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ARTICLE

Impact of Interfacial Composition on Physical Stability and In Vitro Lipase Digestibility of Triacylglycerol Oil Droplets Coated with Lactoferrin and/or Caseinate

Uri Lesmes,[†] Prune Baudot,[§] and David Julian McClements*,[†]

[†]Department of Food Science, University of Massachusetts, Amherst, Massachusetts 01003, and [§]Institut National Superieur Des Sciences Agronomiques de l'alimentation et de l'environnement, Dijon, France

The physicochemical and functional properties of oil-in-water emulsions can be controlled by engineering the interfacial layer coating the oil droplets. This study examined the impact of interfacial deposition of lactoferrin (LF) and/or caseinate (Cas) onto oil droplets stabilized by the opposite protein on emulsion stability and lipase digestibility. Zeta potential measurements show both proteins can be deposited on droplet surfaces coated with the opposite protein. Secondary emulsion formulations containing droplets coated with mixed caseinate−lactoferrin layers (Cas-LF and LF-Cas emulsions) had enhanced stability to flocculation in 3 < pH < 7 and from 0 to 80 mM calcium chloride. The majority of emulsions studied were rapidly digested by lipase in an in vitro digestion model, demonstrating the improved secondary formulations are not expected to alter lipid bioavailability. This work provides information valuable in the design of emulsion formulations for applications in the food, pharmaceutical, and personal care industries.

KEYWORDS: Emulsions; lactoferrin; caseinate; calcium; pH; stability; in vitro digestibility

INTRODUCTION

Colloid-based delivery systems are being used increasingly in the food industry to encapsulate, protect, and deliver active food components, for example, antimicrobials, flavors, colors, vitamins, and nutraceuticals (*1*−*5*). Recently, there has been interest in extending the functional performance of this type of delivery systems by using structural design principles to control the morphology and composition of the colloidal particles (*6*−*8*). For example, the stability of conventional oil-in-water emulsions to environmental stresses and lipid oxidation can be improved by harnessing electrostatic biopolymer interactions to "multilayer" droplets with nanolaminated layers via a layer-by-layer (LbL) approach (*4*, *9*, *10*).

Several studies have shown that various types of electrically charged food-grade biopolymers can be used to form multilayered emulsions (*9*−*16*). In such emulsion formulations, surface active biopolymers, such as globular proteins (e.g., β-lactoglobulin) and flexible proteins (e.g., casein), are typically used to form "primary" emulsions composed of oil droplets surrounded by electrically charged protein coatings. The droplet charge in the protein-stabilized primary emulsions can be controlled by varying the pH either below (positive charge) or above (negative charge) the isoelectric point (p*I*) of the adsorbed protein coating. A "secondary" emulsion can then be formed by mixing the primary emulsion with a solution of oppositely charged biopolymers. Accordingly, multilayer emulsions can be formed by LbL electrostatic deposition of layers around the lipid droplets by sequentially mixing biopolymer-coated lipid droplets with oppositely charged biopolymer solutions (*9*, *16*). This approach has been proposed as a powerful means of controlling the digestibility of emulsified lipids, because the lipid droplets can be coated by dietary fibers that should resist digestion in the mouth, stomach, and small intestine (*4*, *6*, *8*, *11*). The functionality of these multilayer emulsions can be controlled by varying the type of biopolymers used, the number of biopolymer layers within the coating, and the sequence in which the biopolymer layers are deposited (*9*). Consequently, it should be possible to create multilayered delivery systems that can be designed to encapsulate, protect, and deliver bioactive lipophilic components to different regions within the human gastrointestinal tract (GIT).

Previously, polysaccharides have typically been used to form the second and higher level layers around lipid droplets using the electrostatic deposition method (*9*, *10*, *16*). Nevertheless, recent studies suggest that globular proteins can also be used to form the outer layers in multilayer biopolymer coatings, for example, lactoferrin and β-lactoglobulin (*12*, *13*, *17*). Lactoferrin is finding increasing attention as a functional ingredient because it has been shown to have certain health benefits and various potential uses (*18*−*20*), the ability to enhance the physical and chemical stability of oil droplets in emulsions (*13*, *17*, *21*, *22*), and the ability to serve as a targeting moiety for lipophilic drugs (*23*, *24*). Recently, we have demonstrated that in specific formulations, lactoferrin can help promote the physical and chemical stability of omega-3-rich fish oil emulsions (*25*).

In the present study, we utilized the electrostatic LbL deposition approach to formulate emulsions to contain mixed caseinate−lactoferrin layers. We hypothesized that the formation of

*Address correspondence to this author at the Food Biopolymers and Colloids Laboratory, Department of Food Science, University of Massachusetts, Amherst, MA 01003 [telephone (413) 545-1010; fax (413) 545-1262; e-mail mcclements@foodsci.umass.edu].

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heterogeneous protein coatings around lipid droplets would alter their physicochemical properties and stability, as well as the susceptibility of the encapsulated lipid droplets to lipase digestion. The information obtained from this study should be useful for the rational design of emulsion-based food-grade delivery systems for lipophilic active food components.

