Pharm Res (2013) 30:3200–3213
DOI 10.1007/s11095-013-1003-x

RESEARCH PAPER

Potential Biological Fate of Emulsion-Based Delivery Systems: Lipid Particles Nanolaminated with Lactoferrin and β-lactoglobulin Coatings

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Received: 20 July 2012 / Accepted: 6 February 2013 / Published online: 6 March 2013

ABSTRACT

Purpose To develop lipid particles coated by nanolaminated protein coatings as potential oral delivery systems to encapsulate, protect, and deliver lipophilic bioactives.

Methods Nanolaminated protein coatings were formed by sequential electrostatic deposition of cationic lactoferrin (LF) and anionic β-lactoglobulin (BLG) at pH6.5: LF, LF-BLG, LF-BLG-LF, and LF-BLG-LF-BLG. Changes in physicochemical properties were characterized after exposure to environmental stresses (pH2 to 9; 0 to 200 mM NaCl) and simulated gastrointestinal tract (GIT) conditions (mouth, stomach, small intestine). Triglyceride digestion and β-carotene bioaccessibility were also measured.

Results The pH and salt dependence of the electrical charge and aggregation stability of the emulsions were strongly influence by the structure of the interfacial coatings. All emulsions behaved similarly under simulated GIT conditions: extensive droplet aggregation occurred in the stomach and small intestine; triglycerides were rapidly and fully digested after exposure to intestinal fluids; the bioaccessibility of β-carotene was low (< 4%).

Conclusions Nanolaminated protein coatings may be useful for stabilizing encapsulated lipids in functional food and pharmaceutical products during storage, but releasing them after ingestion. Protein coatings had little impact on triglyceride digestion, but they greatly reduced β-carotene bioaccessibility, possibly due to binding to lactoferrin.

KEY WORDS digestion · lactoferrin · β-lactoglobulin · layer-by-layer · multilayer emulsions · nanolaminated · oral delivery systems · stability

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INTRODUCTION

There is currently great interest within the pharmaceutical and food industries in the development of delivery systems to encapsulate, protect, and release lipophilic bioactive components, such as many pharmaceuticals and nutraceuticals (1–6). Consumption of these bioactive lipids has been reported to reduce the incidence of chronic human diseases, such as heart disease, diabetes, and cancer. A promising nano-scale approach that can be used to fabricate delivery systems suitable for oral ingestion of bioactive components is electrostatic layer-by-layer (LbL) deposition (7, 8). This approach relies on sequential deposition of ionic polymers onto oppositely charged colloidal particles, leading to the formation of a particle core surrounded by a nanolaminated polymer shell (9–12). Multilayer emulsions can be produced based on this principle using a multiple-step process (Fig. 1). A "primary" emulsion containing lipid droplets coated with a layer of ionic emulsifier molecules is formed using conventional homogenization procedures. A "secondary" emulsion is then formed by electrostatically depositing an oppositely charged polymer onto the charged lipid droplet surfaces. This latter procedure can be repeated a number of times using two or more ionic polymers to fabricate droplets with multiple layers of laminated polymer coatings (9, 13). The successful utilization of this approach requires careful control of system composition and preparation conditions so as to avoid droplet flocculation due to bridging and depletion mechanisms (9, 11, 12)

Numerous studies have shown that the LbL approach can be used to improve the stability of lipid droplets to environmental stresses, such as pH, ionic strength, thermal processing, freezing, and dehydration (9, 10, 14, 15). Multilayer coatings have also been used to improve the chemical stability of labile encapsulated components, e.g., to retard oxidation of polyunsaturated lipids and carotenoids (16–18).

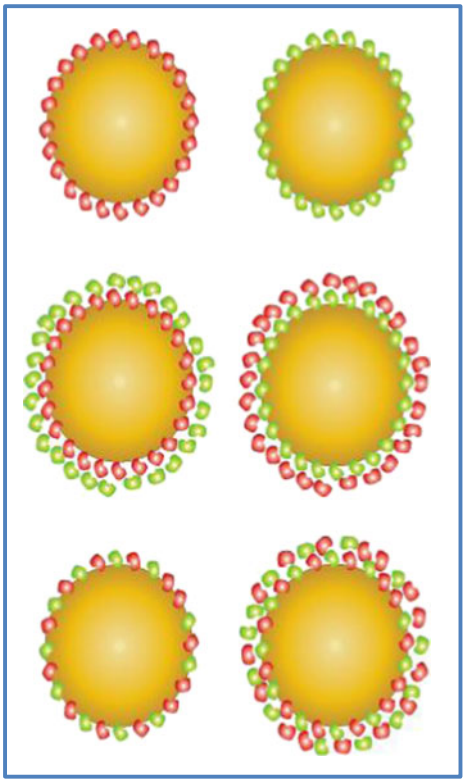

Fig. 1 Possible emulsion types that can be formed using two different types of proteins: (i) mixed emulsions containing two different protein-coated droplet types: (ii) laminated droplets with different interfacial structures; (iii) mixed emulsions with one or more layers.

Recent studies suggest that biopolymer coatings may also alter the fate of lipid droplets within the gastrointestinal tract (GIT), e.g. digestion, release, and absorption (17, 19–22). The influence of polymer coatings on the fate of lipophilic components within the GIT may have either beneficial or detrimental consequences. The ability to control the digestion and release of lipids within the GIT may have advantages for applications such as targeted release of bioactive components to specific regions of the GIT or for inducing satiety through the ileal brake mechanism (23, 24). Conversely, if a polymer coating reduces the release or absorption of a lipophilic component within the GIT, then it may reduce its overall bioactivity, which would be undesirable.

Most previous studies on the development and application of multilayer emulsions suitable for oral ingestion have utilized ionic dietary fibers as the polymers to coat the lipid droplets, such as chitosan, pectin, carrageenan, and alginate (17, 19–21). In the current study, we utilized two globular proteins with different electrical characteristics to form the nanolaminated coatings: lactoferrin (LF) and β-lactoglobulin (BLG). LF is a glycoprotein of the transferrin family, derived from milk and other mammalian fluids. It has an unusually high isoelectric point (pI ~ 8), and therefore tends to be cationic at neutral pH whereas most other major dairy globular proteins are anionic (25, 26). BLG is a globular whey protein derived from the milk of cows and other mammals. It has an isoelectric point (pI) close to 5, which means that it is negatively charged at neutral pH (26). Hence, lipid droplets can be coated with multiple layers of globular proteins by successive electrostatic deposition of anionic BLG molecules and cationic LF molecules at pH values around neutral. The ability to control the composition and structure of the protein coatings around the lipid droplets may be advantageous for a number of reasons. A high local concentration of proteins at lipid droplet surfaces may inhibit surface reactions such as lipid oxidation or digestion. Changing the nature of the outer protein layer will alter droplet charge, which may be important for developing delivery systems that do not interact with anionic ingredients in commercial products that promote precipitation or with anionic mucin molecules in saliva that cause astringency (27, 28). Alternatively, controlling the droplet charge may be important for promoting interactions with anionic biological surfaces in the GIT, e.g., to increase the retention time of bioactive components through mucoadhesion (29).

In this study, we formed nanolaminated coatings around lipid droplets by sequential electrostatic deposition of cationic LF and anionic BLG. Primary emulsions stabilized by a single protein layer (LF), secondary emulsions with two layers (LF-BLG), tertiary emulsions with three layers (LF-BLG-LF), and quaternary emulsions with four layers (LF-BLG- LF-BLG) were prepared by the LbL method. The influence of environmental stresses (pH and ionic strength) and simulated gastrointestinal conditions (mouth, stomach and small intestine) on the physicochemical properties of the nanolaminated lipid particles was then determined. We also studied the influence of the protein coating properties on the rate and extent of triglyceride digestion in the small intestine phase, and on the bioaccessibility of an encapsulated lipophilic component (β-carotene). The term "bioaccessibility" is defined as the fraction of the bioactive component that is solubilized within the mixed micelle phase after digestion of the lipids by lipase in the small intestine. It is assumed that only that fraction of the bioactive component that is solubilized within this mixed micelle phase will be available for absorption. The results from this study should provide useful information for the design of emulsion-based delivery systems to encapsulate and deliver lipophilic bioactive ingredients.

