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Impact of Electrostatic Deposition of Anionic Polysaccharides on the Stability of Oil Droplets Coated by Lactoferrin

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The purpose of this study was to investigate the effects of three anionic polysaccharides (low methoxyl pectin (LMP), high methoxyl pectin (HMP) and alginate) on the physicochemical properties and stability of lactoferrin (LF)-coated lipid droplets. LMP, HMP and alginate were shown to adsorb to the surfaces of LF-coated droplets at neutral pH, which was primarily attributed to electrostatic attraction between anionic groups on the polysaccharide molecules and cationic patches on the protein surfaces. In the absence of polysaccharide, the LF-coated droplets were highly unstable to aggregation when heated above about 60 °C at pH 7, presumably because thermal denaturation of the adsorbed proteins increased droplet attraction. The addition of either LMP or HMP prior to heating greatly improved the thermal stability of the emulsions, with no aggregation being observed from 30 to 90 °C. On the other hand, the presence of anionic polysaccharides had little effect on emulsion stability or even promoted emulsion instability when 0 to 200 mM NaCl or $CaCl_2$ was added. This study shows that the stability of LF-coated lipid droplets can be improved by careful selection of an appropriate type and amount of anionic polysaccharide to incorporate.

KEYWORDS: Lactoferrin; pectin; alginate; polysaccharides; multilayer emulsions; emulsions; electrostatic deposition; stability; heating

INTRODUCTION

There is currently considerable interest in the development of food-grade delivery systems that can encapsulate, protect and deliver lipophilic bioactive components, such as ω-3 fatty acids, carotenoids and phytosterols (*1*−*6*). Adequate consumption of such bioactive lipids can promote human health and help prevent diseases, such as heart disease, diabetes, hypertension, and cancer. However, there are a number of challenges to the development and production of food-grade delivery systems, such as ensuring product safety, stabilization of the bioactive ingredients, and the functionality of the product, e.g. sensory properties, digestibility, and bioactive release. Nanoscale technologies could help overcome some of the shortcomings of current delivery systems, e.g., by designing structured particles that can better encapsulate, protect and deliver encapsulated ingredients, or that can control product stability, rheology or optical properties (*7*, *8*). In recent years, the electrostatic layer-by-layer (LbL) deposition methodology has shown great promise for the development of functionalized delivery systems (*9*−*11*). This simple technique relies on exploiting electrostatic attraction between oppositely charged substances to deposit ionic biopolymers onto the interfaces of oppositely charged lipid droplets (*9*, *10*, *12*, *13*). Numerous studies have shown that applying such an approach can be useful in improving emulsion stability to environmental stresses, such as pH, ionic strength, thermal processing, freezing, and dehydration (*10*, *11*, *14*). Basically, production of such emulsions involves the initial creation of a "primary" emulsion containing lipid droplets coated with a layer of protein molecules. Subsequently, a "secondary" emulsion is formed by adsorbing an oppositely charged biopolymer onto the droplet surfaces. By repeating these procedures one can fabricate droplets with multilayered coatings (*10*). A major challenge associated with developing this type of emulsion is that they are highly susceptible to flocculation, and therefore, it is important to establish the optimum conditions required for their formulation (*10*, *12*, *13*). Moreover, recent reviews also suggest that rationally designing the structure and composition of droplet interfaces can enable control over emulsion functionality, e.g. digestibility and bioavialability in the gastrointestinal tract (*15*−*17*).

Incorporation of natural antioxidants into emulsion formulations has the potential to promote not just the chemical stability of the lipid droplets but also the overall stability of the emulsion (*18*). Recently, much attention has been drawn to the use of bovine lactoferrin (LF) in emulsions. It has been claimed that this globular glycoprotein of the transferrin family (*19*, *20*) has various health benefits and functional applications in commercial products (*20*−*26*). Recently, it has been demonstrated that LF can enhance both the physical and chemical stability of lipid droplets in emulsions (*27*−*30*). LF has also been shown to form multilayer protein emulsions through the deposition of cationic LF molecules onto anionic whey protein-coated lipid droplets (*30*, *31*). Besides its unusually high p*I* compared to other common food proteins, LF also possesses the ability to bind two ferric ions inside two structural lobes, which unfold at two different temperatures (60 and 85 °C) (*32*).

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Few previous studies have been published on the use of LF in multilayered emulsions. To the best of our knowledge, no studies have addressed multilayered emulsion formulations containing LF and food polysaccharides. Such carbohydrate biopolymers have unique molecular characteristics, for example, molecular weight, electrical charge, branching, hydrophobicity, and applications (*2*). Consequently, the interfacial coatings formed by different polysaccharides are expected to have different physicochemical properties (such as thickness, charge, permeability, and environmental responsiveness), which in turn will lead to different functional properties (such as protection, release and stability) (*33*, *34*). It is therefore important to establish the link between the composition and structure of multilayered biopolymer-coated droplets to the functional properties of the emulsions. Hence, the aim of this study was to examine the interfacial adsorption of anionic polysaccharides (sodium alginate, low methoxy pectin, and high methoxy pectin) onto LF-coated lipid droplets and relate them to the physical stability of such emulsion formulations under varying conditions of temperature, pH and salts. The results from this study should provide useful information in the rational design of emulsion-based delivery systems containing mixed protein and polysaccharide-coated droplets with improved or novel functional properties.

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. The manufacturer reported that the corn oil contained approximately 14.3, 28.6, and 57.1 wt % of saturated, monounsaturated, and polyunsaturated fats, respectively. Food grade lactoferrin (Lot 10373317) was supplied by DMV International (Delhi, NY), and the manufacturer reported that it contained 97.7% protein and 0.12% ash. Food grade alginic acid sodium salt was obtained from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO). Low methoxyl pectin (LMP) [DE < 30%] and high methoxyl pectin (HMP) [DE > 50%] were obtained from CP Kelco (Atlanta, GA). Sodium chloride (NaCl), calcium chloride ($CaCl_2$), monobasic phosphate and dibasic phosphate were purchased from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). All solvents and reagents were of analytical grade.