MATERIALS AND METHODS

Materials. *Food Grade Reagents*. Food grade sodium caseinate (Extra grade) was donated by the American Casein Co. (Burlington, NJ), and the manufacturer reported that it contained 96.2% protein, 3.5% ash, and 0.5% fat. Food grade lactoferrin FD was supplied by DMV International (Delhi, NY), and manufacturer analyses showed it contained 97.7% protein and 0.12% ash. Mazola corn oil was purchased at a local supermarket, stored in the refrigerator, and used as received.

Chemical Reagents. Porcine bile extract, type II porcine pancreatic lipase, monobasic phosphate, dibasic phosphate, and $CaCl_2$ were purchased from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). Hydrochloric acid and sodium hydroxide as well as all other solvents and reagents were of analytical grade.

Methodology. A series of primary oil-in-water emulsions was prepared containing lactoferrin and/or caseinate, but with the concentrations and orders of addition of the two proteins being different. In one case, lactoferrin-stabilized primary emulsions (LF emulsions) were prepared, and then different amounts of caseinate were added (LF-Cas emulsions). In the other case, caseinate-stabilized primary emulsions (Cas emulsions) were prepared, and then different amounts of lactoferrin were added (Cas-LF emulsions). For clarity, we refer to a protein as the "primary" protein if it was used to form the primary emulsion and as "secondary" protein if it was used to form the secondary emulsions. The influence of pH, $CaCl_2$, and bile on the physical stability of the LF emulsions, LF-Cas emulsions, Cas emulsions, and Cas-LF emulsions was then monitored. These experiments were conducted to elucidate how individual parameters of the GIT may affect emulsion stability. Thus, emulsion digestibility was also studied in a pH-stat in vitro lipase digestibility assay, where $CaCl_2$ and bile are incorporated into the simulated GIT fluid.

Emulsion Preparation. Sodium caseinate (2.5 wt %) and lactoferrin (2.5 wt %) solutions were prepared with double-distilled water or 5 mM potassium phosphate buffer (pH 7.0) and stirred overnight to ensure proper dissolution. The pH of the protein solutions was adjusted to 7.0 using NaOH or HCl before being added to appropriate quantities of preweighed corn oil. These mixtures were blended at 30% speed for 1 min using a hand-held homogenizer (Tissue Tearor, model 985379-395, Biospec Products Inc.) to form coarse emulsions containing 20% (w/w) corn oil and 2% (w/w) protein. Primary emulsions were formed by passing these coarse emulsions five times through a chilled high-pressure homogenizer (Microfluidizer M-110 L processor, Microfluidics Inc., Newton, MA) operating at 82 MPa and then stored at 4 °C until further use. Secondary emulsions were prepared by incubating primary emulsions for 24 h at 20 °C with aqueous solutions of the secondary protein to yield 10% (w/w) oil emulsions containing 1% (w/w) primary protein and different amounts of secondary protein, specifically 0−8 and 0−2% (w/w) for lactoferrin and sodium caseinate, respectively. Control emulsions were produced by diluting the primary emulsion with double-distilled water or 5 mM phosphate buffer (pH 7.0). Finally, the emulsions were incubated in capped test tubes for 24 h at 20 °C under various pH conditions (3−7) achieved by carefully adjusting the emulsion pH from 7 to the desired value using 0.1 M and/or 0.01 M HCl. Similarly, emulsions were supplemented with $CaCl_2$ solutions to yield samples with various $CaCl_2$ concentrations (0−80 mM), which were stored in closed test tubes for 24 h at 20 °C before analysis.

Emulsion Characterization. The electrical charge of the droplets (ζ-potential) was determined by particle electrophoresis (Zetasizer Nano ZS series, Malvern Instruments, Worcestershire, U.K.). Basically, these measurements record the electrophoretic mobilities of the droplets, which are translated into ζ-potential values using the Smoluchowski model. The mean volume-weighted particle diameter ($d_{4,3}$) and particle size distribution were determined by static laser light scattering (Malvern Mastersizer, Malvern Instruments). In these analyses emulsions were diluted with corresponding solutions with the same ionic strength and pH as the original emulsion to avoid multiple scattering effects.

Adsorption of Bile to Droplet Surfaces. A key step in lipid digestion by lipase is the adsorption of bile salts to lipid droplet surfaces. We therefore examined the effect of bile concentration on oil-in-water emulsions (made 5 mM phosphate buffer, pH 7) incubated for 2 h at 37 °C. Bile concentrations were varied from 0 to 25 mg/mL, as previously described by others to approximate gastrointestinal bile levels (*26*, *27*). An indication of changes in interfacial composition was obtained by measuring changes in droplet ζ-potential as described above.