MATERIALS AND METHODS

Materials

Corn oil was purchased from a commercial food supplier (Mazola, ACH Food Companies, Inc., Memphis, TN) and

stored at 4°C until use. Food grade lactoferrin (Lot 10373317) was supplied by FrieslandCampina Domo (Delhi, NY), and the manufacturer reported that it contained 97.7% protein and 0.12% ash. Powdered β-Lg was obtained from Davisco Foods International (Lot #JE 001-0-415, Le Sueur, 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. Porcine bile extract, type II porcine pancreatic lipase, β-carotene, sodium chloride (NaCl), analytical grade hydrochloric acid (HCl) and sodium hydroxide (NaOH) were purchased from Sigma. The pancreatic lipase used was reported to have an activity of 100–400 units/mg protein (using olive oil (30 min incubation)) and 30–90 units/mg protein (using triacetin) by the supplier. Double distilled water was used to make all solutions.

Primary Emulsion Preparation

Aqueous emulsifier solutions were prepared by dissolving powdered LF (0.4% w/w) or BLG (0.8% w/w) in double distilled water and stirring for 3 h. The LF solution had to be filtered to remove some insoluble particles. Corn oil was added to each protein solution so that the final system contained: 2% (w/w) oil; 0.4% (w/w) LF or 0.8% (w/w) BLG; with the remainder being double distilled water. This mixture was homogenized for 2 min using a hand blender (Tissue Tearor, model 985379–395, Biospec Products Inc.) and then passed 3 times through a high pressure homogenizer (Microfluidizer M-110 L processor, Microfluidics Inc., Newton, MA) operating at 11,000 psi (75.8 MPa). For the bioaccessibility studies, a lipophilic bioactive compound (0.5% (w/w) β-carotene) was incorporated into the oil phase prior to homogenization.

Establishment of Optimum Fabrication Conditions

Initially, a series of experiments was carried out to determine the optimum protein concentrations to use to form the nanolaminated coatings in each of the multilayer emulsions. In particular, we used ζ-potential and particle size measurements to determine the minimum amount of protein required to cover the lipid droplet surfaces, without having large amounts of excess protein in the continuous phase and without promoting extensive droplet aggregation. A primary emulsion containing 2% (w/w) oil and 0.4% (w/w) LF was prepared as described in the previous section. Solutions containing different BLG concentrations (0 to 2% w/w) were added to this emulsion (1:1 ratio) to form secondary emulsions. These emulsions were then mixed using a magnetic stirrer and heated at 80°C for 30 min to crosslink the adsorbed proteins. Previous studies have shown that adsorbed BLG molecules form covalent cross-links with each other when they are heated above their thermal denaturation temperature due to disulfide bond formation (30). The secondary emulsion with the highest concentration of BLG (1% w/w) was then mixed with solutions (1:1 ratio) containing different amounts of LF (0 to 2% w/w) to form tertiary emulsions. The tertiary emulsion with the highest LF concentration (1% w/w) was mixed with solutions (1:1 ratio) containing different BLG concentrations (0 to 2% w/w) to form quaternary emulsions. These emulsions were heated at 80°C for 30 min to crosslink the adsorbed proteins. At each stage of protein addition the samples were analyzed for particle size and ζ-potential as described later.

The protein concentrations that gave saturation coverage and little particle aggregation were used to prepare the various multilayer emulsions in the remainder of the studies. To compare measurements made on different emulsion systems the data was plotted as a function of the protein-to-oil ratio (POR).

Multilayer Emulsion Preparation

Secondary Emulsion

A secondary emulsion containing 2% (w/w) oil, 0.4% (w/w) LF, 1% (w/w) BLG was prepared by mixing 2% (w/w) BLG solution with primary LF emulsion (4% w/w oil, 0.8% w/w LF) at a 1:1 mass ratio. This BLG concentration was selected from the previous protein addition experiment as it produced a stable emulsion. For the environmental stress tests, the secondary emulsions were heated at 80°C for 30 min to cross-link LF and BLG layers.

Tertiary Emulsion

A secondary emulsion containing 4% oil, 0.8% LF, 2% BLG was mixed with 4% LF solution in a 1:1 mass ratio to produce a tertiary emulsion with 2% oil, 0.4% LF, 1% BLG, and 2% LF. Heating was avoided at this stage since LF was thought to form the outer layer in the protein coating, and it becomes thermally denatured at temperatures higher than 60°C thereby undergoing aggregation.

Quaternary Emulsion

This emulsion contained 2% oil, 0.4% LF, 1% BLG, 2% LF, 2% BLG and was produced by mixing a tertiary emulsion (4% oil, 0.8% LF, 2% BLG, 4% LF) with 4% BLG solution at a 1:1 mass ratio. For the environmental stress tests, quaternary emulsions were heated at 80°C for 30 min to cross-link LF and BLG layers.

Particle Characterization

Particle size distributions were determined using a dynamic light scattering (DLS) instrument (Zetasizer Nano ZS series, Malvern Instruments, Worcestershire, U.K.). Emulsion samples were diluted in pH-adjusted double distilled water at a ratio of 1:200 (v/v) and then placed in a capillary test tube that was loaded into the instrument. Particle sizes were reported as the Z-average mean diameter calculated from the particle size distribution. In some cases, extensive droplet aggregation occurred and the emulsions were too large to analyze using dynamic light scattering. For these samples, the particle size was measured using static light scattering after dilution with appropriate buffer solution (MasterSizer 2000, Malvern Instruments, Worcestershire, U.K.). The electrical charge (ζ-potential) of the particles was determined using electrophoretic mobility measurements on diluted emulsion samples (Zetasizer Nano ZS series, Malvern Instruments, Worcestershire, U.K.). Emulsion samples, diluted in double distilled water (1:200 v/v) at the appropriate pH, were placed in a capillary test tube that was loaded into the instrument. Samples were equilibrated for 1 min inside the instrument before data was collected over at least 10 sequential readings and processed using the Smoluchowski model.

Stability to Environmental Stresses

Emulsions were subjected to a number of environmental stresses (pH and/or ionic composition,) and then their physicochemical properties were characterized. The influence of pH on emulsion stability was determined by preparing samples with aqueous phases adjusted to pH2 to 9 using either HCl or NaOH. The influence of ionic strength on emulsion stability was examined by adding 0 to 200 mM NaCl to the emulsions after preparation (pH7.0).

Behavior in Simulated Gastrointestinal Tract

Emulsions containing one, two, three, or four protein layers were formed as described above, except that the heating step was not used to cross-link the protein layers. This step was omitted to simplify the preparation conditions for the structured emulsions used in the simulated GIT model. We did not observe any major changes in the initial size or charge of the lipid droplets coated by different protein layers with or without cross-linking. All emulsions were then passed through a full simulated GIT model that consisted of mouth, gastric, and small intestines phases. The rate and extent of lipid digestion was measured during the small intestine phase using a pH-stat method. Measurements of the microstructure, particle size distribution, and particle charge were measured at each stage.

Oral Stage

Artificial saliva (pH6.8) containing 3% mucin was prepared according to the composition reported previously (31). The *in vitro* oral model consisted of a conical flask (125 mL) containing artificial saliva maintained at 37°C with continuous shaking at 100 rev min^{-1} 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 initial emulsion (2% fat) was mixed with artificial saliva (ratio 1:1 w/w). The resulting mixture contained 1% (w/w) oil and was taken for characterization at the end of the incubation period.