Primary Emulsion Preparation. An aqueous emulsifier solution was prepared by dissolving 1 g of powdered LF in 89 g phosphate buffer solution (10 mM, pH 7) and stirring for at least 3 h. The LF solution was then filtered to remove any insoluble particles. Ten grams of corn oil was added to the filtered LF solution so that the final system contained 10% oil, 1% LF and 90% buffer (w/w). This mixture was coarsely 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.

Secondary Emulsion Preparation. Three types of secondary emulsions were produced by mixing the primary emulsion with alginate, low methoxy pectin (LMP), or high methoxy pectin (HMP) solutions. For each polysaccharide type, a 2% (w/w) solution was prepared by dissolving 2 g of powdered ingredient into 98 g of phosphate buffer solution (10 mM phosphate, pH 7) and stirring for 2 to 3 h to ensure dissolution. These solutions were then diluted further to obtain a series of aqueous solutions with varying polysaccharide concentrations (0, 0.4, 0.8, 1.2, 1.6 and 2%). Five milliliter aliquots of each polysaccharide solution were placed in separate glass test tubes, and then 5 mL of primary emulsion was added to each tube. The final composition of the secondary emulsions was 5% oil, 0.5% LF and 0, 0.2, 0.4, 0.6, 0.8, or 1% polysaccharide. The tubes were then vortexed and stored overnight at 20 °C at ambient temperature prior to analysis.

Physical Characterization. The physical properties and stability of the emulsions were monitored by measuring their particle size distribution and charge. Particle size distributions were measured using static light scattering by injecting diluted emulsion samples into an optical measurement cell and measuring the angular scattering pattern (Malvern Mastersizer 2000, Malvern Instruments, Worcestershire, U.K.). Background corrections and system alignment were performed prior to each measurement when the measurement cell was filled with the appropriate buffer solution. Particle sizes are reported as the volume-averaged mean diameters (d_{43}) calculated from the particle size distribution.

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 were diluted in 10 mM phosphate buffer at the appropriate pH at a ratio of 1:200 (v/v) and then 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. The ζ-potential measurements were used in this study to provide some information about changes in interfacial properties. It should be noted that the ζ-potential depends on interfacial composition and structure, as well as on aqueous phase composition, and so measured changes in ζ-potential only give a rough indication of overall changes in interfacial properties.

Photographs of the emulsion samples were taken over a period of 7 days using a digital camera to illustrate macroscopic instability.

Stability to Environmental Stresses

1. Thermal Stability. Secondary emulsions were formed by adding 5 mL aliquots of a primary emulsion (10% corn oil-in-water emulsion) to test tubes containing 5 mL aliquots of buffer, 1% alginate, LMP, or HMP solutions. The final emulsions therefore contained 5% oil, 0.5% LF and 0.5% polysaccharide. The tubes were then vortexed and stored overnight at 20 °C before being heat treated. Samples were then placed in a preheated water bath for 30 min at temperatures of 30, 40, 50, 60, 70, 80, and 90 °C. After cooling of the tubes to room temperature using an ice bath, they were stored overnight at 20 °C before being analyzed.

2. pH Stability. Secondary emulsions were formed by adding 5 mL aliquots of a primary emulsion (10% corn oil-in-water emulsion) to test tubes containing 5 mL aliquots of buffer, 1% alginate, LMP, or HMP solutions. The pH of the emulsions was then adjusted to values ranging from 2 to 9 using HCl and/or NaOH solutions, and the samples were stored overnight at 20 °C before further analysis.

3. Salt Stability. Emulsions were prepared with 6% oil, 0.6% LF and either 0 or 0.6% polysaccharide. One milliliter of various NaCl or $CaCl_2$ solutions was then added to test tubes containing 5 mL of emulsions to give a range of salt concentrations. Samples were vortexed and stored overnight at 20 °C before analysis. Finally, the emulsions contained 5% oil, 0.5% LF, 0.5% polysaccharide, and salt concentrations varying from 0 to 200 mM.

After being subjected to these environmental stress tests, the emulsion samples were analyzed for particle size distribution, ζ-potential and visual appearance as described above.

RESULTS AND DISCUSSION

Previous studies have shown that LF-stabilized lipid droplets are unstable to various kinds of environmental stresses, including pH changes, high ionic strengths, and thermal processing (*35*). A major objective of the present study was therefore to determine whether the properties of LF-stabilized lipid droplets could be improved by coating them with food-grade anionic polysaccharides.

Emulsion Formation and Stability. Initially, we examined the impact of polysaccharide type and concentration on the formation and stability of emulsions containing LF-stabilized lipid droplets. Emulsions were prepared by adding LF-stabilized lipid droplets to solutions (pH 7) containing three different kinds of anionic polysaccharides: alginate; low methoxy pectin (LMP); high methoxy pectin (HMP). The impact of polysaccharide type and concentration on particle size and charge (ζ-potential) was then measured (**Figures 1** and **2**). At pH 7, the ζ-potential of the LF-stabilized lipid droplets in the primary emulsions was slightly negative ($\zeta = -13$ mV), despite this pH being below the reported isoelectric point of lactoferrin ($pI \approx 8$). The most likely explanation for this effect is that the cationic groups on the adsorbed LF bound some anionic phosphate ions present in the buffer. Indeed,