In Vitro Determination of Lipase Digestibility of Emulsions. A key functional trait of emulsions is their ability to be digested in the GIT, which is mainly done in the small intestine by pancreatic lipases. Thus, the in vitro digestion of the emulsified lipids was monitored using a simulated GIT fluid and a method previously described (*11*, *28*−*31*). Practically, samples were incubated for 2 h at 37 °C in a simulated small intestinal fluid containing pancreatic lipase and the free fatty acids (FFA) released were monitored over time by determining the amount of 0.25 M NaOH needed to maintain a constant pH of 7.0 within the reaction chamber. This was achieved using an autotitration unit (Metrohm USA, Inc.) controlled by dedicated software (Tiamo 1.2.1 software, Metrohm GA, Switzerland). The final digestion sample contained 6 mL of emulsion diluted to a final volume of 37.5 mL using simulated small intestinal fluid. The final composition within the reaction cell was 1.6% oil, 0.16% primary protein, various amounts of secondary protein, 5 mg/mL porcine bile extract, 20 mM $CaCl_2$, and 3.2 mg/mL porcine lipase. All additives were individually dissolved in phosphate buffer (5 mM, pH 7.0) before use. Lipase addition and initialization of the titration program were done only after the addition of all other predissolved ingredients and careful balancing of the pH to 7.00.

Experimental Design. All experiments were carried out at least in duplicates using freshly prepared samples, and the results are presented as the calculated mean and standard deviation.

RESULTS AND DISCUSSION

Currently, much attention has been given to establishing guiding principles for the design of interfacial structures to affect emulsion digestibility. This study explored how lactoferrin and sodium caseinate can be used to modulate interfacial properties and thereby alter lipid droplet stability and digestibility in simulated GIT conditions. The interfacial composition was varied by altering the order of addition and relative amounts of lactoferrin (LF) and caseinate (Cas) in the emulsions.

Formation of Secondary Emulsions. Initially, we examined the impact of adding a secondary protein to an emulsion containing lipid droplets initially stabilized by a primary protein at pH 7. At this pH, the LF is positively charged (p$I \approx 8.5$) and the caseinate is negatively charged (p$I \approx 4.5$) (*18*, *32*, *33*). Changes in interfacial composition were monitored by measuring changes in droplet surface charge (ζ-potential) when either LF was added to Cas-coated droplets or Cas was added to LF-coated droplets (**Figure 1**). In the absence of LF, the Cas-coated droplets were negatively charged at pH 7 ($\zeta = -56$ mV), because the adsorbed caseinate was above its isoelectric point. When increasing amounts of LF were added to this emulsion, the ζ-potential became progressively less negative and eventually became positive, indicating that either LF was incorporated into the interfacial layer with caseinate or that LF displaced the caseinate from the droplet surfaces. Our later experiments suggest that LF was incorporated into the interfacial layer, presumably as a secondary layer on top of the caseinate (see Influence of $CaCl_2$ on Droplet Charge and Stability). A relatively high amount of LF (between 8 and 9% w/w) had to be added to the Cas-stabilized emulsions to reach charge neutrality, and the droplets never reached a constant ζ-potential level indicative of saturation with the secondary protein (**Figure 1A**). Interestingly, these emulsions were stable to droplet aggregation and creaming at all LF levels (data not shown), even in the region where the particle charge was relatively low. This suggests that the emulsions were stabilized by steric repulsion, rather than by electrostatic repulsion (*34*).

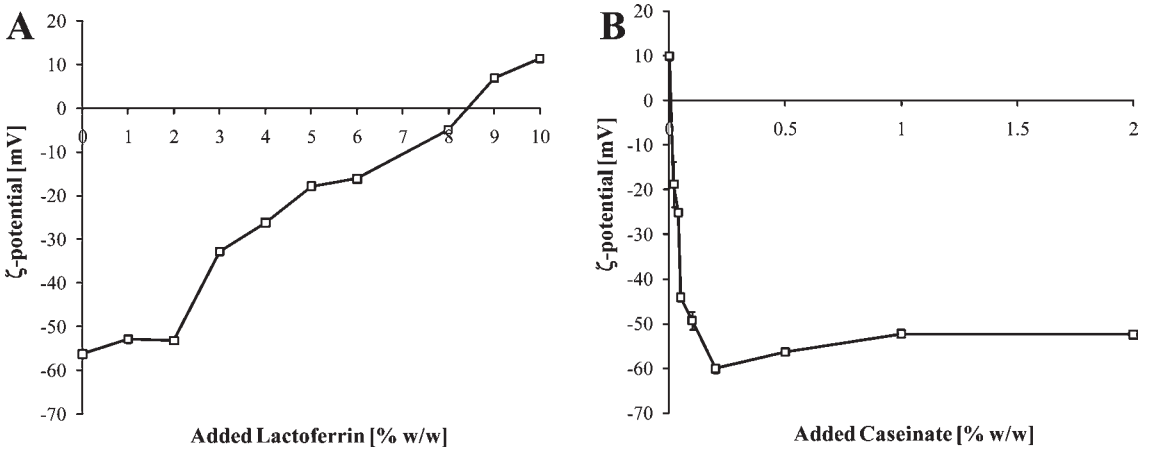

Figure 1. Electrokinetic charge (ζ-potential) of oil droplets coated with caseinate and then supplemented with lactoferrin (**A**) and of droplets coated with lactoferrin and then supplemented with caseinate (**B**). Error bars are given but may lay within data points.

