cdot Na$	0.021
Urea	H_2NCONH_2	0.198
Lactic acid sodium salt	$C_3H_5O_3Na$	0.146
Porcine gastric mucin (Type II)	—	30
Water	H_2O	Remainder

taken for physicochemical and structural characterization at the end of the 2 h digestion period.

The volume of NaOH (0.25 M) added to the emulsion was recorded and used to calculate the concentration of free fatty acids generated by lipolysis. The amount of free fatty acids released was calculated using the following equations:

$$V_{max} = 2 \times \left[\frac{m_{oil}}{MW_{oil}} \times \frac{1000}{C_{NaOH}}\right]$$

$$\%FFA\ released = \frac{V_{Exp}}{V_{Max}} \times 100\%$$

where, m_{oil} is the total mass of oil present in the reaction vessel (g), MW_{oil} is the molecular weight of the oil ($g\ mo^{-1}$), C_{NaOH} is the concentration of sodium hydroxide in the titration burette ($mol\ dm^{-3}$), and V_{Max} is the volume of NaOH titrated into the reaction vessel to neutralize the FFA released assuming that all the triacylglycerols are converted into two free fatty acids. Finally, V_{Exp} is the actual volume of NaOH titrated into the reaction vessel to neutralize the FFA released during the experiment.

2.5 Particle size, charge and rheological measurements

Particle size measurement. The particle size distribution of the emulsions was measured using a laser diffraction particle size analyzer (Mastersizer 2000, Malvern Instruments, Ltd., Worcestershire, UK). To avoid multiple scattering effects the emulsions were diluted to a droplet concentration of approximately 0.005 wt% using pH-adjusted water at the same pH as the sample. The emulsions were stirred continuously throughout the measurements to ensure the samples were homogenous. Measurements are reported as the volume-weighted mean diameter: $d_{43} = \sum d_i n_i^4 / \sum d_i n_i^3$, where n_i is the number of droplets of diameter d_i. We note that particle size measurements made by static light scattering on highly flocculated emulsions should be treated with caution. First, the theory (Mie theory) used to interpret light scattering data assumes that the scattering particles are homogeneous spheres with well-defined refractive indices. In reality, flocs are non-spherical and non-homogeneous particles, with ill-defined refractive indices. Second, the process of dilution and stirring may have altered the dimensions and structural organization of the flocs. Consequently, the reported particle sizes should only be treated as an indication of strong droplet association rather than a measure of the actual size of any aggregates present in the original non-diluted samples.

Particle charge measurements. The ζ-potential of emulsions was determined using a particle electrophoresis instrument (Zetasizer Nano ZS series, Malvern Instruments, Worcestershire, UK). Emulsions were diluted to a droplet concentration of approximately 0.001 wt% using pH-adjusted water to avoid multiple scattering effects. The pH of the water used was adjusted to the same pH as the initial emulsion sample. After loading the samples into the instrument they were equilibrated for approximately 120 s before particle charge data was collected over 20 continuous readings.

Rheological properties. The rheological behavior of samples was measured using a dynamic shear rheometer (Kinexus Rotational Rheometer, Malvern, UK). A "cup and bob" geometry consisting of a rotating inner cylinder (diameter 25 mm) and static outer cylinder (diameter 27.5 mm) was used. The samples were loaded into the rheometer measurement cell and allowed to equilibrate at 25 °C for 5 min before beginning all experiments. Samples underwent a constant shearing treatment ($1\ s^{-1}$ for 10 min) prior to analysis to remove history effects. The shear stress of the emulsions was then measured over a range of shear rates (0.01 to 50 s^{-1}), and the apparent viscosity was calculated from this data.

2.6 Emulsion microstructure

Confocal imaging of emulsion microstructures was carried out with a 60× oil immersion objective lens using a Nikon confocal microscope (C1 Digital Eclipse) at ambient temperature. Samples were imaged at various stages of digestion: (i) immediately after preparation, (ii) after 15 min incubation at pH 6.8 in simulated saliva, (iii) after 2 h incubation at pH 2.5 in SGF, and (iv) after 2 h incubation at pH 7 in SI. Emulsion samples were stained with the fluorescent dye Nile red (0.1 wt% dissolved in 100% ethanol). The fluorescent dye was excited by an argon 476 nm laser and emitted light was collected between 555–620 nm.