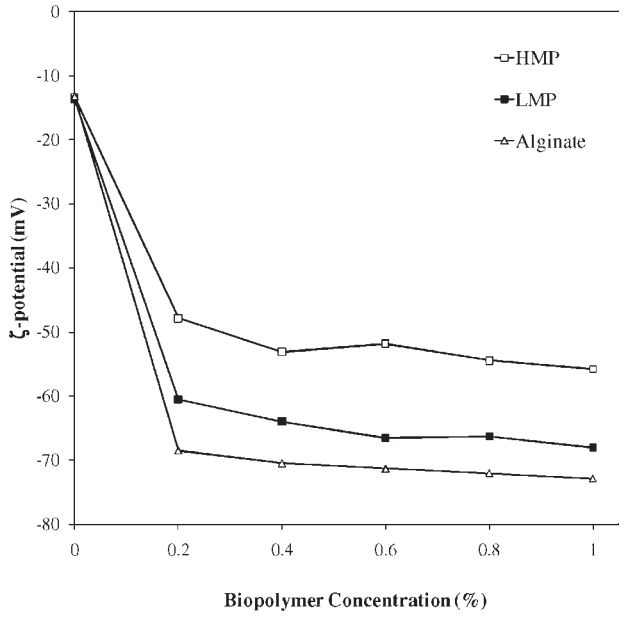

Figure 1. Dependence of droplet charge on added biopolymer concentration in 5% corn oil -in-water emulsions (0.5 wt % LF, 10 mM phosphate buffer, pH 7).

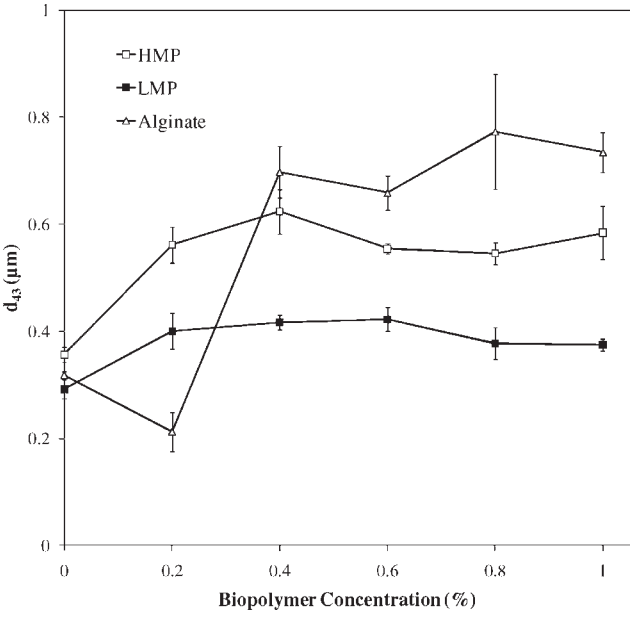

Figure 2. Dependence of mean droplet size on added biopolymer concentration in 5% corn oil-in-water emulsions (0.5 wt % LF, 10 mM phosphate buffer, pH 7).

when measurements were made at the same pH in distilled water rather than buffer solution, the LF-stabilized lipid droplets did have a positive charge (+19 mV). As the polysaccharide concentration was increased in the secondary emulsions, the droplet ζ-potential became increasingly negative until relatively constant values were attained at 0.2% (w/w) polysaccharide and higher (**Figure 1**). The final ζ-potentials attained for the emulsions were ~−50, −60, and −70 mV in the presence of 0.2% LMP, HMP and alginate, respectively, which reflects their relative linear charge densities (*36*).

Previously, such changes in the ζ-potential of emulsions have been accounted for by electrostatic deposition of charged polysaccharides onto lipid droplet surfaces (*7*, *10*, *33*, *37*). Our results suggest that all three anionic polysaccharides were able to adsorb onto the surfaces of the anionic LF-coated droplets at neutral pH. This effect may be attributed to electrostatic binding of anionic groups on the polysaccharide chains to cationic patches on the electrically heterogeneous surface of the LF molecules, as has been observed with other pairs of polysaccharides and proteins (*38*−*41*). Nevertheless, other types of attractive interactions may also play a role in causing the pectin molecules to bind to the LF-coated lipid droplets, such as hydrophobic interactions, hydrogen bonding, and van der Waals forces.

Laser scattering-based particle size measurements indicated that there was some increase in the mean particle diameter of the emulsions upon addition of the anionic polysaccharides, particularly alginate (**Figure 2**). This effect may be due to the formation of polymeric bridges between the anionic polysaccharides and cationic patches on different droplet surfaces when the LF-stabilized lipid droplets and polysaccharide solution are mixed together. A mathematical analysis of the conditions where bridging flocculation occurs in mixed systems containing charged colloidal particles and oppositely charged polymer molecules has recently been carried out (*42*). Interestingly, there was a slight decrease in mean particle diameter when low amounts of alginate were added (**Figure 2**). This effect may be due to the fact that there was some flocculation in the emulsions containing only LF-coated lipid droplets, which was disrupted when low amounts of alginate were added. In the remainder of the experiments we used a LF-to-polysaccharide ratio of 1:1 [w/w] to prepare the secondary emulsions for the environmental stress tests, since this was sufficient to coat the LF-stabilized lipid droplets (**Figure 1**).

Thermal Stability of Emulsions. Food emulsions may be subjected to various kinds of thermal treatments during or after processing, such as pasteurization, sterilization, baking, grilling, boiling or microwaving. It is therefore important to understand the influence of heating on the stability and physicochemical properties of emulsions. Hence, we studied the effect of holding emulsions at different temperatures (30 to 90 °C, 30 min) on particle charge (**Figure 3**) and size (**Figure 4**). The secondary emulsions used contained 5% corn oil, 0.5% LF and 0.5% alginate, LMP, or HMP, while the primary emulsions used contained 5% corn oil and 0.5% LF.