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 pH1.2 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 the final mixture contained 0.5% (w/w) oil. This mixture was then adjusted to pH2.5 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 mgmL^{-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 (pH7.0) before use. Pancreatic lipase solutions were prepared 2 h prior to the experiments by dissolving powdered lipase into distilled water. 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 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:

$$V_{max} = 2 \times \left[\frac{m_{oil}}{MW_{oil}} \times \frac{1000}{C_{NaOH}} \right] \quad (1)$$

$$\%\text{FFA Released} = \frac{V_{Exp}}{V_{Max}} \times 100\% \quad (2)$$

Here, m_{oil} is the total mass of oil present in the reaction vessel (g), MW_{oil} is the molecular weight of the oil (g per mol), C_{NaOH} is the concentration of sodium hydroxide in the titration burette (mol per 1000 cm^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.

Bioaccessibility Determination

Bioaccessibility is defined as the fraction of the bioactive component that is solubilized within the mixed micelle phase after digestion of the lipids by lipase in the small intestine. The bioaccessibility of a model lipophilic bioactive molecule (0.5% β-carotene per 100 g of oil phase) in the different emulsions was determined. After *in vitro* digestion, 10 ml of sample were collected and a portion was centrifuged (4000 rpm, Thermo Scientific, CL10 centrifuge) at 25°C for 40 min. Centrifuged samples separated into an orange-white sediment layer at the bottom and a clear solution at the top. The upper phase was assumed to contain mixed micelles capable of solubilizing the bioactive molecule. Aliquots (5 mL) were collected directly from the total digesta or from the micelle phase of centrifuged samples. A syringe filter (0.45 μm) was used to filter the micelle phase to remove any residual large particles. Aliquots were vortexed with 5 mL chloroform and centrifuged at 1750 rpm at 25°C for 10 min. The bottom chloroform phase was collected while the top layer was vortexed with another 5 mL of chloroform and centrifuged at 1750 rpm for another 10 min. The bottom chloroform phase was added to the first chloroform phase, mixed and analyzed using a UV-visible spectrophotometer (Ultrospec 3000 pro, GE Health Sciences, USA) at 450 nm. A cuvette containing pure chloroform was used as a reference cell to zero the spectrophotometer.

The concentration of β-carotene extracted from a sample was determined from a calibration curve of absorbance *versus* β-carotene concentration in chloroform. The bioaccessibility was calculated using the following equation:

$$Bioaccessibility(\%) = \frac{C_{micelle\ Phase}}{C_{Total\ Digesta}} \times 100\% \quad (3)$$

Here, $C_{Micelle\ Phase}$ is the concentration of the bioactive component determined in the micelle phase, and $C_{Total\ Digesta}$ is the concentration determined in the total digesta.

Emulsion Microstructure

Confocal imaging of emulsion microstructures was carried out with a 60× oil immersion objective lens using a confocal microscope (C1 Digital Eclipse, Nikon) at ambient temperature. Samples were imaged at various stages of digestion: (i) immediately after preparation, (ii) after 15 min incubation at pH6.8 in simulated saliva (iii) after 2 h incubation at pH2.5 in SGF, and (iv) after 2 h incubation at pH7 in SIF. 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.

Statistical Analysis

All experiments were performed at least twice on newly prepared samples, with two to three measurements being made per sample. Results are reported as averages and standard deviations of these measurements.

RESULTS AND DISCUSSION

In principle, lipid-based delivery systems with a variety of different structures can be formed using two or more different globular proteins as emulsifiers (Fig. 1). In this study, we utilized the LbL electrostatic deposition method to prepare lipid droplets coated by nanolaminated protein layers. We then determined the influence of these nanolaminated coatings on the properties of lipid droplets exposed to different environmental stresses and *in vitro* digestion conditions.

Formation of Nanolaminated Lipid Particles

These experiments were carried out at pH6.5 to ensure that the β-lactoglobulin molecules were negative (pH > pI) and the lactoferrin molecules were positive (pH < pI), so that there would be an electrostatic attraction between them.

Primary Emulsions

The LF-coated droplets in the primary emulsion had a high positive charge (≈ +39 mV), which can be attributed to the fact that the pH was well below the pI of LF (32). Particle size measurements indicated that the primary emulsions contained small droplets that were stable to aggregation (d=179 nm), which can be attributed to strong electrostatic and steric repulsion between the LF-coated lipid droplets (32, 33). LF has a high molecular weight (≈ 80 kDa) and has hydrophilic carbohydrate groups covalently attached to its polypeptide backbone, leading to the formation of a thick hydrophilic interfacial layer that generates a strong steric repulsion (33). In addition, at pH values where the adsorbed protein layer has an appreciable charge there will also be a strong electrostatic repulsion between the droplets.

Secondary Emulsions

Secondary emulsions were formed by mixing the primary emulsions with β-lactoglobulin solutions. Addition of increasing amounts of BLG to the primary emulsions caused the electrical charge to change from highly positive (≈ +39 mV at 0% BLG) to highly negative (≈ -54 mV at 2% BLG), which suggested that anionic BLG molecules adsorbed onto the surfaces of cationic LF-coated droplets (Fig. 2a). The addition of 0.25% BLG to the primary emulsions (POR=0.325) led to an appreciable increase in particle size (Fig. 2b), which was attributed to droplet aggregation caused by charge neutralization and electrostatic bridging effects (34). The addition of 2% BLG (POR=1.2) to the primary emulsions led to a mean particle diameter of the secondary emulsions (d=182.3 nm) that was only slightly larger than that of the primary emulsions (d=178.5 nm), suggesting that the β-lactoglobulin molecules formed a thin coating (thickness≈1.9 nm) around the LF-coated lipid droplets. This dimension is similar to the diameter of a β-lactoglobulin molecule in solution and similar to the thickness of β-lactoglobulin monolayers formed at interfaces (35, 36).

Tertiary Emulsions

Tertiary emulsions were formed by mixing lactoferrin solutions with secondary emulsions. The ζ-potential measurements indicated that the electrical charge on the droplets changed from −52.8 to −5.1 mV when the amount of LF added was increased from 0 to 2% (POR=1.2 to 3.2) (Fig. 2a). These results suggest that cationic LF molecules electrostatically adsorbed onto the surfaces of the anionic LF-BLG-coated lipid droplets forming a third protein layer. It is interesting to note that the droplets did not become positively charged even at the highest levels of LF used. In contrast, studies have shown that charge reversal (from negative to positive) occurs when high levels of a cationic polyelectrolyte (such as chitosan) are added to an emulsion containing anionic lipid droplets (37, 38). The difference in behavior of these two cationic biopolymers may be