In the absence of caseinate, the LF-coated droplets were positively charged at pH 7 ($\zeta = +10$ mV), because the adsorbed protein was below its isoelectric point (**Figure 1B**). When increasing amounts of caseinate were added to the emulsion initially containing LF-coated droplets, the ζ-potential became progressively less positive and eventually became negative, indicating that either the caseinate was incorporated into the original interfacial layer or that the caseinate displaced the LF from the droplet surfaces. The amount of caseinate required to reach charge neutrality (< 0.02% w/w) was much lower (**Figure 1B**) than the amount of LF required for the emulsions initially stabilized by caseinate (**Figure 1A**). The LF-Cas emulsions were also stable to droplet aggregation and creaming at all caseinate levels, even in the region where the particle charge was close to zero (data not shown), again suggesting that steric repulsion may be more important than electrostatic repulsion.

A number of studies have shown that ionic polysaccharides can adsorb onto the surfaces of oppositely charged protein-coated lipid droplets and cause charge reversal (*35*−*40*). Recent studies have shown that globular proteins can also adsorb onto the surfaces of oppositely charged protein-coated lipid droplets and promote charge reversal, for example, LF onto β-lactoglobulin-coated droplets (*12*, *13*). The fact that much less caseinate is required to saturate the LF-coated droplets (**Figure 1B**) than LF is required to saturate the Cas-coated droplets (**Figure 1A**) suggests that the driving force for caseinate adsorption is much greater than that for LF adsorption. Caseinate molecules have a flexible structure and relatively low molecular weight (≈20 kDa), whereas LF molecules have a globular structure and relatively high molecular weight (≈80 kDa). These differences in molecular characteristics may account for their different relative affinities for the droplet surfaces.

Changes in the organization and/or composition of the protein molecules at the oil droplet surfaces would be expected to alter emulsion physical stability and functionality. We therefore examined the influence of interfacial characteristics on the physical stability of the emulsions to environmental stressors (e.g., pH and calcium ions) and on lipid digestibility in the following sections. For the remainder of the experiments, the properties of primary emulsions were compared with those of secondary emulsions. Primary emulsion controls were formulated to contain 10% (w/w) oil and 1% (w/w) primary protein. Secondary emulsions were formulated so as to form emulsions with 10% oil (w/w), 1% (w/w) primary protein, and specific levels of secondary protein, which yielded surface charge values (zeta-potentials) of about −30 mV or lower without exceeding surface charge saturation. Such

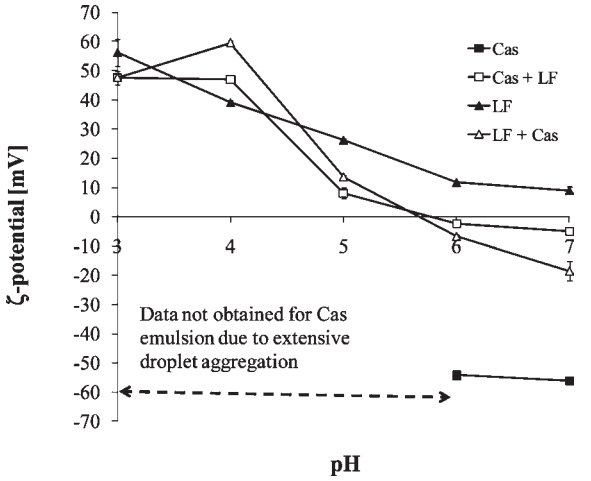

Figure 2. Electrokinetic charge (ζ-potential) of primary and secondary emulsions after 24 h of incubation in different pH conditions. Secondary emulsions were produced by mixing a primary emulsion with a solution of the second protein; for example, Cas-LF emulsion was a caseinate-stabilized emulsion mixed with a lactoferrin solution. Error bars are given but may lay within data points.

formulations are considered to be stable food emulsions (*41*) and are not likely to contain significant amounts of secondary protein in the continuous phase. The Cas-LF emulsions were formed by adding 8% (w/w) LF to Cas-stabilized emulsions containing 1% (w/w) Cas; thus, the final emulsion formulation was 10% (w/w) oil, 4% (w/w) LF, and 1% Cas. Similarly, LF-Cas emulsions were formulated to contain 10% (w/w) oil, 0.05% Cas, and 1% LF.

Influence of pH on Droplet Charge and Stability. Alterations in the sign and magnitude of particle charge occur when protein-coated oil droplets are exposed to different pH conditions, which usually alters their stability to aggregation and creaming (*41*, *42*). In this study, primary and secondary emulsions were incubated overnight at various pH conditions, and the droplet ζ-potential and particle size were measured (**Figures 2** and **3**). The Cas-coated oil droplets had a relatively high negative charged (−50 to −60 mV) at pH 6 and 7, which can be attributed to the fact that the adsorbed caseinate was above its isoelectric point ($pI \approx 4.6$). Reliable ζ-potential measurements could not be made for this emulsion at lower pH values because considerable droplet aggregation occurred (**Figure 3**). LF-coated oil droplets were positively charged across the entire pH range studied, with the