The ζ-potential of the primary emulsion increased from −20 to −10 mV when the holding temperature was increased from 20 to 60 °C (**Figure 3**), which suggested that there was some change in the interfacial composition or structure upon heating. At higher temperatures it was not possible to measure the droplet charge using the microelectrophoresis instrument because the emulsions were so highly aggregated. Prior to thermal treatment, the ζ-potentials on the droplets in the secondary emulsions were different: $\zeta \approx -70$, −70, and −55 for alginate, LMP, and HMP, respectively. These differences can be attributed to differences in the electrical characteristics of the added biopolymers (*36*). LMP and alginate are known to have higher linear charge densities than HMP, i.e., more carboxylic acid groups per unit chain length (*36*). The electrical charge on the particles in all of the secondary emulsions did not change when the temperature was increased from 30 to 90 °C, which suggested that the polysaccharides remained attached to the lipid droplet surfaces after thermal processing. These results also suggest that there was little change in interfacial properties after heating, but further studies using analytical techniques that provide more direct information about interfacial structure or composition should be used to confirm this, such as spectroscopy, calorimetry, or microscopy.

The mean particle diameter in the primary emulsion increased from around 0.7 to 2.8 μm upon heating from 30 to 60 °C, indicating that some droplet aggregation occurred (**Figure 4**).

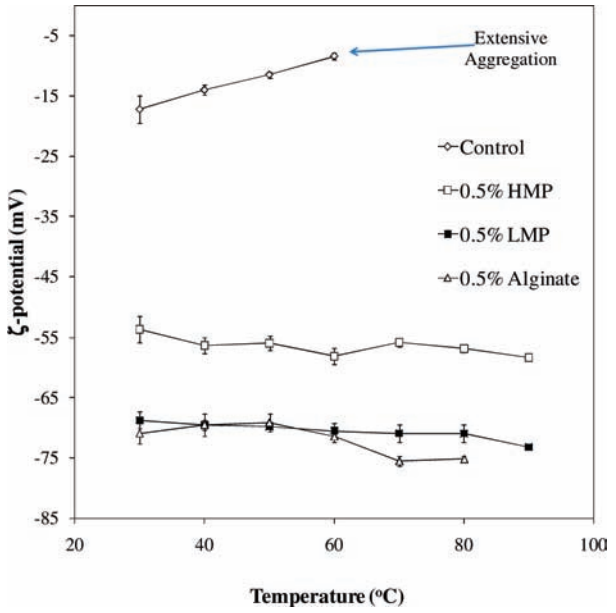

Figure 3. Effect of varying temperatures on the droplet charge of secondary 5% corn oil emulsions (0.5 wt % LF; 0.5 wt % alginate, LMP or HMP; 10 mM phosphate buffer; pH 7).

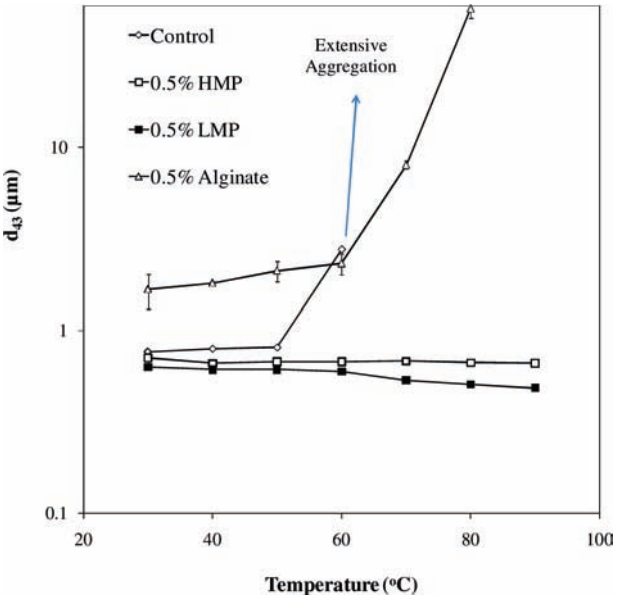

Figure 4. Effect of varying temperatures on the mean droplet size of secondary 5% corn oil emulsions (0.5 wt % LF; 0.5 wt % alginate, LMP 0r HMP; 10 mM phosphate buffer; pH 7).

At higher holding temperatures, the primary emulsion changed into a solid mass with a pastelike consistency, indicating that extensive droplet aggregation and emulsion gelation had occurred. Consequently, it was not possible to measure the particle size distribution of these emulsions at higher temperatures. Lactoferrin is a globular protein that contains two lobes that are each capable of binding one ferric ion. When LF is adsorbed at an oil−water interface, one of the lobes unfolds at around 60 °C, while the other unfolds at around 85 °C (*32*). Therefore, on holding the primary emulsion at 60 °C, one of the lobes of LF probably unfolds, causing conformational change in the LF molecules. As a result, there could be an increase in hydrophobic interactions and disulfide bond formation between LF molecules adsorbed onto different lipid droplets leading to droplet aggregation as has been observed for other types of globular proteins adsorbed to droplet surfaces (*43*, *44*). This conformational change may also be responsible for the observed change in the ζ-potential of the droplets after heating (**Figure 3**). On heating the primary emulsion at temperatures higher than 60 °C, both the lobes of LF are likely to unfold, leading to more extensive aggregation, which would account for the formation of a gel-like material in the primary emulsions after holding at higher temperatures.