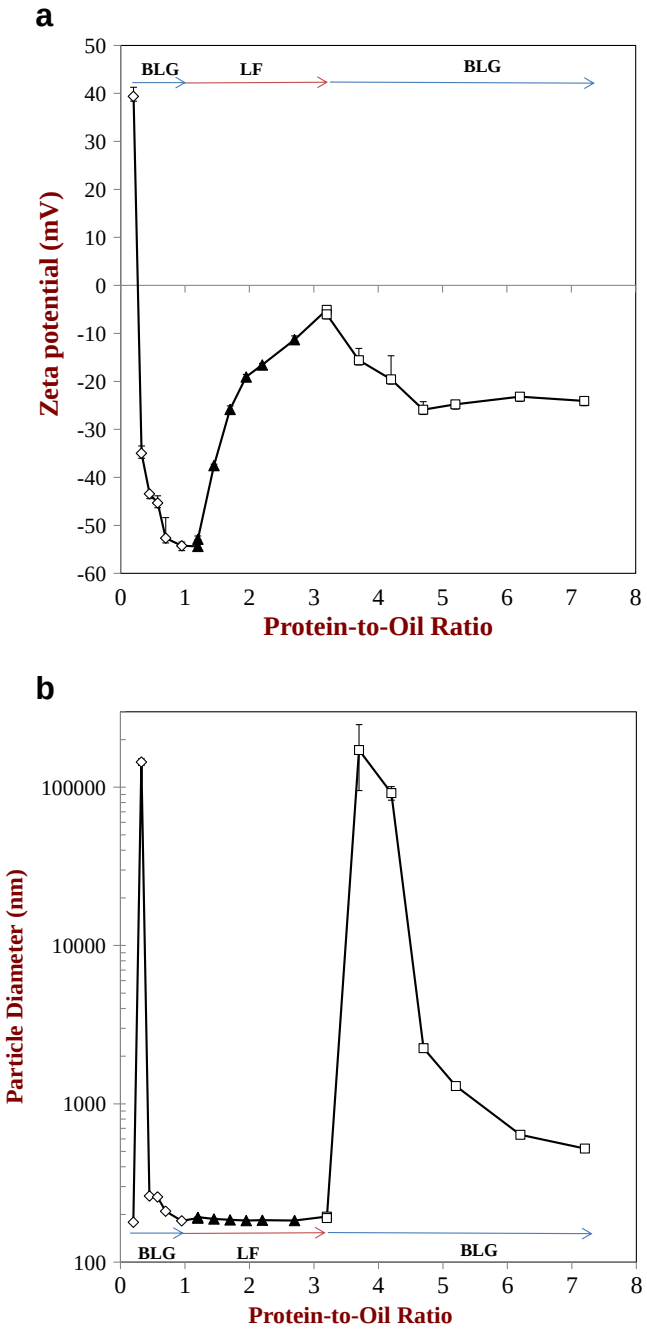

Fig. 2 (**a**) Dependence of particle ζ-potential on the protein-to-oil ratio when increasing amounts of BLG or LF are added to oil-in-water emulsions initially containing LF-coated lipid particles. (**b**) Dependence of mean particle diameter on the protein-to-oil ratio when increasing amounts of BLG or LF are added to oil-in-water emulsions initially containing LF-coated lipid particles. The particle sizes were measured by dynamic (d < 10,000 nm) or static (d > 10,000 nm) light scattering.

attributed to differences in their molecular structures: chitosan is a polyelectrolyte with a flexible open structure, whereas LF is a globular protein with a rigid compact structure. When chitosan adsorbs to an anionic surface some of its positively charged groups bind to anionic surface groups but others dangle into the surrounding aqueous phase, thereby leading to an overall net positive charge (37). When LF adsorbs to an anionic surface it adopts an orientation where the majority of cationic groups face the anionic surface and are neutralized by anionic groups. The side of the LF molecules exposed to the aqueous phase may have a mixture of cationic and anionic groups on its surface leading to little net charge. In addition, there may have been some steric hindrance effects reducing the ability of the LF molecules to pack into a uniform monolayer. Interestingly, the mean particle diameters of all the tertiary emulsions were found to be fairly similar ($d \sim 190$ nm) indicating that the droplets were stable to aggregation and that a thin layer of LF molecules (thickness≈4 nm) was formed around the droplets (Fig. 2b). This could be due to the ability of LF molecules to increase the repulsive interactions between the droplets, through a combination of electrostatic and steric repulsion, thereby inhibiting droplet aggregation (as discussed above).

Quaternary Emulsions

Quaternary emulsions were formed by mixing tertiary emulsions with β-lactoglobulin solutions. The droplet charge became more negative as the concentration of BLG added to the systems increased, reaching a relatively steady value above 0.75% BLG (POR = 4.7) (Fig. 2a). This increase in negative change on the droplets suggests that anionic BLG molecules adsorbed to the surfaces of anionic LF-BLG-LF-coated lipid droplets forming a fourth protein layer. The BLG molecules may have adsorbed onto cationic patches on the surfaces of the LF molecules, and has been reported for various globular protein and polyelectrolyte systems (39). When increasing amounts of BLG were added to the tertiary emulsions there was a large increase in particle diameter with a maximum around POR≈3.7, which indicated that extensive droplet aggregation had occurred (Fig. 2b). The origin of this effect is probably a bridging mechanism, i.e., BLG molecules were shared between more than two or more droplets (34). As the protein-to-oil ratio was increased further the particle size decreased, but it was still considerably higher than the initial tertiary emulsion. For example, the mean particle diameter of the quaternary emulsions with the highest BLG concentration was around 520 nm, compared to 190 nm for the initial tertiary emulsions. The particles in the quaternary emulsions may therefore exist as microclusters of a number of droplets, rather than as individual droplets.

This series of experiments enabled us to establish the optimum protein concentrations for preparation of primary, secondary, tertiary and quaternary emulsions for the environmental stress and digestion studies (see "Materials and Methods" section).

Influence of pH on Particle Stability

Emulsion-based delivery systems may be incorporated into commercial products with different pH values, and they experience varying pH conditions as they pass through the mouth, stomach, and small intestine after ingestion. It is therefore important to establish the influence of pH on the stability and physicochemical properties of nanolaminated lipid particles. Primary, secondary, tertiary, and quaternary emulsions were prepared as described previously, adjusted to pH values from 2 to 9, and then analyzed.

The ζ-potential of the primary emulsion containing LF-coated droplets decreased from +67.4 to −21.6 mV when the pH was increased from 2 to 9, with the point of zero charge being around pH 8, which is close to the reported isoelectric point of LF (40). Although the ζ-potential would be expected to increase in magnitude as the pH was decreased from 3 to 2 (due to increasing ionization of positive charges), the measured ζ-potential actually become less positive. This phenomenon can be attributed to the increase in ionic strength associated with the increase in free chloride ion (Cl^-) concentration when HCl is added to reduce the pH. The chloride ions will generate an electrostatic screening effect that reduces the measured ζ-potential (41). A similar behavior was observed in all the other emulsions studied, i.e., the magnitude of the ζ-potential was higher at pH 3 than 2. A primary emulsion containing BLG-coated droplets was also analyzed for the sake of comparison. The ζ-potential of this emulsion went from +46 mV at pH 2 to −72 mV at pH 9 (Fig. 3). The point of zero charge was around pH 4.5 which is close to the pI of BLG.

The ζ-potential *versus* pH profiles of the secondary, tertiary, and quaternary emulsions were qualitatively similar, moving from highly positive at low pH to highly negative at high pH, with a point of zero charge around pH 5 (Fig. 3). The emulsions with BLG as an outer coating (i.e., BLG, LF-BLG, and LF-BLG- LF-BLG) tended to be more negative at higher pH values than the emulsions with LF as an outer coating (i.e., LF and LF-BLG-LF). These results suggest that the net electrical characteristics of the protein-coated droplets were mainly determined by the outer layer in this pH range. At pH values below the pI of BLG (pH < 5), the BLG and LF should both be positively charged and there should be an electrostatic repulsion between them. In the absence of interfacial cross-linking one would therefore expect the outer layers in multilayer protein coatings to become detached from the droplet surfaces at low pH values.

However, we used thermal treatments to cross-link the proteins in those emulsions with BLG as the outer coating, and therefore we would not have expected the protein layers to desorb in these systems. The fact that the secondary, tertiary, and quaternary emulsions all had fairly similar charge characteristics at low pH values may therefore have been because they all had a BLG outer coating (Fig. 3).