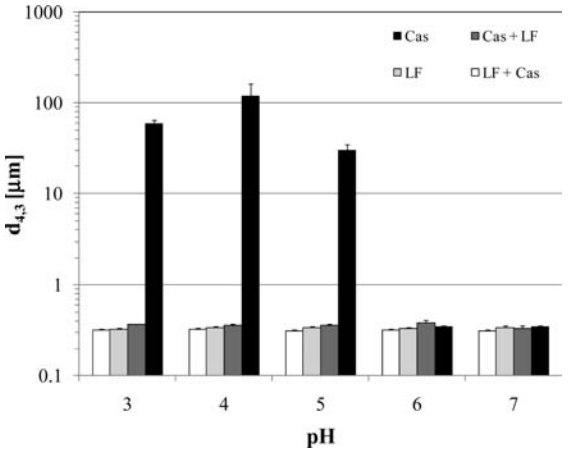

Figure 3. Static laser light scattering based volume weighed mean droplet size ($d_{4,3}$) of primary and secondary emulsions after 24 h of incubation in different pH conditions. Secondary emulsions were produced by mixing a primary emulsion with a solution of the second protein; for example, Cas-LF emulsion was a caseinate-stabilized emulsion mixed with a lactoferrin solution. Data are presented as duplicate means.

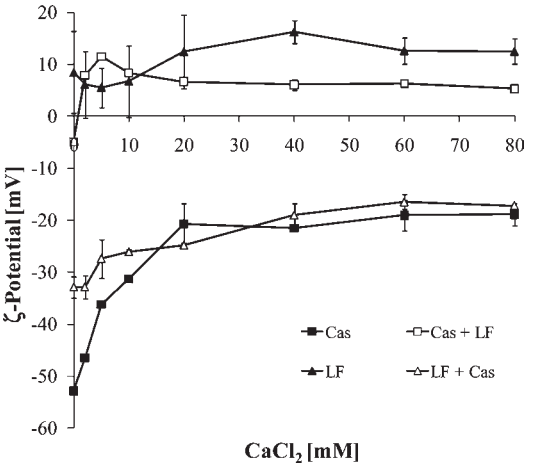

Figure 4. Electrokinetic charge (ζ-potential) of primary and secondary emulsions after 24 h of incubation in different concentrations of $CaCl_2$. Secondary emulsions were produced by mixing a primary emulsion with a solution of the second protein; for example, Cas-LF emulsion was a caseinate-stabilized emulsion mixed with a lactoferrin solution.

magnitude of the charge becoming more positive as the solution was decreased from pH 7 to 3, which can be attributed to the fact that the adsorbed LF was always below its isoelectric point (p$I \approx$ 8.5). The LF-coated droplets remained stable to droplet aggregation from pH 3 to 7 (**Figure 3**), which indicates that the overall repulsive interactions (mainly electrostatic and steric) dominated the overall attractive interactions (mainly van der Waals and hydrophobic) (*34*). The relatively high pI of LF meant that it was positively charged across the entire pH range, which meant there was always some level of electrostatic repulsion. In addition, LF has a relatively high molecular weight compared to other globular food proteins, which may increase the range of the steric repulsion between droplets. Finally, LF is a glycoprotein that has some sugar groups covalently attached to its peptide backbone (*43*), which may also increase the steric repulsion. In the future, it would be useful to carry out a theoretical analysis of the relative magnitude and range of the various kinds of colloidal interaction operating in the system (e.g., van der Waals, steric, electrostatic, and depletion). Nevertheless, more detailed information would be needed about the structural organization, composition, charge distribution, and thickness of the interfacial layers.

The pH dependence of the particle charge and stability of the secondary emulsions was appreciably different from that of the primary emulsions (**Figures 2** and **3**). The secondary emulsion formed by adding LF to Cas-coated oil droplets (Cas-LF emulsion) had a relatively low negative charge at pH 7 and 6, but became increasingly positively charged as the pH was decreased further (**Figure 2**). At pH 7, the fact that the droplets in the Cas-LF emulsion are much less negative than those in the Cas emulsion can be attributed to adsorption of cationic LF molecules to the droplet surfaces. As the pH is decreased, LF and caseinate become more positively charged or less negatively charged for caseinate at pH > pI. This would account for the increase in net positive charge on the particles when the pH was decreased. One would expect that there would be some electrostatic repulsion between the caseinate and LF molecules at the droplet surface once the pH became appreciably below the pI of the caseinate, because then both proteins would have a net positive charge. This would be expected to cause some of the protein molecules to become desorbed from the oil droplet surfaces; however, this could not be determined from the ζ-potential measurements. Interestingly, the Cas-LF emulsion was stable to droplet aggregation from pH 7 to 3 (**Figure 3**), even under conditions where the net particle charge was relatively low, for example, $\zeta = -5$ mV at pH 7. Again, this suggests that steric repulsion may play a more important role in stabilizing these emulsions against droplet aggregation than electrostatic repulsion.

The secondary emulsion formed by adding caseinate to LF-coated oil droplets (LF-Cas emulsion) had a fairly high negative charge (−19 mV) at pH 7, but became positively charged when the pH was decreased (**Figure 2**). At pH 7, the particles in the LF-caseinate emulsions were much more negative than those in the LF emulsions, which can be attributed to adsorption of anionic caseinate molecules onto the cationic LF-coated droplet surfaces. The increase in positive charge in the LF-Cas emulsions with decreasing pH can again be attributed to the changes in the electrical properties of the adsorbed LF and caseinate molecules associated with protonation of the carboxyl and amine groups. As mentioned earlier, one might expect that there would be some electrostatic repulsion between the caseinate and LF molecules at the droplet surface at pH values less than the pI of caseinate, which may have promoted some of the proteins to be desorbed from the droplet surfaces. The LF-Cas emulsions were also stable to droplet aggregation from pH 7 to 3 (**Figure 3**), suggesting that steric repulsion played an important role in stabilizing the oil droplets.