The secondary emulsions containing added LMP and HMP had good thermal stability, i.e., there was little change in mean particle diameter after holding the emulsions at temperatures ranging from 30 to 90 °C. These results indicate that addition of these polysaccharides to the LF-stabilized emulsions was able to prevent extensive droplet aggregation during heating. On the other hand, the secondary emulsions containing alginate only remained stable from about 30 to 60 °C, after which there was an appreciable increase in mean particle diameter, indicating that extensive droplet flocculation had occurred. After holding at 90 °C, the emulsion containing alginate changed into a solid mass with a pastelike consistency. The differences in the ability of the polysaccharides to improve the thermal stability of the LF-stabilized emulsions may be attributed to differences in their molecular characteristics. Pectins have a linear anionic backbone with some neutral side chains branching off (*36*), which may provide good steric stabilization as well as electrostatic stabilization. On the other hand, alginates are linear anionic molecules with no branches (*36*), and therefore they may lie flatter against the droplet surfaces. Consequently, when the adsorbed LF molecules unfold they may be able to come into close contact with each other in the presence of alginate, which leads to more droplet aggregation.

pH-Stability of Emulsions. It is also important to establish the impact of pH on the stability and physicochemical properties of emulsions. In commercial applications, lipid droplets may be surrounded by an aqueous phase that is acidic, neutral or alkaline depending on the precise nature of the product. After consumption, an emulsion is exposed to various pH changes as it passes through the human gastrointestinal tract. In this section, we therefore examine the influence of pH on the electrical characteristics and stability of primary and secondary emulsions.

The influence of pH on the electrical charge (ζ-potential) of the droplets in the primary and secondary emulsions was measured (**Figure 5a**). The ζ-potential in the primary emulsion was highly positive (+40 to 50 mV) from pH 2 to 4, moderately positive (+14 mV) at pH 5, and negative at pH ≥ 6. As noted earlier, the point of zero charge for the LF-stabilized droplets (pH ≈ 6) was somewhat less than the reported isoelectric point (pI ≈ 8) of lactoferrin, which was attributed to electrostatic binding of anionic phosphate groups from the buffer solution to cationic groups on the adsorbed LF molecules. In the presence of the anionic polysaccharides, the electrical charge on the droplets was more negative than in their absence across the entire pH range. The effect of polysaccharide adsorption on droplet charge was highlighted by plotting the difference in ζ-potential between the primary and secondary emulsions: $\Delta\zeta = \zeta_{1^\circ} - \zeta_{2^\circ}$ (**Figure 5b**). The difference in ζ-potential became increasingly negative when the aqueous phase was adjusted from pH 9 to 4, which suggested an increased adsorption of anionic polysaccharides to the lipid droplet surfaces. This may have occurred because the number of positively charged binding sites on the surface of the LF-stabilized lipid droplets increased with decreasing pH due to progressive ionization of the amino groups ($-NH_2 + H^+ \leftrightarrow -NH_3^+$). Δζ became less negative when the aqueous phase was adjusted from pH 4 to 2 (**Figure 5b**), suggesting that the coated droplets lost some of their negative charge. The pK_a values of the carboxylic acid side groups on pectin and alginate are around pH 3.5, and

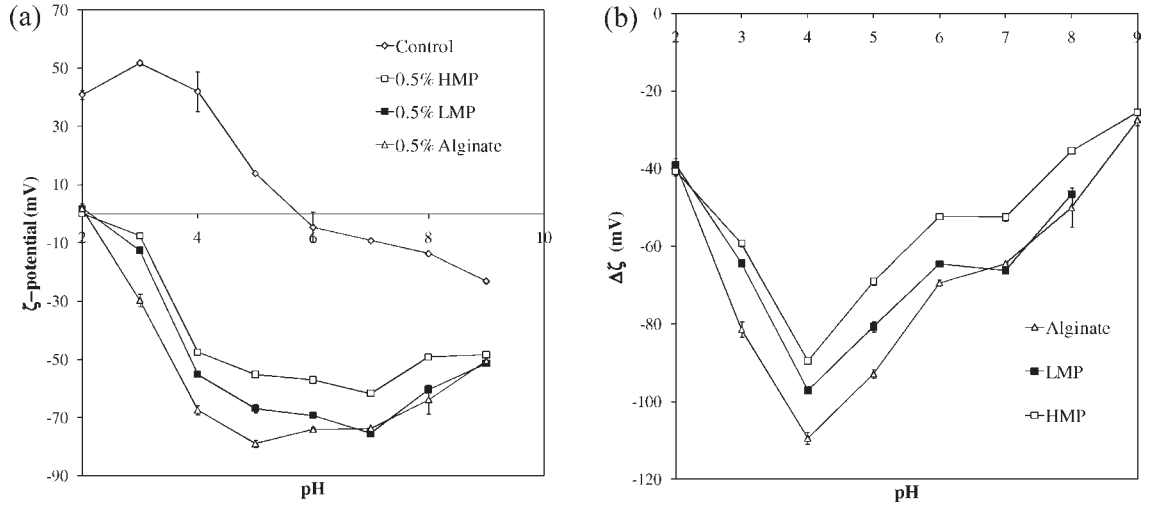

Figure 5. (**a**) Effect of varying pH values on the droplet charge of secondary 5% corn oil emulsions (0.5 wt % LF; 0.5 wt % alginate, LMP or HMP; 10 mM phosphate buffer; pH 7). (**b**) Effect of varying pH values on the difference in droplet charge between the primary emulsion (5% corn oil, 0.5% LF, 10 mM phosphate buffer, pH 7) and the secondary emulsions (5% corn oil; 0.5% LF; 0.5% alginate, LMP or HMP; 10 mM phosphate buffer, pH 7).