3. Results and discussion

3.1 Influence of initial emulsion composition and microstructure on behavior within a model GIT

3.1.1 Initial emulsions. Initially, the single-protein emulsions (pH 7) contained relatively small droplets ($d_{43} < 0.4$ μm) after preparation (Fig. 1a and 2b and c), which suggested that they were stable to droplet aggregation under these conditions. As

expected, the β-Lg-coated droplets were highly negative because they were above their isoelectric point, whereas the LF-coated droplets were highly positive because they were below their isoelectric point (Fig. 1b). On the other hand, the mixed emulsions contained relatively large particles ($d_{43} \approx 13$ μm) (Fig. 1a), suggesting that the oppositely charged lipid droplets were attracted to each other and formed microclusters. The particle size distribution measurements (Fig. 2a) and confocal microscopy images (Fig. 3) suggested that the microclusters ranged in size from around 1 to 100 μm. The microclusters which formed in the mixed emulsions had a slight negative charge, which suggests that the anionic β-Lg-coated droplets may have been preferentially located at their outer edges, or that there were more anionic β-Lg-coated droplets than cationic LF-coated droplets in each cluster. These measurements indicated that there were appreciable differences between the microstructural and electrical properties of the initial emulsions. We hypothesized that these differences would alter the subsequent fate of the emulsions within the GIT model.

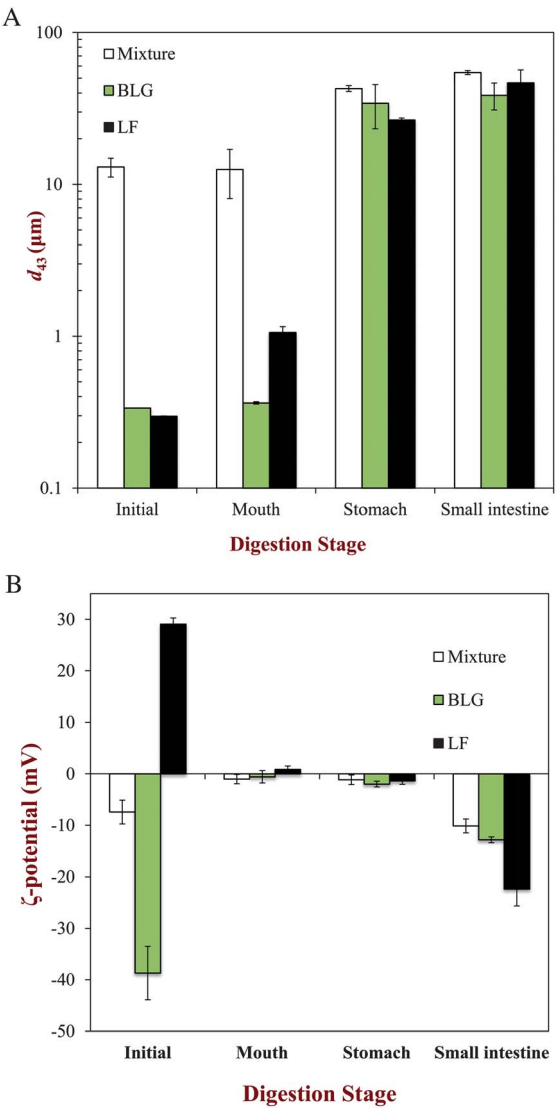

Fig. 1 Influence of initial emulsion type on (A) the mean particle diameter (d_{43}), and (B) the droplet charge, measured at various stages in an *in vitro* gastrointestinal tract model. The emulsions initially contained β-lactoglobulin-coated droplets, lactoferrin-coated droplets, or a mixture of the two.