The primary emulsions containing LF-coated lipid droplets were stable to droplet aggregation across the entire pH range studied, with a mean particle diameter of ~180 nm (Fig. 4). One might have expected some droplet aggregation at pH values around the isoelectric point of the LF molecules (pH≈8), since then the droplets have little net charge and the electrostatic repulsion between them is weak (42). The fact that we did not observe any droplet aggregation in these emulsions at this pH suggests that there was a strong steric repulsion between the LF-coated lipid droplets, which has been attributed to their high molecular weight and the presence of carbohydrate moieties leading to a thick hydrophilic interfacial layer (33). The primary emulsions containing BLG-coated lipid droplets were stable to droplet aggregation at low and high pH values, which can be attributed to the strong electrostatic repulsion between them. However, they exhibited extensive droplet aggregation at pH values near their isoelectric point (pH4 and 5) due to the decrease in electrostatic repulsion (11). BLG molecules have a lower molecular weight that LF molecules and they do not have carbohydrate moieties, and therefore they would be expected to form thinner layers with less steric repulsion (32). The secondary, tertiary, and quaternary emulsions all had fairly similar particle size *versus* pH profiles (Fig. 4), being stable to droplet aggregation at low (≤ 3) and high (≥ 6) pH values, but highly unstable at intermediate values (pH5). At pH5, the nanolaminated lipid droplets all have little net charge (Fig. 3), which suggests that droplet aggregation occurred due to the reduction in the electrostatic repulsion between the droplets. Interestingly, the tertiary emulsion (LF-BLG-LF) exhibited extensive droplet aggregation even though it had LF as an outer coating, which suggests that steric repulsion was less important in this system than in the primary emulsion containing LF-coated droplets.

Overall, these results indicate that emulsion stability cannot simply be related to the thickness or charge of the interfacial coatings. Some emulsions had little net charge but were stable to aggregation (e.g., LF-coated droplets at pH8), whereas others were unstable (e.g., BLG-coated droplets at pH4). Some emulsions had similar charges, but different interfacial thicknesses, but were still all unstable to aggregation (e.g., LF-BLG, LF-BLG-LF, and LF-BLG-LF-BLG coated droplets at pH5).

Influence of Ionic Strength on Particle Stability

Emulsion-based delivery systems may be used in commercial products that have different ionic compositions or they may pass through regions of the GI tract that have different ionic contents. It is therefore important to establish the effects of ionic composition on the stability and physicochemical properties of nanolaminated lipid particles. A series of emulsions was prepared at pH6.5 containing

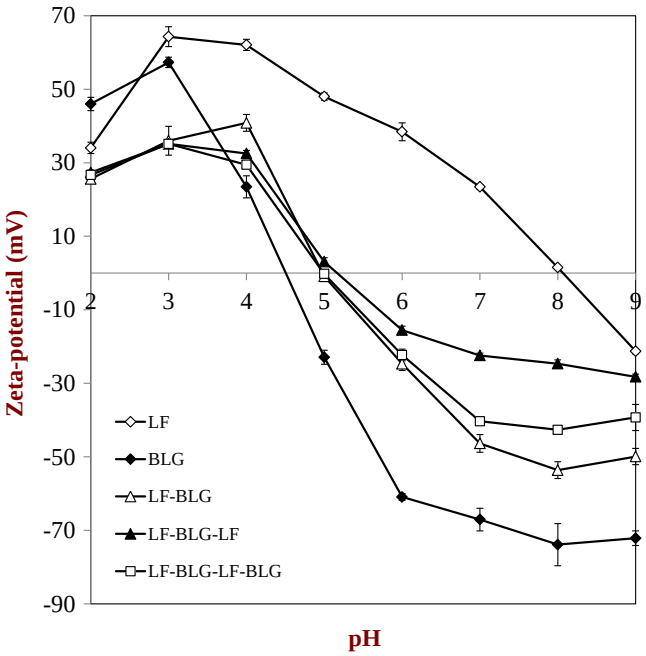

Fig. 3 pH-Dependence of the particle charge (ζ-potential) on the nature of the protein coatings surrounding lipid droplets in oil-in-water emulsions.

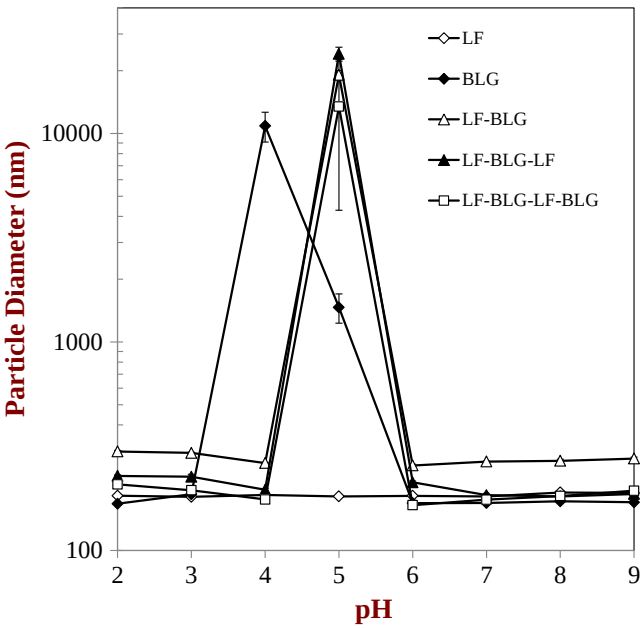

Fig. 4 pH-Dependence of the mean particle diameter on the nature of the protein coatings surrounding lipid droplets in oil-in-water emulsions.

different amounts of sodium chloride and then their physicochemical properties were analyzed.

For all of the emulsions, the magnitude of the ζ-potential decreased with increasing salt concentration (Fig. 5), which can be attributed to electrostatic screening effects, i.e., accumulation of counter-ions around charged particles (41). However, we observed charge reversal in the primary emulsions containing LF-coated droplets at higher salt concentrations (> 100 mM NaCl), which suggests that ion binding may also have been important in this system, e.g., binding of anionic chloride ions to cationic groups on the LF surface (41).

The primary emulsion containing LF-coated droplets was stable to droplet aggregation at low salt levels (≤ 10 mM NaCl), but exhibited extensive droplet aggregation at higher salt levels (Fig. 6). As already mentioned, the addition of salt would have screened the electrostatic repulsion between droplets, and it may also have promoted bridging flocculation between negative patches on one droplet and positive patches another. For example, chloride ions may have acted as ionic bridges between cationic groups on different LF-coated droplets. All the other emulsions were stable to the addition of 0 to 200 mM NaCl, which suggests that the repulsive interactions (e.g., electrostatic and steric) were large enough to overcome the attractive interactions (e.g., van der Waals and hydrophobic). In particular, we postulate that the formation of a thick interfacial protein coating around the lipid droplets generated a strong steric repulsion between them, which was large enough to overcome the attractive interactions. In a previous study, the good stability of lipid droplets coated by a BLG-pectin layer at high ionic strengths was also attributed to an increase in the steric repulsion between the droplets due to the formation of a thick interfacial coating around them (10). These results suggest that nano-lamination of the lipid particles may improve their stability to high ionic strength environments.

Influence of Simulated Gastrointestinal Conditions

In this series of experiments we examined the influence of interfacial coating properties on the behavior of the nanolaminated lipid particles under simulated gastrointestinal tract (GIT) conditions. The mean particle size, charge, and aggregation state of the emulsions was measured after incubation in simulated mouth, stomach, and small intestinal fluids.