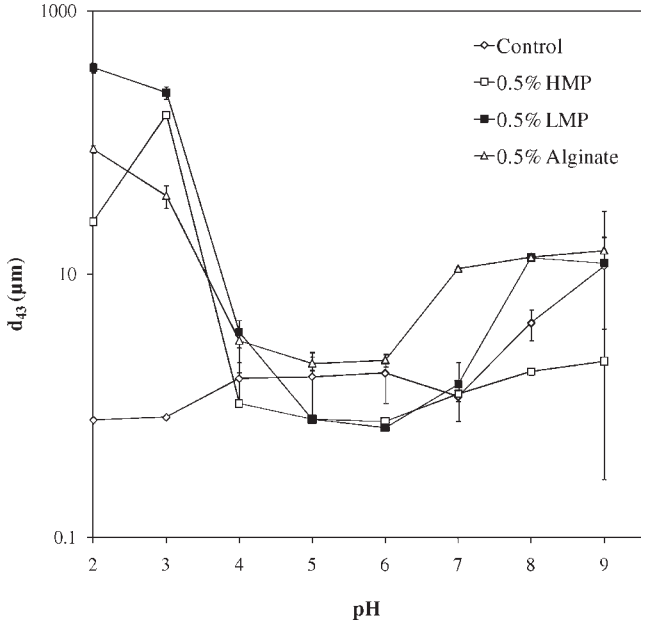

Figure 6. Effect of varying pH values on the mean droplet size of secondary 5% corn oil emulsions (0.5 wt % LF; 0.5 wt % alginate, LMP or HMP; 10 mM phosphate buffer; pH 7).

therefore these anionic polysaccharides become less negatively charged as the pH is adjusted below this value ($-CO_2^- + H^+ \leftrightarrow -CO_2H$). The net charge on the lipid droplets coated by alginate, LMP and HMP was slightly positive at pH 2 with ζ-potentials of 1.8, 1.8, and 0.1 mV, respectively (**Figure 5a**), however they were negative at pH 3 and above.

The primary emulsion had a relatively low mean particle diameter (d_{43} = 0.7 to 1.7 μm) from pH 2 to 7 (**Figure 6**). However, there was an appreciable increase in mean particle diameter to 4 and 11 μm when the emulsions were adjusted to pH 8 and 9, respectively. One might expect the greatest degree of droplet aggregation to occur around the point of zero charge, since this would be the pH where the electrostatic repulsion between the droplets would be weakest. Nevertheless, we observed greater droplet aggregation at higher pH values, even though the droplets became more negatively charged. The reported p*I* of LF is around pH 8, which may account for the observed increase in aggregation around this pH. It is possible that any bound phosphate buffer ions helped prevent droplet aggregation by increasing the hydration repulsion between the lipid droplets. When the pH is increased, the phosphate ions are released, which reduces this effect. Despite the differences in particle size, all the primary emulsions were stable to creaming even after 7 days storage at ambient temperature with no visible evidence of phase separation (data not shown).

The addition of anionic polysaccharides to the emulsions had a major impact on their pH-stability (**Figure 6**). At pH 2 and 3, all of the secondary emulsions had larger particle diameters (d_{43} > 20 μm) than the primary emulsions (d_{43} < 1 μm) indicating that extensive droplet aggregation occurred. A possible reason for droplet aggregation at acidic pH is the relatively weak electrostatic repulsion between the droplets when they have a low net charge (**Figure 5a**). In addition, the electrostatic attraction between the anionic polysaccharides and the cationic LF-coated lipid droplet surfaces will be weakened in this pH range because of the partial loss of negative charge on the polysaccharides below their pK_a values. Hence, a single polysaccharide molecule may have partially detached from one lipid droplet surface and become attached to another lipid droplet, leading to bridging flocculation. At pH 4 to 6, the presence of the anionic polysaccharides led to similar or smaller particle sizes than in the primary emulsions, indicating that the polysaccharide coatings either improved droplet stability or at least did not adversely affect it (**Figure 6**). At pH 7 and 9, there were appreciable differences between the primary and secondary emulsions depending on polysaccharide type. Alginate and LMP both led to an increase in mean particle diameter over that of the primary emulsion, whereas HMP led to a decrease (**Figure 6**). At these higher pH values the LF-coated droplets become slightly negatively charged (**Figure 5a**), but there is still evidence of adsorption of anionic polysaccharides to their surfaces since Δζ is negative (**Figure 5b**). The net charge on the droplets in the secondary emulsions is relatively high (|ζ| > 30 mV), and therefore droplet aggregation cannot be attributed to a reduction in electrostatic repulsion. It is possible that the affinity of the anionic polysaccharides for the anionic LF-coated lipid droplets in this pH range is relatively weak, which leads to some bridging flocculation as discussed earlier for low pH values. LMP and alginate have a higher linear charge density than HMP, which may account for

the greater tendency for droplet aggregation to occur by this bridging mechanism.

Salt-Stability of Emulsions. Practically, it is important to understand how various kinds of salts impact the stability and physical properties of emulsions. For example, there is a tendency to reduce the sodium content in some foods to reduce the risk of hypertension, whereas there is a tendency to increase the calcium content of some foods to improve bone health. In this section, we therefore examine the impact of sodium and calcium on the stability and physicochemical properties of primary and secondary emulsions.

The effects of varying NaCl concentration on the ζ-potential and mean particle diameter of the emulsions were measured at pH 7 (**Figure 7**). In the absence of salt, the initial charge on the lipid droplets was determined by their interfacial composition: $\zeta = -17$, -37, -54, and -74 mV for primary, HMP, LMP and alginate, respectively. There was a slight decrease in the magnitude of the ζ-potential on the droplets in the primary emulsions when the NaCl concentration was increased from 0 to 200 mM (**Figure 7a**), which can be attributed to electrostatic screening effects (*45*). On the other hand, there was little change in the ζ-potential on the droplets in the secondary emulsions when the salt concentration was increased. This may have occurred because electrostatic screening effects were compensated by changes in interfacial structure or composition induced by alterations in the magnitude and range of electrostatic interactions (*37*). There was also little change in the mean particle diameters (**Figure 7b**) or any evidence of visible creaming (data not shown) of the primary or secondary emulsions with increasing salt concentration, which suggests they were all stable to droplet aggregation.