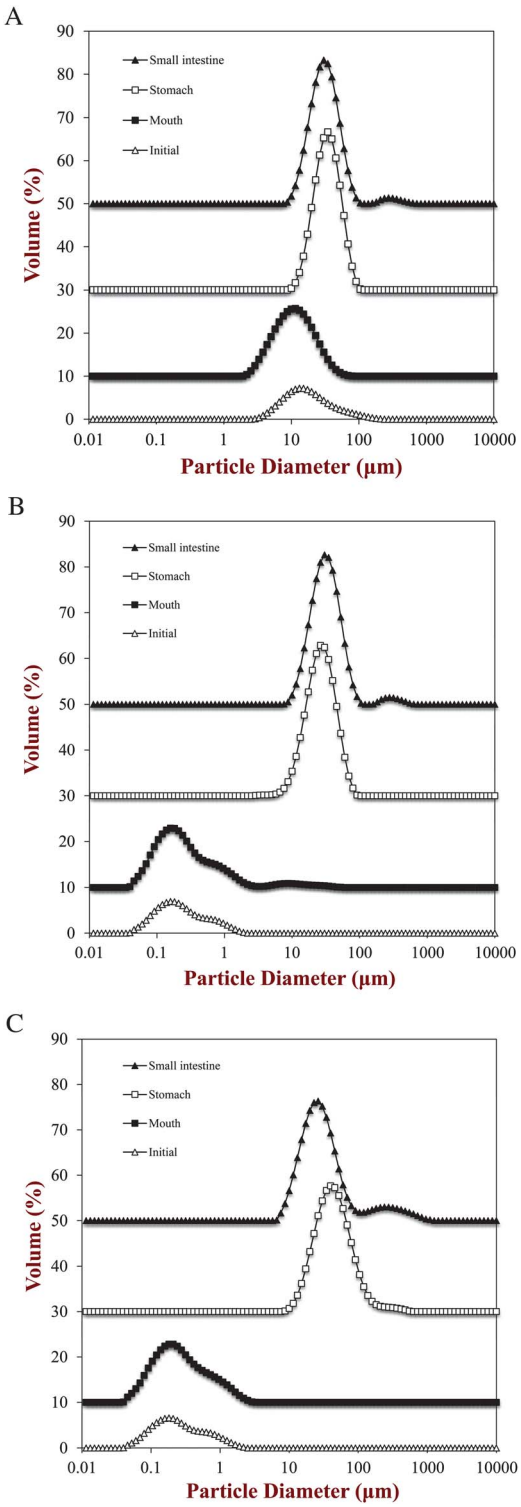

Fig. 2 Influence of initial emulsion type on the particle size distribution measured at various stages in an *in vitro* gastrointestinal tract model: (A) mixed emulsion; (B) lactoferrin stabilized emulsion; (C) β-lactoglobulin stabilized emulsion.

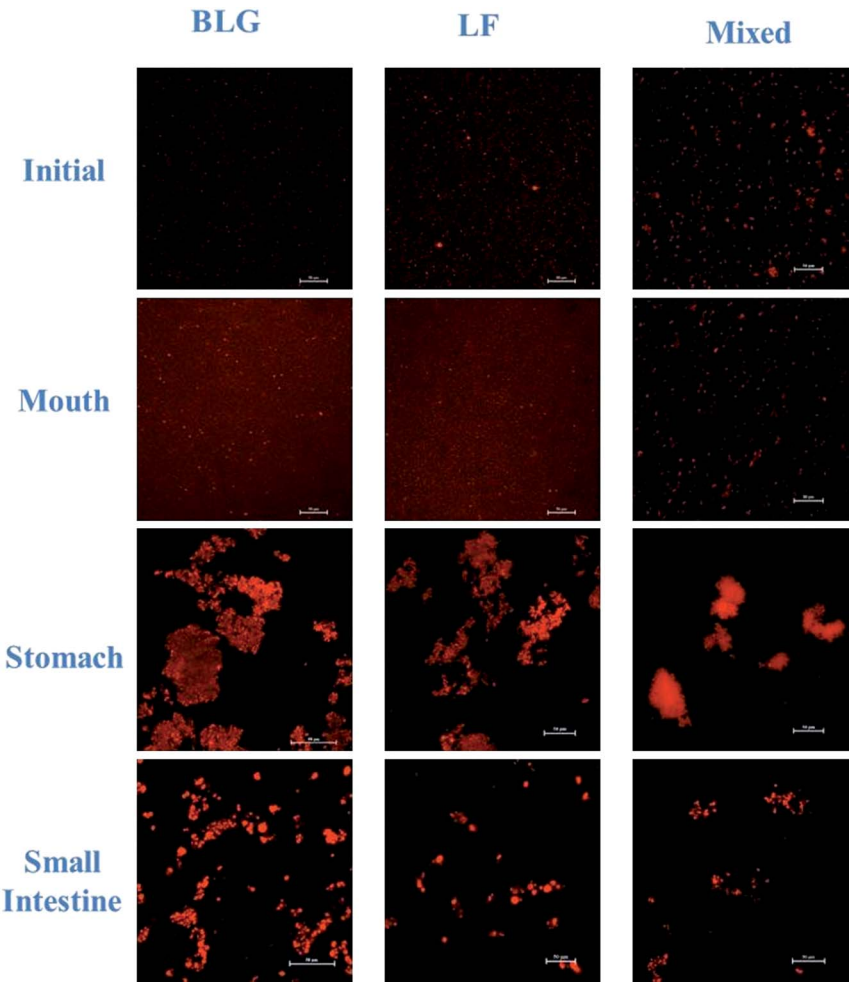

Fig. 3 Influence of initial emulsion type on the microstructure measured by confocal fluorescence microscopy at various stages in an *in vitro* gastrointestinal tract model. The emulsions studied were mixed, lactoferrin stabilized, and β-lactoglobulin stabilized emulsions, respectively. The fat phase was stained red, and the scale bars are 50 μm long.