Initially, all of the emulsions contained small particles (d<350 nm) prior to subjection to the simulated GIT conditions (Fig. 7). There was an appreciable increase in mean particle size (Fig. 7) and some evidence of particle flocculation in the confocal microscopy images (Fig. 8) after exposure to simulated oral fluids, suggesting that all of the protein-coated droplets were unstable to mouth conditions. Presumably, flocculation was induced by the presence of salts and polymers (mucin) in the simulated oral fluid, as has been reported by other workers for protein-coated droplets (43, 44). Salts reduce *repulsive* interactions between droplets by screening electrostatic forces, whereas polymers increase *attractive* interactions by promoting bridging or depletion flocculation. The extent of droplet aggregation

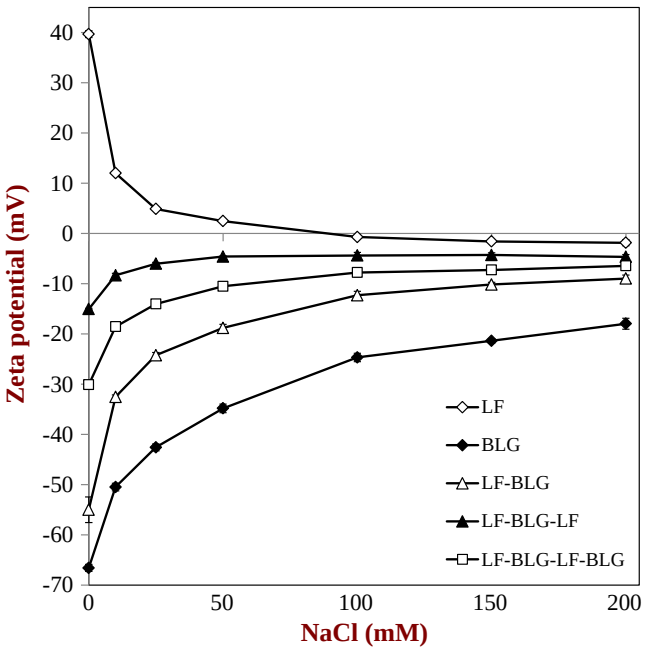

Fig. 5 Salt-dependence of the particle charge (ζ-potential) on the nature of the protein coatings surrounding lipid droplets in oil-in-water emulsions.

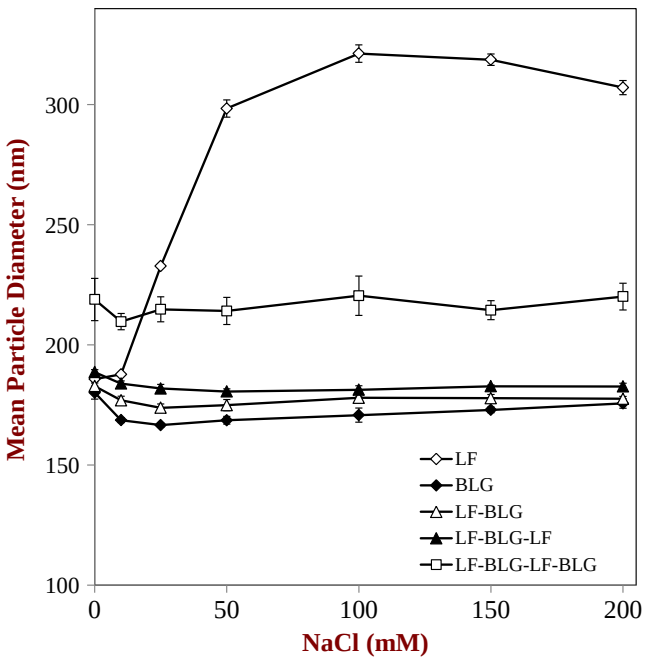

Fig. 6 Salt-dependence of the mean particle diameter on the nature of the protein coatings surrounding lipid droplets in oil-in-water emulsions.

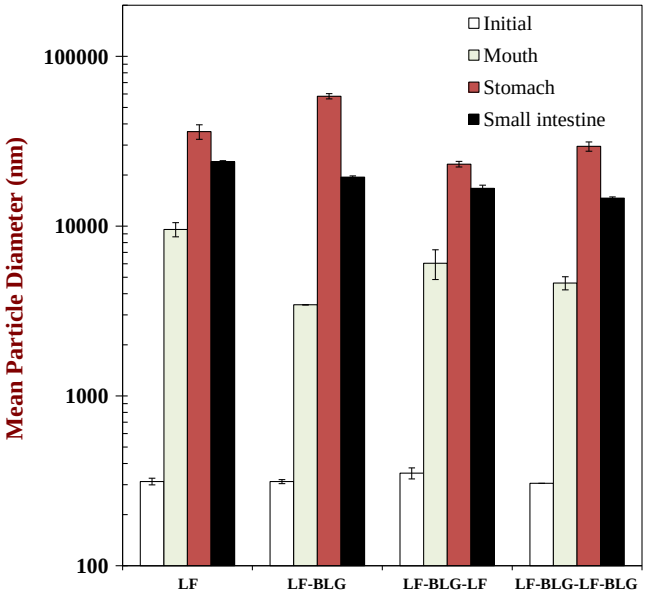

Fig. 7 Change in mean particle diameter of oil-in-water emulsions containing lipid particles coated by one, two, three or four layers of protein as they pass through different stages of a simulated gastrointestinal tract model.

increased after exposure to simulated gastric fluids, suggesting that all of the protein-coated droplets were highly unstable to stomach conditions. This effect may be attributed to the high acidity, ionic strength, and protease activity of the simulated gastric fluids (43, 45, 46). The low pH of the stomach means that both LF and BLG had a net positive charge (Fig. 3), and therefore the multilayer interfacial coatings may fall apart due to electrostatic repulsion between neighboring protein layers. In addition, proteases promote hydrolysis of adsorbed protein layers, which may also reduce interfacial layer integrity (43). The particles in all systems were large ($d > 10$ μm) after exposure to simulated small intestinal fluids (Fig. 7) and there was evidence of large clumps in the confocal microscopy images (Fig. 8). We postulate that these clumps consisted of aggregates that contained some lipid digestion products, i.e., free fatty acids (FFAs) and monoacylglycerols (MAGs). The presence of these insoluble clumps suggests that there may have been some interaction between anionic FFAs and cationic fragments of the original LF molecules in the simulated intestinal fluid.

Indirect information about changes in interfacial composition of the lipid particles as they passed through the

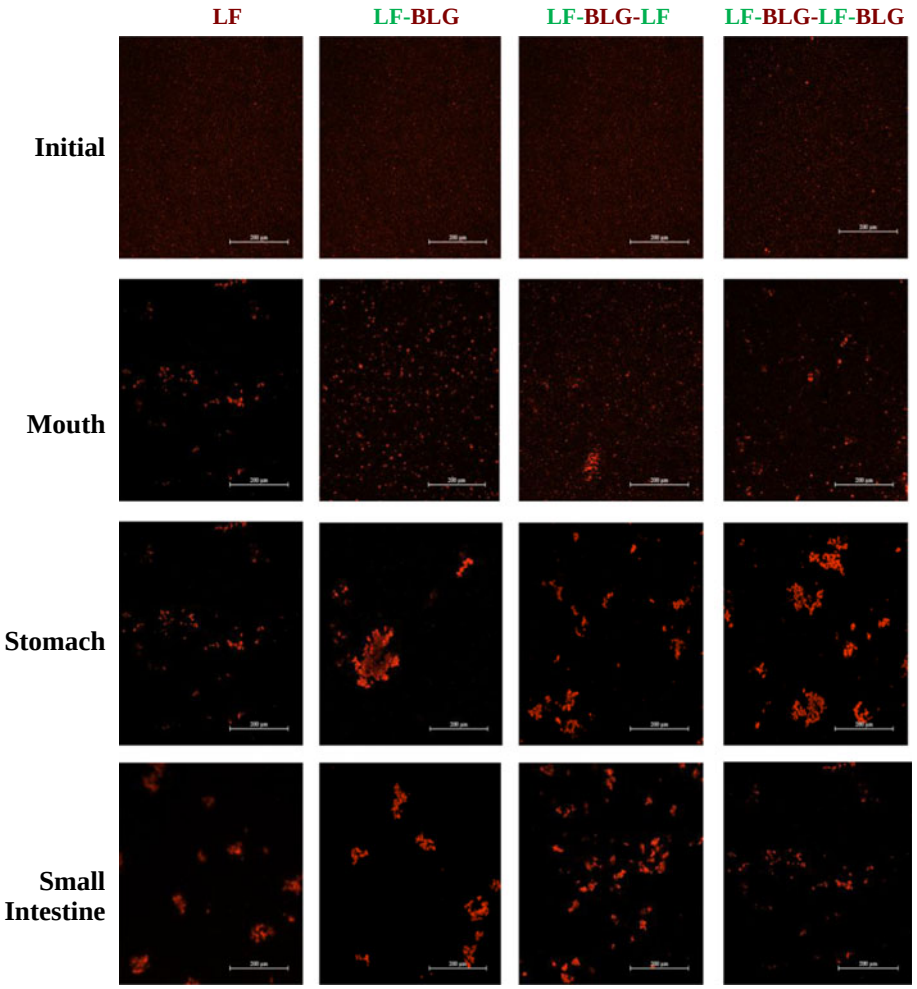

Fig. 8 Change in microstructure of oil-in-water emulsions containing lipid particles coated by one, two, three or four layers of protein as they pass through different stages of a simulated gastrointestinal tract model monitored using confocal fluorescent microscopy. The red areas represent non-polar regions. The scale bars represent a length of 200 μm.