The influence of $CaCl_2$ concentration on the ζ-potential and mean particle diameter of the emulsions was also measured at pH 7 (**Figure 8**). There was little change in the ζ-potential of the primary or secondary emulsions with increasing $CaCl_2$ concentration (**Figure 8a**). A number of physicochemical mechanisms may account for this: (i) little or no ion binding occurred to the droplet surfaces; (ii) both cationic calcium and anionic chloride ions bound, so that there was some charge compensation; (iii) ion binding changed the thickness or structure of the polysaccharide layer at the droplet interface; (iv) ion binding caused an alteration in the composition of the polysaccharide layer in the case of secondary emulsions.

The influence of calcium concentration on the stability of the emulsions was strongly dependent on their initial interfacial composition (**Figure 8b**). The primary emulsions, containing LF-coated lipid droplets, exhibited an appreciable increase in mean particle diameter with increasing calcium concentration: after 1 day storage d_{43} increased from around 1 to 4 μm when $CaCl_2$ increased from 0 to 200 mM. After 3 days storage, considerable destabilization and phase separation were observed in primary emulsions containing 50 mM or more of $CaCl_2$ (data not shown). The instability of the emulsions to calcium addition may be attributed to electrostatic screening and ion binding effects, which decrease the electrostatic repulsion and promote bridging flocculation (*45*). The alginate-secondary emulsion was stable up to 50 mM $CaCl_2$, having a particle size of around 0.6 to 1 μm; but further addition of $CaCl_2$ caused the droplets to aggregate and the emulsion to form a solid mass which could not be analyzed by light scattering. The LMP-secondary emulsions could also not be analyzed by light scattering because they also formed a solid mass upon addition of 25 mM $CaCl_2$ or higher. The HMP-secondary emulsions were stable up to 50 mM $CaCl_2$, having particle diameters ranging from 1 to 3 μm, but they also exhibited extensive flocculation at higher $CaCl_2$ levels. The extensive droplet aggregation observed at high $CaCl_2$ concentrations

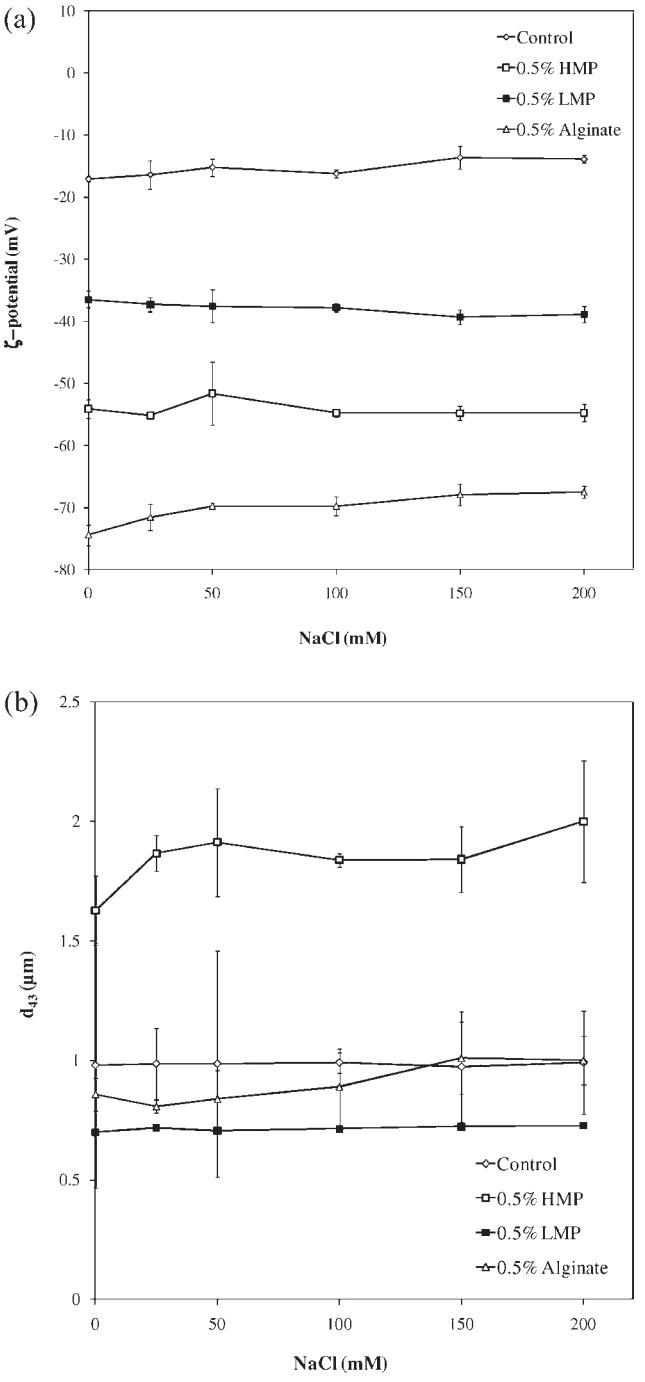

Figure 7. (**a**) Effect of varying NaCl concentrations on the droplet charge of secondary 5% corn oil emulsions (0.5 wt % LF; 0.5 wt % alginate, LMP or HMP; 10 mM phosphate buffer; pH 7). (**b**) Effect of varying NaCl concentrations on the mean droplet size of secondary 5% corn oil emulsions (0.5 wt % LF; 0.5 wt % alginate, LMP or HMP; 10 mM phosphate buffer; pH 7).