3.1.2 Response to oral conditions. There were considerable alterations in the microstructures and electrical properties of the various emulsions after they were incubated in artificial saliva. There was a pronounced decrease in the magnitude of the electrical charge on the particles in all the emulsions: from +29 to +0.6 mV for the LF emulsion; from −39 to −0.6 mV for the β-Lg emulsion; and, from −7 to −1 mV for the mixed system (Fig. 1b). This decrease in droplet charge may have originated from a number of physicochemical phenomena associated with specific components within the saliva: (i) electrostatic screening by mineral ions; (ii) binding of multivalent counter-ions to droplet surfaces; (iii) adsorption of polyelectrolytes (mucin) to the droplet surfaces. Interestingly, the large reduction in the magnitude of the ζ-potential did not promote droplet aggregation in the β-Lg-emulsions or any further aggregation in the mixed emulsions (Fig. 1a), which might have been expected due to a reduction in the electrostatic repulsion between particles. It is possible that some of the mucin molecules formed a coating around the lipid droplets, thereby generating a steric repulsion that prevented aggregation. There was an appreciable increase in the mean particle diameter of the emulsions containing LF-coated lipid droplets after incubation in simulated oral conditions (Fig. 1a). This effect may have been because of bridging flocculation caused by binding of anionic mucin molecules to the cationic lipid droplets. These structures would be more difficult to breakdown within the light scattering instrument, which is possibly why the particle sizing data indicated that they were larger. Previous research has also found that lactoferrin-coated lipid droplets undergo extensive aggregation when mixed with artificial saliva,[19] which was attributed to electrostatic screening and bridging effects by salts and polyelectrolytes (mucin) in the saliva.

3.1.3 Response to gastric conditions. There were major changes in the particle size and microstructure of all the emulsions after they were incubated in simulated gastric fluid (SGF). There was a large increase in mean particle diameter (Fig. 1a). Larger particles were observed in the particle size distribution (Fig. 2), and there was evidence of extensive flocculation in the confocal microscopy images (Fig. 3). Droplet aggregation within the gastric stage can be attributed to the combined effects of various constituents within the SGF, *e.g.* acids, salts, and pepsin.

The pH is reduced from around neutral in the oral stage to highly acidic in the gastric stage, which will influence the electrical characteristics of the various proteins in the system. The lactoferrin should remain highly positively charged as the pH is reduced, whereas the β-lactoglobulin should go from negative, to neutral, to positive as the pH is altered from above to below its isoelectric point. Previous studies have shown that protein-coated droplets may undergo irreversible aggregation when they are passed through their isoelectric points, due to the reduction in electrical charge.[23] Some of the droplet aggregation in the systems containing β-lactoglobulin may therefore be attributed to this effect. In the mixed systems, at pH values below the isoelectric point of β-lactoglobulin (pI ≈ 5), both proteins should have positive charges and should therefore be repelled from each other, which may alter the nature of the aggregation process in the stomach.

There were appreciable amounts of dissolved ions in the stomach stage arising from the NaCl present in the SGF and the various salts added in the oral phase. Salt ions are known to promote droplet flocculation in protein-stabilized emulsions due to their ability to screen electrostatic interactions or bind to droplet surfaces causing charge neutralization or bridging effects.[24] Some of the droplet aggregation observed may therefore be due to the increase in ionic strength of the aqueous phase surrounding the lipid droplets in the stomach. Finally, SGF contains pepsin, which is an enzyme capable of catalyzing the hydrolysis of proteins under acidic conditions.[25] The hydrolysis of the adsorbed or free proteins in the emulsions may have altered their stability to droplet aggregation.[12,20] For example, the peptides that remain at the interface may be unable to provide sufficient electrostatic and/or steric repulsions. As a result, emulsions are highly susceptible to flocculation and coalescence. Previous researchers have also reported that extensive droplet aggregation occurs when protein-stabilized emulsions are exposed to simulated gastric conditions.[20,26]

3.1.4 Response to small intestine conditions. Finally, we compared the behavior of the three different emulsions after incubation under small intestine conditions. For all three systems, the mean particle diameter was relatively high (Fig. 1a), there was evidence of large particles in the particle size distributions (Fig. 2), and extensive droplet aggregation was observed in the confocal microscopy images (Fig. 3). The microscopy images indicated that there were smaller amounts of lipid droplets present in the small intestine than in the stomach, which is due to dilution and digestion of the droplets. The confocal images also suggest that some droplet coalescence had occurred, since the size of the individual droplets in the small intestine stage appears to be larger than in the stomach stage. Coalescence may have been a result of protein and lipid hydrolysis during digestion, thus reducing the resistance of the interfacial membranes to rupturing.[20] The electrical charge on the particles after the small intestine phase was much more negative than after the gastric stage, which is probably due to adsorption of anionic species to the surfaces of non-digested fat and/or due to the presence of anionic species generated by the digestion process (such as mixed micelles and vesicles). Bile salts, phospholipids and free fatty acids are all negatively charged at neutral pH and may therefore have contributed to the formation of anionic colloidal particles. Surprisingly the negative charge was greatest in the emulsions that initially contained only lactoferrin, which should be positively charged at this pH. One would expect that the proteins would be fully or partly digested at the end of the small intestine phase. The protein digestion products may therefore have influenced the nature of the colloidal structures formed within the small intestine fluid after digestion.