simulated gastrointestinal tract was obtained by measuring their ζ-potentials (Fig. 9). Initially, the LF-coated droplets were highly positive, but all of the multilayer emulsions contained droplets that were negative. The initial pH in the emulsions was close to neutral, which meant that the LF was cationic whereas the BLG was anionic (Fig. 3). These results suggest that BLG dominated the overall electrical charge in the multilayer emulsions, which would be expected in the LF-BLG and LF-BLG-LG-BLG emulsions, since it formed the outer layer. However, the net charge was also negative in the LF-BLG-LF emulsions, which suggested that BLG even dominated the charge in systems where LF formed the outer layer. After exposure to oral conditions all of the emulsions had a high net negative charge (−27 to −33 mV). In the case of the LF-coated lipid droplets this is probably due to the adsorption of anionic mucin molecules onto the cationic droplet surfaces. In the case of the multilayer emulsions, this may be due to the inherent negative charge associated with the interfacial protein coating (Fig. 3) and/or due to adsorption of anionic mucin molecules to the droplet surfaces. After exposure to gastric conditions the magnitude of the particle charge decreased appreciably, and was either slightly negative (LF and LF-BLG) or slightly positive (LF-BLG-LF and LF-BLG-LF-BLG). Measurements of the ζ-potential as a function of pH in the absence of simulated GI tract conditions showed that all the protein-coated lipid droplets were highly positively charged at low pH values (Fig. 3). The particle charge may have been altered in the presence of simulated gastric juices due to a number of reasons: (i) hydrolysis of the adsorbed protein layers; (ii) displacement of protein molecules by anionic surface active substances; (iii) co-adsorption of anionic molecules to the droplet surfaces (such as mucin); (iv) electrostatic screening by salts from the simulated oral and gastric phases. After exposure to the simulated small intestinal conditions, the particles in all of the systems were highly negatively charged (−15 to −18 mV), irrespective of the nature of the initial protein coating. The negative charge on the particles present within the simulated small intestinal fluids after digestion may arise from a variety of sources. First, the digestion of triacylglcyerols produces free fatty acids that have a negative charge. Second, bile salts and phospholipids present within the simulated small intestinal fluids also have a negative charge. Third, any remaining BLG molecules will have a small negative charge, while any remaining LF molecules will have a small net positive charge under neutral pH conditions and high salt concentrations.

The influence of the initial protein coating on the digestion of the lipid droplets was determined using the pH-stat method after they had been exposed to the mouth and stomach stages of the simulated GI tract model. Qualitatively, we found that all of the protein-coated lipid droplets behaved similarly regardless of their initial interfacial structure (Fig. 10). There was a steep increase in free fatty acids released during the first 20 min of digestion, followed by a more gradual increase at longer incubation times. These results suggest that the initial protein coating structure had little influence on the digestion of the triglycerides in the emulsions. Recently, we developed a simple

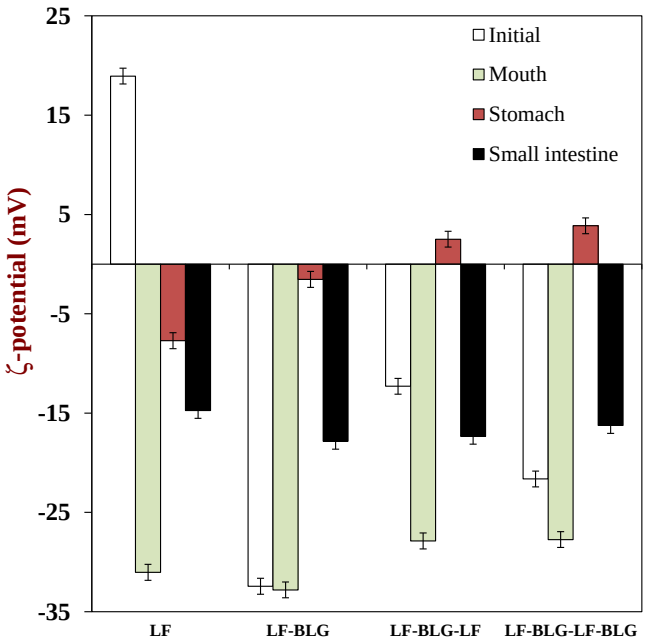

Fig. 9 Change in droplet charge of oil-in-water emulsions containing lipid particles coated by one, two, three or four layers of protein as they pass through different stages of a simulated gastrointestinal tract model.

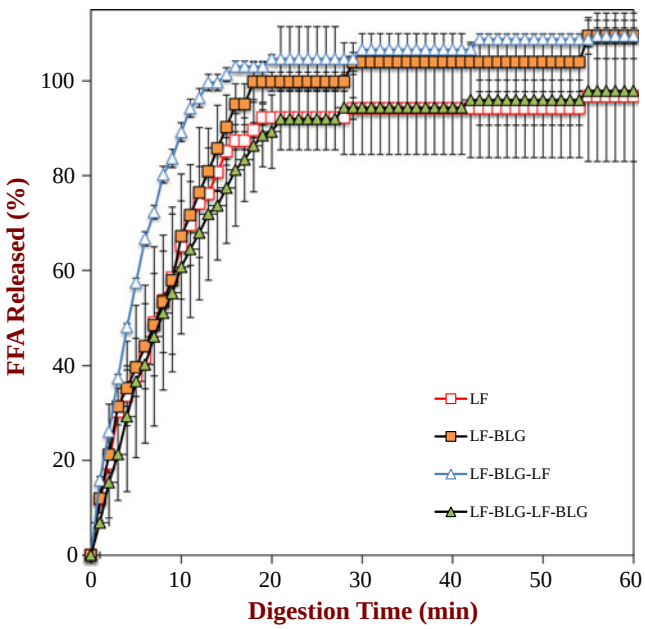

Fig. 10 Titration curves (FFA *versus* time) obtained for oil-in-water emulsions containing lipid particles coated by one, two, three or four layers of protein (pH7.0).

mathematical model to describe the FFA *versus* time profiles obtained using the pH stat method (47). The percentage of the total free fatty acids that are released (Φ) from an emulsified lipid as a function of time (t) as measured by the pH-stat method is modeled by the following equation:

$$\Phi = \phi_{\max}\left(1 - \left(1 + \frac{3kMt}{2d_0\rho_0}\right)^{-2}\right) \quad (4)$$

Here, $\Phi_{\max}$ provides a measure of the total *extent* of digestion (i.e., the percentage of FFA released by the end of the reaction), k provides a measure of the *rate* of digestion (i.e., μmols of FFA released per unit droplet surface area per unit time), d_0 is the initial droplet diameter, ρ_0 is the oil droplet density, and M is the molar mass of the oil. A pH-stat profile can then be characterized in terms of just two parameters: $\Phi_{\max}$ and k, which can be determined by finding the values which give the best fit between the experimental data and the mathematical model. This equation can also be used to calculate the "digestion time", which is the time required for the FFAs released to increase to 50% (47). This model was used to analyze the pH-stat data presented for the lipid droplets coated by different interfacial layers, and the parameters derived are tabulated in Table I. It was assumed that M=800 gmol^{-1}, ρ_O=920 kg m^{-3}, and d_0=313 nm (measured value for primary emulsion). The calculated digestion rate did not depend strongly on the number of layers originally coating the lipid droplets (varying from around 0.2 to 0.5 μmols s^{-1}m^{-2}). In addition, all of the lipid phase appeared to be fully digested by the end of the intestinal phase (> 100%) for all systems. The fact that the final amount of FFA released from the samples appeared to be higher than 100% may be for a number of reasons. For example, there may have been other components within the simulated gastrointestinal tract that decomposed during the pH-stat titration (e.g., proteins or phospholipids), or some of the monoacylglycerols may have broken down to glycerol and free fatty acids (47).