Influence of $CaCl_2$ on Droplet Charge and Stability. Calcium chloride, commonly used as an additive in food and drug formulations, often has destabilizing effects on caseinate and caseinate-stabilized emulsions, which can be attributed to its ability to bind to anionic protein groups and to reduce electrostatic interactions (*44*−*46*). This salt is also present in the human GIT and so is typically used in GIT in vitro digestion models. In this part of the study, the destabilizing effects of calcium chloride on the primary and secondary emulsions were studied by adding increasing levels of $CaCl_2$ to the emulsions and monitoring their ζ-potential and particle size distribution after overnight incubation at room temperature (**Figures 4** and **5**).

There were distinct differences in the influence of $CaCl_2$ addition on the physicochemical properties of the two primary emulsions. For the Cas emulsions, there was an appreciable change in the particle ζ-potential, from around −55 to −21 mV, when the $CaCl_2$ concentration was increased from 0 to 20 mM, which can be attributed to the binding of cationic Ca^{2+} ions to anionic groups on the caseinate molecules, as well as some

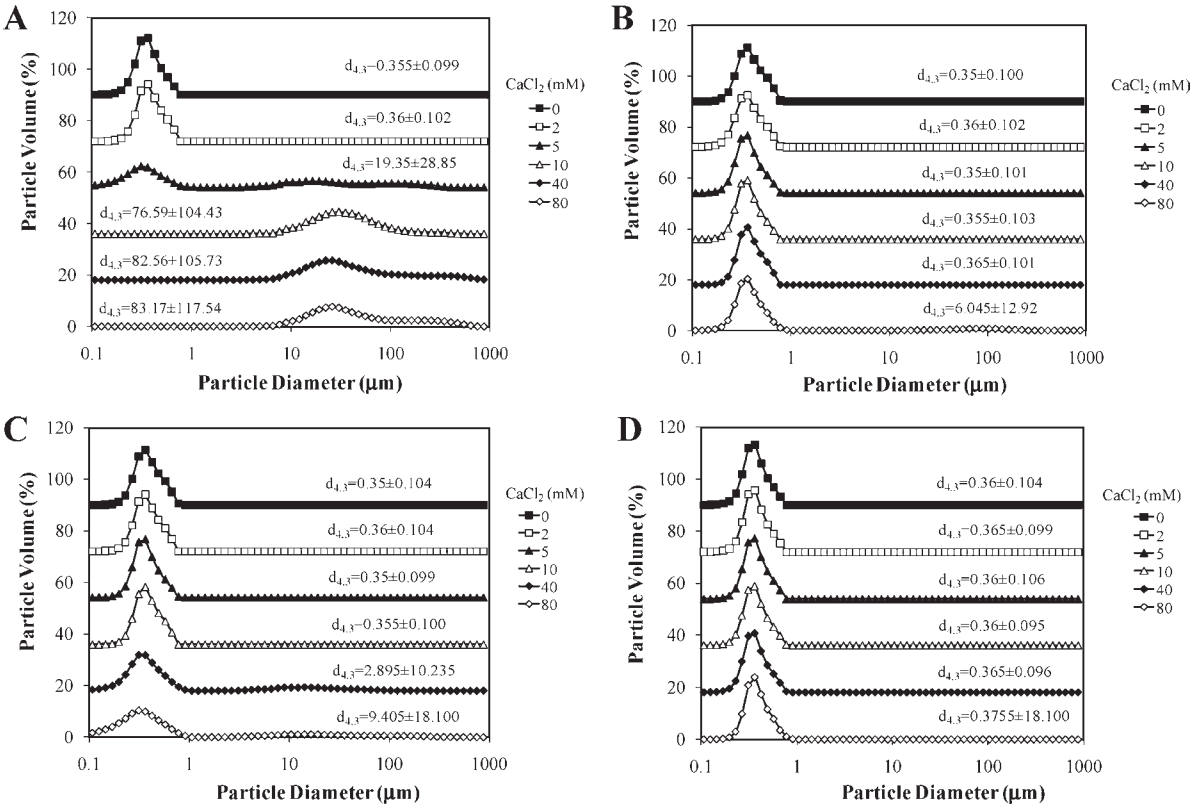

Figure 5. Static laser light scattering based particle size distribution curves (volume weighed) of primary and secondary emulsions after 24 h of incubation in different concentrations of $CaCl_2$: (**A**) Cas emulsion; (**B**) Cas-LF emulsion; (**C**) LF emulsion; (**D**) LF-Cas emulsion.