can partly be attributed to ion binding effects and screening of the electrostatic repulsion between oil droplets (*46*−*49*). However, there may also be some additional physicochemical phenomenon contributing to emulsion instability and gelation in the presence of anionic polysaccharides. Calcium ions can bind to alginate and LMP to form gels in aqueous solutions, and may also promote some self-association of HMP. Consequently, part of the observed aggregation in the emulsions may be attributed to the direct effect of the calcium ions on the anionic polysaccharides. Indeed, the primary emulsions had better stability to calcium

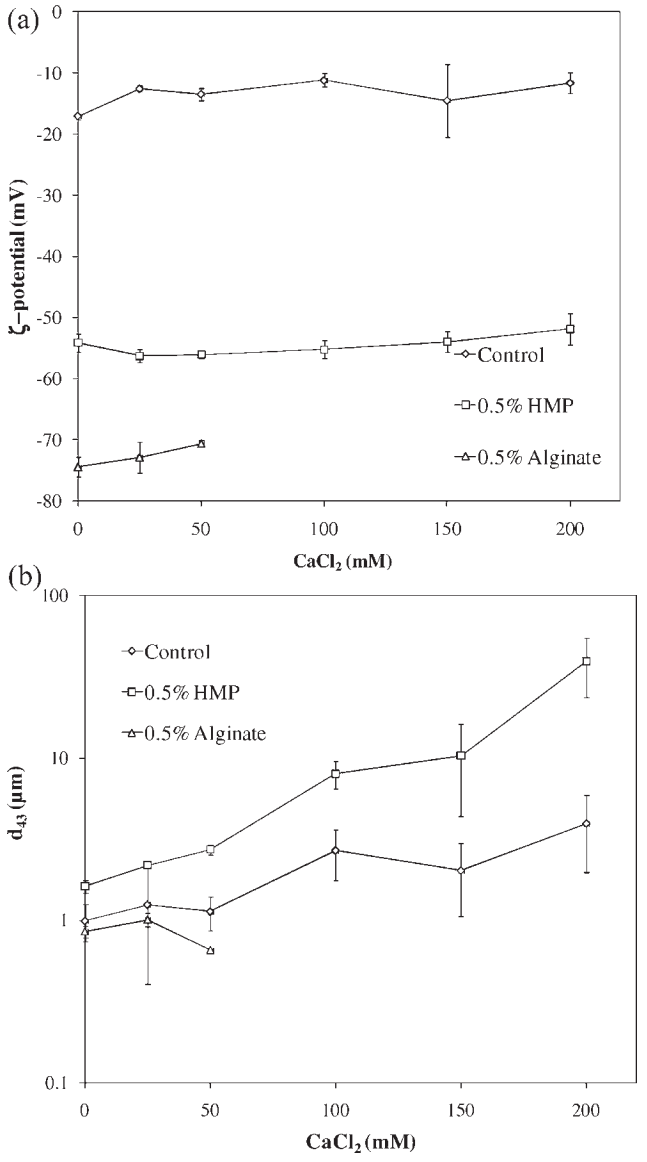

Figure 8. (**a**) Effect of varying $CaCl_2$ concentrations on the droplet charge of secondary 5% corn oil emulsions (0.5 wt % LF; 0.5 wt % alginate, LMP or HMP; 10 mM phosphate buffer; pH 7). (**b**) Effect of varying $CaCl_2$ concentrations on the mean droplet size of secondary 5% corn oil emulsions (0.5 wt % LF; 0.5 wt % alginate, LMP or HMP; 10 mM phosphate buffer; pH 7).

addition than the secondary emulsions. If one wants to prepare stable emulsions containing calcium, it may therefore be important to avoid the use of anionic polysaccharides. On the other hand, if the emulsions are going to be utilized in a highly viscous product, or if gelation is desirable, then this may not be a problem.

This study has shown that the stability and functional performance of oil-in-water emulsions containing lactoferrin-stabilized lipid droplets can be altered by adding anionic polysaccharides to form electrostatic interfacial complexes. Covering LF-stabilized droplets with polysaccharide coatings led to the formation of emulsions that contained anionic droplets across a wide range of pH values (pH 3 to 9), which may have important implications for certain practical applications. For example, cationic droplets may precipitate when added to systems containing anionic ingredients, or they may promote bitterness/astringency due to interactions with anionic mucin molecules in the mouth. Thus the addition of anionic polysaccharides to LF-stabilized emulsions may be used to overcome these negative effects. We also found that polysaccharide coatings improved the thermal stability of LF-stabilized emulsions. On the other hand, polysaccharide coatings could promote droplet aggregation in the presence of high calcium levels and at certain pH values, which may limit their application in certain products. High methoxyl pectin provided improved emulsion stability over a wider range of environmental stresses than either low methoxyl pectin or alginate.

In future studies, it would be useful to establish the phase diagrams of lactoferrin−anionic polysaccharide pairs in aqueous solutions, i.e., the impact of pH, ionic strength, lactoferrin: polysaccharide ratios, and temperature on complex formation. This information would be useful in interpreting the data on more complex systems, such as the emulsion−polysaccharide systems studied in this work. In particular, it could be used to design polysaccharide−LF coated lipid droplets in a more systematic fashion. In this study, we formed electrostatic complexes between the protein and polysaccharide after the emulsions were formed. In future studies, it would be interesting to examine the impact of forming the electrostatic complexes before homogenization on the formation and stability of the resulting emulsions. Overall, this study and other such studies provide insights into the formulation of complex food emulsions which could be used for food and biotechnological applications.

ABBREVIATIONS USED

LF, lactoferrin; IP, isoelectric point; PSD, particle size distribution; LMP, low methoxyl pectin; HMP, high methoxyl pectin.

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