3.2 Influence of initial emulsion composition and microstructure on lipid digestion

The rate of digestion of the emulsions during the small intestine stage was monitored using a pH-stat method, *i.e.*, by measuring the amount of alkali solution that had to be added to maintain a neutral pH.[27] In systems containing both lipids and proteins the pH may change due to lipid hydrolysis (producing fatty acids and monoacylglycerols) or protein hydrolysis (producing amino acids and peptides). We therefore measured the amount of alkali solution that had to be added to the digestion reaction chamber for the three emulsions and for protein solutions that contained the same type and amount of proteins as the corresponding emulsions. The amount of free fatty acids released was then calculated by subtracting the data for the emulsion from the data for the corresponding protein solution, assuming that the protein was digested similarly in both systems. In practice, some globular proteins are digested at different rates when they are present in solution or at oil–water interfaces.[28,29]

For all three protein solutions (β-Lg, LF, and mixed), there was an appreciable increase in the amount of alkaline solution added to the samples during digestion (Fig. 4a), which can be attributed to protein hydrolysis. Bile extracts similar to the ones used in this study are known to have protease activity,[20] and there may also be some residual protease (pepsin) remaining in the samples from the gastric stage. The total amount of alkali added to the samples was fairly similar for all three proteins, which suggests that they were all digested by a similar amount. We also found that the amount of alkali added to the three different emulsions during digestion was fairly similar (Fig. 4a), which suggests the lipids were also digested at a similar rate. Indeed, we observed no major differences in the rate or extent of lipid digestion in mixed emulsions or single-protein emulsions (Fig. 4b). These experiments suggest that the initial microstructure of the emulsions had little impact on their subsequent digestion in the small intestine. Presumably, this occurs since the original structure of the emulsions was progressively destroyed as they passed through the GIT. For example, there were changes in the structural organization of the lipid droplets in the system, their particle sizes, and their interfacial compositions (Fig. 1–3), which may be attributed to interactions with the various components in the intestinal fluids they encounter. It should be noted that none of the emulsions was completely digested at the end of the small intestine stage, which we attribute to the relatively high amount of fat (2.5 wt%) that was initially present during the lipid digestion stage. In reality, the human body would respond to a high fat meal by producing more lipase to digest the lipids and more bile salts and phospholipids to solubilize the digestion products (free fatty acids and monoacylglycerols) produced.

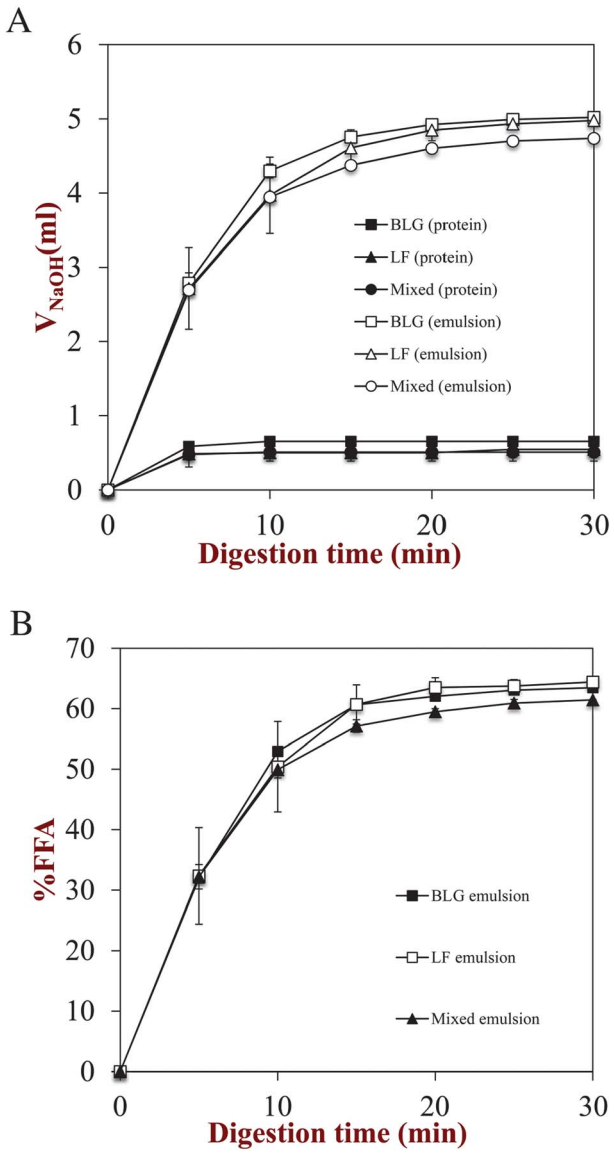

Fig. 4 (a) Volume of NaOH solution titrated into the digestion vessel to maintain the pH at 7.0 for emulsions and protein solutions. The emulsions contained different kinds of protein-coated oil droplets (β-lactoglobulin, lactoferrin, or mixed). The aqueous solutions had the same type and concentration of protein as the corresponding emulsions. (b) Calculated free fatty acids release due to lipid digestion for emulsions containing different kinds of protein-coated droplets (β-lactoglobulin, lactoferrin, or mixed).