electrostatic screening effects (*34*). Addition of ≥5 mM $CaCl_2$ induced appreciable droplet flocculation as demonstrated by the appearance of a population of large particles in the particle size distribution (**Figure 5**). These results are in agreement with previous studies of the effects of calcium addition on the properties of caseinate-stabilized emulsions and can be attributed to droplet flocculation induced by charge neutralization and ion bridging effects (*44*). For the LF emulsions, there appeared to be slight changes in the positive charge on the droplets from around +8 to +13 mV (**Figure 4**), however without being statistically significant. Addition of counterions to an emulsion containing electrically charged droplets usually leads to a reduction in the magnitude of the ζ-potential due to electrostatic screening effects (*34*). The slight changes in the LF emulsion positive charge may therefore be attributed to weak binding of cationic Ca^{2+} ions to anionic groups (e.g., $-CO_2^-$) on the adsorbed LF molecules. Thus, although the net droplet charge of LF emulsions was relatively low (< +10 mV), slight aggregation was only observed at 40−80 mM $CaCl_2$, which can support the hypothesis that these emulsions were mainly stabilized by steric repulsion, rather than electrostatic repulsion. Accordingly, increasing $CaCl_2$ levels increased the electrostatic screening effects leading to the collapse of the protein structures around the droplets and consequently diminishing the stabilizing effects of steric repulsion between the droplets.

We also measured the influence of $CaCl_2$ on the electrical charge and stability of the droplets in the secondary emulsions (**Figures 4** and **5**). For the LF-Cas emulsions, there was a slight decrease in the magnitude of the negative charge on the droplets when the $CaCl_2$ was increased, which can be attributed to Ca^{2+} binding and electrostatic screening effects (*34*). The overall decrease in charge was less for the LF-Cas emulsions than for the Cas emulsions, which also had a relatively high initial negative charge (**Figure 4**). There was little change in the particle size distribution of the LF-Cas emulsions with increasing $CaCl_2$ concentration (**Figure 5D**), which indicates that these emulsions were also stable to calcium-induced flocculation even though they initially had a relatively high negative charge. A possible explanation for this phenomenon is that the emulsions were stabilized primarily by steric repulsion, rather than electrostatic repulsion. For the Cas-LF emulsions, the droplet charge went from slightly negative (−5 mV) in the absence of calcium to slightly positive (+5 mV) in the presence of high levels of calcium (**Figure 4**), which suggests that some Ca^{2+} ions bound to exposed anionic groups (e.g., $-CO_2^-$) on the adsorbed caseinate or LF molecules. These emulsions were relatively stable to droplet aggregation up to 10 mM $CaCl_2$, but showed some evidence of aggregation at higher calcium ion levels, with a small population of large particles being observed in the particle size distribution (**Figure 5B**).

Influence of Bile Salts on Droplet Charge and Stability. There is increasing interest in the design of emulsions to control the digestion and release of lipophilic substances in the GIT (*6*, *8*, *47*). It is therefore useful to examine the impact of bile salts, which are biological surface active substances, on the properties of protein-coated oil droplets. Emulsion samples were incubated with various levels of bile extracts for 2 h at 37 °C, and then their ζ-potential was measured. These experimental conditions have previously been shown to be sufficient for bile to exert its effect on droplet size and charge (*26*). ζ-Potential measurements can provide useful insights into the impact of experimental variables on the interfacial composition of oil droplets.

Measurements of the dependence of ζ-potential of primary and secondary emulsions on bile extract concentration are shown in **Figure 6**. It is important to note that no significant creaming or aggregation was observed for all emulsions after 24 h, which concurs with previous results (*26*, *48*). For Cas emulsions, the ζ-potential remained relatively constant at all bile levels studied

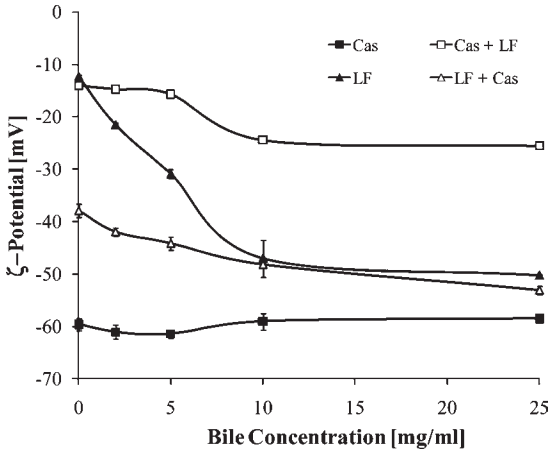

Figure 6. Changes in the electrokinetic charge (ζ-potential) of primary and secondary emulsions after 2 h of incubation at 37 °C with various levels of bile. All values are expressed as duplicate means. Error bars are given but may lay within data points.

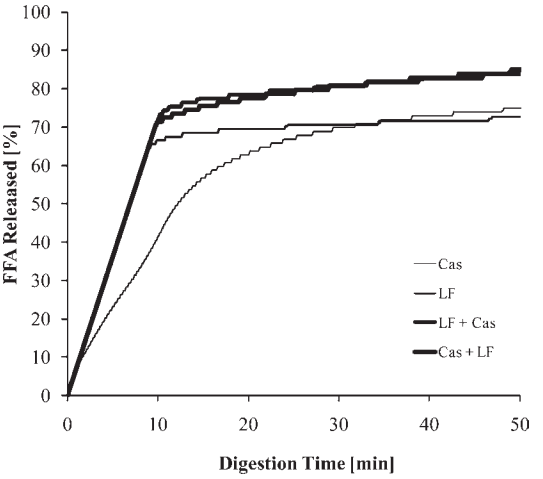

Figure 7. Timed release of free fatty acids from the various emulsion formulations by pancreatic lipase measured in a pH-stat in vitro digestion model.