The fact that the initial interfacial structure or composition did not have a major impact on the final digestibility of the triglycerides in the lipid phase, suggests that the protein coatings were effectively removed from the lipid droplet surfaces as they passed through the gastrointestinal tract. This may have occurred due to a number of reasons. Protein multilayers may have dissociated at high salt concentrations due to electrostatic screening effects or at low pH values due to the two proteins having similar net charges (both positive). Protein layers may have been removed due to competitive adsorption between the protein molecules and other surface active substances in the gastrointestinal fluids, such as bile salts, phospholipids, and free fatty acids. Protein layers may have been hydrolyzed by proteases in the gastric and small intestinal fluids, which caused them to lose their surface activity.

Table I Parameters obtained by fitting the digestion model (Eq. 4) to the pH-stat data during the first 30 min of digestion. It was assumed that the initial oil droplet diameter was 313 nm, the molar mass of the oil (corn oil) was 800 gmol^{-1}, and the density of the oil was 920 kgm^{-3}

Interfacial Coating	Digestion extent Φ_{max} (%)	Digestion Rate k (μmols s^{-1}m^{-2})	Digestion Time (min)
LF	105.9 ± 2.3	0.29 ± 0.03	5.7 ± 0.5
LF-BLG	120.0 ± 4.1	0.24 ± 0.01	6.8 ± 0.1
LF-BLG-LF	113.5 ± 0.2	0.49 ± 0.16	3.0 ± 0.4
LF-BLG- LF-BLG	112.5 ± 2.0	0.22 ± 0.06	7.7 ± 2.1

Influence of Protein Coatings on Bioaccessibility of Lipophilic Bioactive

Finally, we examined the influence of interfacial protein coatings on the bioaccessibility of a model lipophilic compound (β-carotene), i.e., the fraction released into the mixed micelle phase after digestion in the small intestine. After the small intestinal phase of the simulated gastrointestinal model we centrifuged the samples and they separated into an orange-white sediment at the bottom and a clear aqueous solution at the top (Fig. 11). The bioaccessibility was estimated by measuring the concentrations of β-carotene in the micelle phase and the sediment phase (Eq. 3). We found that

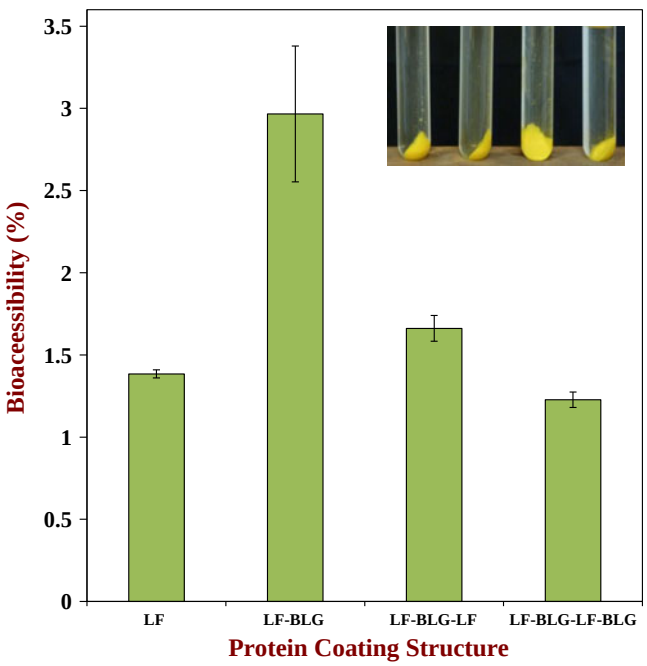

Fig. 11 Bioaccessibility of β-carotene determined after centrifugation of digested oil-in-water emulsions containing lipid particles initially coated by one, two, three or four layers of protein. The inset is a photograph of samples after digestion and centrifugation.

the β-carotene bioaccessibility was low (1–3%) in all of the emulsions studied, which was surprising since previous researchers have reported a much higher β-carotene bioaccessibility (> 70%) in oil-in-water emulsions containing protein-coated droplets (46). Indeed, when we repeated the experiment using a non-ionic surfactant (Tween 20) as the emulsifier instead of proteins we obtained a bioaccessibility of 72.5%±1.4%. The low bioaccessibility of the β-carotene in the protein-coated lipid droplets found in our study was supported by the overall visual appearance of the digesta after centrifugation (Fig. 11): the micelle layer had little orange color but the sediment layer was strongly orange. A possible reason for this finding is that all of our emulsions contained lactoferrin (which is cationic at neutral pH), whereas previous studies have used whey proteins (which are anionic at neutral pH). It is possible that β-carotene bound to LF molecules or digestion products of LF that accumulated within the sediment after lipid hydrolysis and centrifugation, and were therefore not detected in the micelle phase. This may have occurred due to a direct interaction between the carotenoid and the protein molecules, or it may have been due to an interaction between the anionic mixed micelles and cationic protein molecules. Overall these results suggest that lactoferrin decreased the bioavailability of β-carotene in a simulated GIT, but further studies are required to identify the nature of the interaction between this carotenoid and lactoferrin, and to determine if similar results are also observed *in vivo*. This effect may have important consequences for the utilization of lactoferrin coatings for delivering certain kinds of bioactive components.

CONCLUSIONS

We have shown that lipid droplets can be nanolaminated with globular protein coatings by sequential electrostatic deposition of anionic and cationic proteins onto their surfaces. This approach enables one to control the composition and structure of the interfacial region surrounding lipid droplets, and thus control the stability and functionality of emulsion-based delivery systems. We have shown that interfacial properties influence the stability of nanolaminated lipid droplets to changes in environmental conditions (pH and ionic strength). However, we found that lipid droplets coated by single or multiple layers of proteins behaved fairly similarly in a simulated gastrointestinal tract. In particular, the rate and extent of triglyceride digestion was fairly similar for lipid droplets initially coated by one, two, three or four layers of protein. We attribute this effect to removal of the protein layers from the droplet surfaces as they pass through the different stages of the gastrointestinal tract. Interestingly, we found that the bioaccessibility of an encapsulated lipophilic compound (β-carotene) was low (< 4%) in all of the emulsions tested, which we suggested was due to its interaction with cationic lactoferrin or its peptide digestion products. This result has important implications for designing effective encapsulation and delivery systems for lipophilic bioactive components. Nanolaminated protein coatings may be used to increase the physical or chemical stability of an encapsulated lipophilic compound within a commercial product (such as a functional food or a drug preparation), but their ability to release it within the GI tract may depend on the nature of the compound encapsulated and the interfacial coatings used.

ACKNOWLEDGMENTS AND DISCLOSURES

This material is partly based upon work supported by grants from the United States Department of Agriculture (CREES, NRI Grant and NSF-EPA-USDA Grant) and the Massachusetts Department of Agricultural Resources. We also acknowledge funding from the University of Massachusetts (Hatch).

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