3.3 Influence of initial emulsion composition rheological properties in simulated GIT conditions

The rheological properties of ingested foods may influence their behavior within the gastrointestinal tract.[30] The mixed emulsions initially had much higher viscosities than the two single-protein emulsions, and so we examined the influence of passage through the GIT on their rheological properties.

The dependence of the apparent viscosity on the shear rate of the mixed emulsions at different stages of the simulated GIT is shown in Fig. 5a. In general, the viscosity of the mixed emulsions decreased as they passed through each stage of the GIT model: initial > oral > gastric > small intestine. The main reason for this decrease is the progressive dilution of the emulsions with the various intestinal fluids, since a decrease in droplet concentration is known to cause a reduction in emulsion viscosity.[24] This dilution effect appears to be larger than any increase in viscosity due to the presence of biopolymers (such as mucin in the mouth) or increased aggregation (*e.g.*, in the stomach). For the mixed emulsions, there was an appreciable decrease in apparent viscosity with increasing shear rate, particularly in the initial samples. This shear thinning behavior can be attributed to

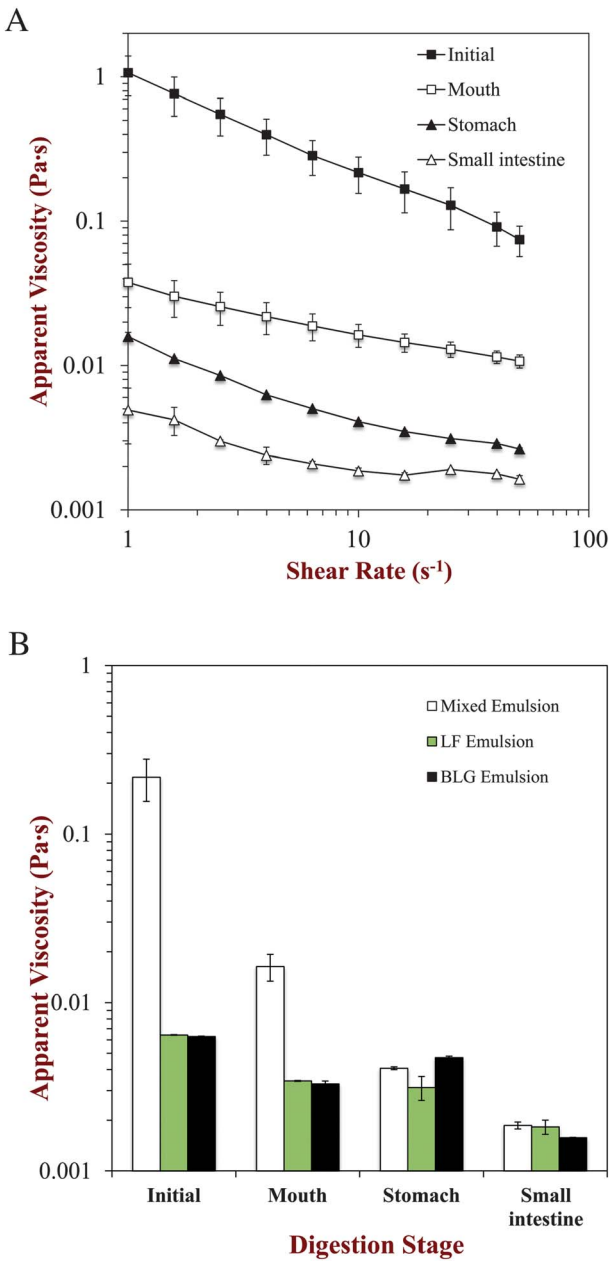

Fig. 5 (a) Apparent shear viscosity *versus* shear rate profiles of mixed emulsions at various stages in an *in vitro* gastrointestinal tract model. (b) Influence of initial emulsion type on the apparent shear viscosity (at 10 s^{-1}) of emulsions at different stages in an *in vitro* gastrointestinal tract model. The emulsions initially contained β-lactoglobulin-coated droplets, lactoferrin-coated droplets, or a mixture of the two.

progressive disruption of flocculated droplets with increasing applied shear force.[31] Shear thinning behavior was also observed in the samples collected from the single-protein emulsions (data not shown).

The apparent viscosities of the mixed emulsions were compared with those of the single-protein emulsions at different stages of the GIT at a constant applied shear rate of 10 s^{-1} (Fig. 5b). In general, there was a decrease in viscosity as the emulsions passed through each stage of the GIT model for all three emulsions, which can be attributed to the dilution effect mentioned earlier. The only exceptions to this trend were the LF emulsion and β-Lg emulsion where the viscosities were either similar or higher in the gastric stage than in the oral stage. This effect may be attributed to the extensive droplet flocculation that was observed in the simulated gastric fluids, since flocculation is known to increase viscosity.[31] The mixed emulsions had much higher viscosities than the single-protein emulsions in the oral stage, but fairly similar viscosities in the gastric and small intestinal stages (Fig. 5b). This behavior can be attributed to the fact that the mixed emulsion was highly aggregated in the oral phase, but the single-protein emulsions were not, and that all the emulsions were highly aggregated in the SGF and SIF. This effect may have important implications for the design of functional foods that have highly viscous textures in the mouth, but that behave like conventional emulsions within the stomach and small intestine. Thus, it may be possible to use this approach to modulate the sensory perception of emulsion-based foods, without altering their physiological and nutritional function.