(0−25 mg/mL), which may suggest that either the caseinate molecules were not displaced from the droplet surfaces or that bile-coated oil droplets had a similar ζ-potential. Indeed, previous work (*48*) has shown the ζ-potential of bile-coated oil droplets is −54 mV, which suggested that caseinate displacement by bile can hardly be detected through ζ-potential measurements. For LF emulsions, the ζ-potential went from slightly negative (−12 mV) to highly negative (−50 mV) when the bile concentration was increased from 0 to 25 mg/mL (**Figure 6**), which suggests that bile molecules adsorbed to the droplet surfaces. Interestingly, these results indicate LF emulsion had an unexpected slight negative charge unlike the corresponding emulsion produced in double-distilled water. As divalent ions are known to closely interact with proteins in solution, one could hold that the apparent negative droplet charge could arise from the binding of phosphate ions (present in the buffer) to the LF on the droplet surface; however, further experiments should establish such a hypothesis. The bile molecules could adsorb to the droplet surfaces in a number of ways: (i) they could partially or fully displace the original LF molecules from the droplet surfaces; (ii) they could adsorb between the LF molecules; or (iii) they could form a secondary layer on top of the layer of LF molecules. At present, the precise physicochemical mechanism is currently unknown and requires further studies using a variety of analytical instruments to measure changes in surface composition and structure.

The impact of bile concentration on the ζ-potential of the droplets in the secondary emulsions was also measured. For both Cas-LF and LF-Cas emulsions, the droplets became slightly more negatively charged as the bile concentration increased, which suggested that some anionic bile salts adsorbed to the droplet surfaces. Nevertheless, the final ζ-potential was appreciably less negative than droplets coated only by bile salts, which indicates that the proteins were not fully displaced from the droplet surfaces. These results indicate that there are distinct differences between the behaviors of protein-coated lipid droplets to bile salts, depending on the nature of the proteins at the oil−water interface.

In Vitro Digestibility of Oil Droplets by Lipase. Finally, we examined the impact of interfacial properties on the in vitro digestion of the emulsified oils in simulated GIT conditions. Practically, it is useful to know if a delivery system adversely affects the bioavailability of encapsulated substances by preventing their release during digestion or if a delivery system can be designed to control the release of encapsulated substances at different loci along the GIT (*6*, *8*, *47*). An in vitro digestion model was therefore used to monitor the impact of interfacial properties on the lipase-induced digestion of the emulsified oil (**Figure 7**). The lipase-catalyzed digestion reaction was followed by measuring the release of FFA from emulsions during lipolysis (*11*, *28*).

All four emulsions were digested relatively rapidly in the in vitro digestion model, with the majority of the lipids being digested within the first 10−20 min (**Figure 7**). These results suggest that the lipase molecules were able to access the emulsified lipids in all emulsion systems and catalyze the conversion of triacylglycerols into FFA and monoacylglycerols. Nevertheless, there were some differences between the extent and digestion rates of the different emulsions, with the Cas emulsion being digested to a slightly smaller extent and at a somewhat slower rate than the other emulsions. The lipase digestion of the Cas emulsions may have been delayed for the following reasons: (i) the adsorbed caseinate layer may have retarded the adsorption of bile salts and/or lipase to the droplet surfaces, thereby restricting the access of the enzyme to the substrate; (ii) the droplets in these emulsions were highly susceptible to flocculation at the calcium level (20 mM $CaCl_2$) used in the in vitro digestion model (**Figure 5**); hence, the enzyme had to diffuse between the outer droplets of the flocs before it could reach the inner droplets and digest them. Nevertheless, these results suggest that within the typical time taken for a food to pass through the small intestine (≈2 h) the lipids in all of the emulsions should be digested and released. However, recent studies (*30*, *49*) show the passage of emulsions through the stomach involves physical destabilization of emulsions and consequently is expected to affect the formulations reaching the small intestine for lipid digestion. Further work is needed to elucidate the physicochemical changes emulsion formulations undergo as the pass through the GIT.

Conclusions. This study focused on understanding the properties of oil-in-water emulsions containing caseinate and/or lactoferrin at the droplet interface. Emulsions containing caseinate and lactoferrin at the oil−water interface could be prepared and had good stability to pH (3−7) and calcium (0−80 mM) addition. Nevertheless, the oil droplets in these emulsions could still be digested by lipase in an in vitro digestion model, which has important implications for the formulation of delivery systems to encapsulate, protect, and deliver lipophilic functional components. Overall, this works highlights the possibility of utilizing more than one protein component to enhance the physical stability of lipid droplets in emulsions.

ABBREVIATIONS USED

Cas, caseinate; LF, lactoferrin; LbL, layer by layer; GIT, gastrointestinal tract; FFA, free fatty acids.

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