3.4 Comparison of full and simplified *in vitro* digestion models

Finally, we compared the results obtained with the full simulated GIT model that included oral, gastric and small intestine stages, with a simplified GIT model that only included the small intestine stage. We did this because the simplified GIT model is often used in screening studies designed to determine the influence of sample composition or structure on lipid digestion.[21,32]

The particle sizes, charges and microstructures of the three emulsions after being passed through the simple and full GIT models were compared. We observed little difference in the mean particle size or microstructure of the three emulsions using either GIT model (Fig. 6a and 7). The lipid droplets appeared to be highly aggregated in all three protein-stabilized emulsions, which may have been due to oil droplet flocculation, coalescence, and digestion, as well as due to complexation with other components in the digestion medium, such as bile salts, calcium, and other ions. Other researchers have also reported a high degree of droplet aggregation after the small intestinal phase.[20,33] Nevertheless, we did observe some differences in the electrical characteristics of the particles (Fig. 6b). The emulsions that had been through the simple model all had fairly similar high negative charges (−30 to −32 mV), whereas the emulsions that had been through the full model had lower negative charges that were more dependent on initial emulsion type (−10 to −22 mV). This suggests that there were some components within the artificial saliva or the gastric fluids that contributed to the electrical characteristics of the particles present after digestion in the small

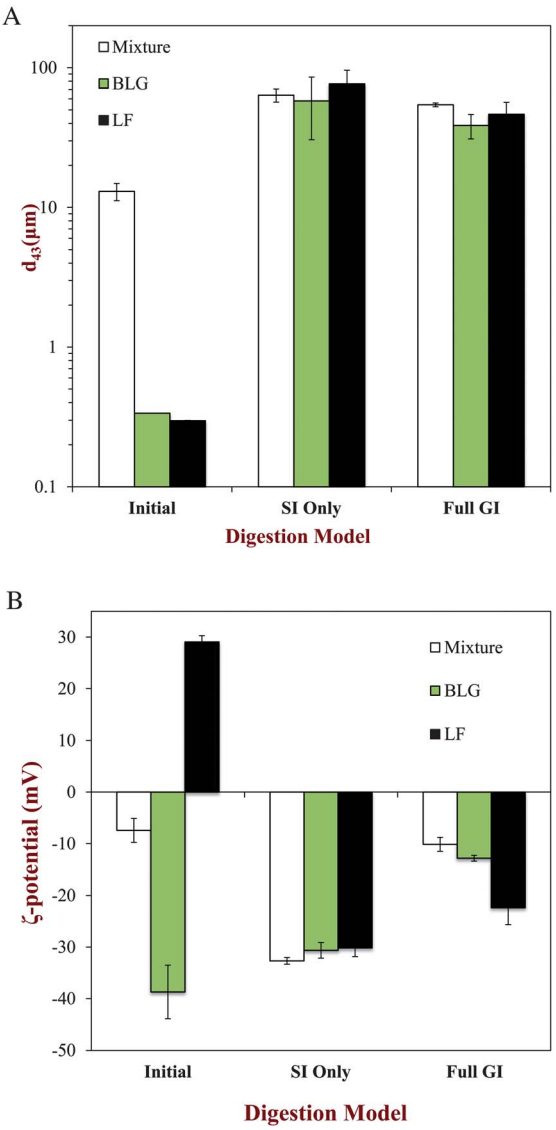

Fig. 6 (a) Influence of initial emulsion type on the mean particle diameter (d_{43}) measured after passage through two different *in vitro* gastrointestinal tract models: (i) full GI model (oral, gastric and small intestine); (ii) small intestine only. (b) Influence of initial emulsion type on the droplet charge measured after passage through two different *in vitro* gastrointestinal tract models: (i) full GI model (oral, gastric and small intestine); (ii) small intestine only.

intestine. Some of the ionic components within the intestinal fluids may have become incorporated into the particles formed after the small intestine stage, *e.g.*, mineral ions, biopolymers (*e.g.*, mucin), or enzymes (*e.g.*, pepsin). Alternatively, the difference in the electrical characteristics of the particles may have been due to digestion of some or all of the proteins by pepsin leading to the generation of different charged species in the system.

We also observed some differences in the rate of lipid digestion in the three emulsions when they were subjected to either the simple or full GIT models (Fig. 8). For all three emulsions, the rate and extent of lipid digestion was somewhat less for the full model, than for the simple model. This suggests that there were

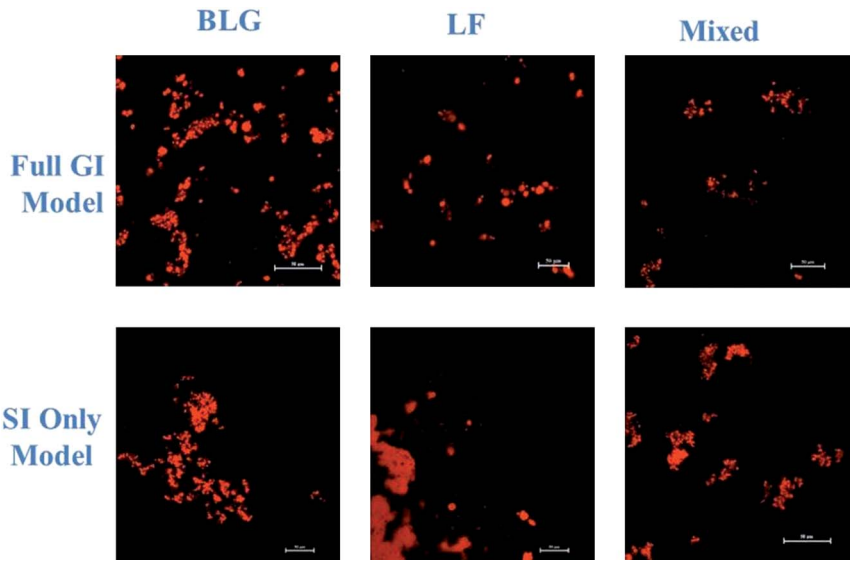

Fig. 7 Influence of initial emulsion type on the microstructure measured by confocal fluorescence microscopy after passage through two different *in vitro* gastrointestinal tract models: (i) full GI model (oral, gastric and small intestine); (ii) small intestine only. The fat phase was stained red, and the scale bars are 50 μm long.

some changes in the structural organization or interfacial composition of the emulsions as a result of passage through the oral and gastric stages that altered their susceptibility to digestion. Having said this, the pH-stat measurements indicated that all the samples were digested at a fairly similar rate and extent. In other words, there were only slight quantitative differences between the two methods, but the results obtained were qualitatively fairly similar. This suggests that the simple digestion model may be appropriate for studying the digestion of protein-stabilized emulsions.

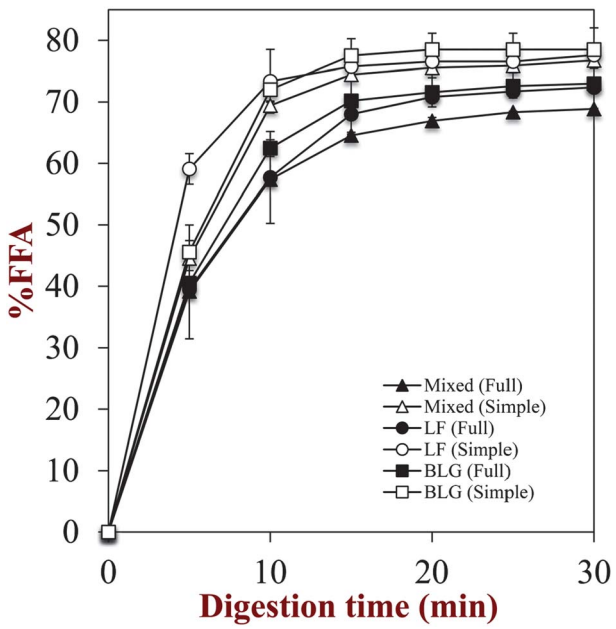

Fig. 8 Influence of initial emulsion type on the calculated release of free fatty acids after passage through two different *in vitro* gastrointestinal tract models: (i) full GI model (oral, gastric and small intestine); (ii) small intestine only.

4. Conclusions

The purpose of this study was to examine the influence of heteroaggregation on the behavior of protein-coated lipid droplets within simulated gastrointestinal conditions. Initially, we hypothesized that differences in the microstructural and rheological properties of the original emulsions would influence their behavior in the GIT model. We found that the mixed emulsions, containing a mixture of anionic and cationic protein-coated lipid droplets, were initially much more viscous than single-protein emulsions with similar fat contents. The mixed emulsions remained much more viscous than the single-protein emulsions in simulated oral conditions, but the electrical characteristics, aggregation state and viscosity of all the emulsions were fairly similar in the stomach and small intestine stages. As a result, all of the emulsions were hydrolyzed at a similar rate and to a similar extent by intestinal enzymes. Overall, our results suggest that the initial microstructural organization of protein-stabilized emulsions does not have a major impact on their subsequent digestion under simulated small intestinal conditions. This information is important for the design of functional foods that have desirable physicochemical and sensory characteristics, and are also fully digested in the gastrointestinal tract.

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