**Characterization of mucin – lipid droplet interactions:
Influence on potential fate of fish oil-in-water emulsions under
simulated gastrointestinal conditions**

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Abstract

The influence of mucin upon the properties and stability of oil-in-water emulsions containing lipid droplets coated by either a protein (caseinate) or non-ionic surfactant (Tween 80) was studied. At pH values below the isoelectric point of emulsion (*e.g.*, stomach conditions), anionic mucin molecules bound to the surfaces of cationic caseinate-coated lipid droplets, which led to extensive bridging flocculation and droplet coalescence. Conversely, at pH values **above** the isoelectric point (*e.g.*, mouth conditions), there was an electrostatic repulsion between anionic mucin molecules and anionic caseinate-coated lipid droplets. At this pH, relatively high levels of mucin promoted droplet flocculation through a depletion mechanism. The extent of droplet flocculation and/or coalescence in the systems depended on both the droplet and mucin concentration. Emulsions stabilized by the non-ionic surfactant were not susceptible to bridging flocculation, and only exhibited depletion flocculation at high mucin levels, which was attributed to the strong steric repulsion between them. These results have important implications for understanding the gastrointestinal fate of lipid droplets, which is critical for the development of foods with improved nutritional attributes.

Keywords

Emulsion; Mucin; Depletion; Bridging; Gastrointestinal tract; Delivery systems

1. Introduction

Emulsified lipid droplets are integral components of many commonly consumed food products, such as beverages, creams, desserts, dips, dressings, and sauces. In addition, lipid droplets may be formed within the mouth or gastrointestinal tract after ingestion of other types of lipid-rich foods, such as butters, chocolates, margarines, and meats. An understanding of the gastrointestinal fate of lipids may lead to the design of foods with improved sensory characteristics and nutritional benefits. For example, foods can be designed to have a creamy mouth-feel at a reduced total fat level, to induce satiety at lower calorie levels, or to improve the bioavailability profile of nutrients, vitamins, or nutraceuticals.

The behavior of lipids within the human gastrointestinal tract (GIT) is influenced by their interactions with mucins, which are a family of heavily glycosylated and relatively large proteins (Bansil & Turner, 2006; Strous & Dekker, 1992). These glycoproteins are the principal organic constituents of the mucus layer that coats the surfaces of the GIT (Dekker, Rossen, Buller, & Einerhand, 2002), and are typically present in saliva and gastric fluids secreted by the mouth and stomach. Mucins play an important role in the oral processing and digestion of foods, such as lubrication and hydration (Tabak, 1990). Mucins typically consist of oligosaccharide side-chains ($\approx 80\%$) attached to a protein core ($\approx 20\%$) in a “bottle brush” configuration (Bansil & Turner, 2006). The oligosaccharide chains contain a complex mixture of constituents *e.g.* N-acetylgalactosamine, N-acetylglucosamine, fucose, galactose, sialic acid, and traces of mannose and sulfate. The steric repulsion between the hydrated carbohydrate groups and between carbohydrates and the peptide backbone result in an extended conformation of mucins (Jentoft, 1990).

Nevertheless, mucins still have sufficient flexibility to form loops and hairpin turns (Round, et al., 2002). Mucins may also associate with each other via disulfide bonds or non-covalent bonds (Sheehan, et al., 2004), leading to aggregates with large molar weights, *i.e.* 0.5 to 20 MDa (Coles, Chang, & Zauscher, 2010).

The interactions of lipid droplets and mucin have been the subject of a number of previous studies. Mucin was shown to promote the flocculation of β -lactoglobulin or β -casein coated lipid droplets at pH 6.7, which was attributed to a depletion mechanism (Vingerhoeds, Blijdenstein, Zoet, & van Aken, 2005). Mucin can improve the salt-stability of lactoferrin-stabilized lipid droplets (pH 6.8) as a result of the electrostatic binding of this anionic glycoprotein to the cationic protein-coated droplet surfaces (Sarkar, Goh, & Singh, 2009). The addition of mucin was reported to promote the flocculation of β -lactoglobulin-coated lipid droplets under simulated gastric conditions (pH 1.5 and with pepsin) (Sarkar, Goh, & Singh, 2010). A study investigated the influence of pH on the aggregation of caseinate-coated lipid droplets in the presence of mucin, and reported that droplet flocculation occurred at pH 7 through a bridging mechanism, but at pH 1 or 3 due to a bridging mechanism (Ritzoulis, et al., 2012).

The aim of the current study was to provide further insights into the impact of mucin on the potential gastrointestinal fate of lipid droplets. In particular, we investigated the impact of emulsifier type (caseinate or Tween 80), pH, lipid droplet concentration, and mucin level on mucin-droplet interactions. The influence of mucin was evaluated using a combination of creaming stability, particle size, particle charge (ζ -potential), and confocal microscopy. This study provides information that can be used to provide a more

thorough understanding of the potential behavior of lipid droplets in the GIT, which may lead to the development of food products with improved sensory or nutritional profiles.

2. Experimental Methods

2.1. Materials

Commercial porcine gastric mucin was obtained from Sigma-Aldrich (Type II, Lot number: SLBM5328V; St. Louis, MO). Porcine gastric mucin has been reported to consist primarily of MUC5AC and MUC6 (Nordman, et al., 2002). Although it is desirable to perform experiments on human mucin, porcine gastric mucin has been widely utilized as a model system due to the similarities between porcine and human gastric mucin MUC5AC (Turner, Bhaskar, Hadzopoulou-Cladaras, & LaMont, 1999). Besides, porcine MUC5AC also has large similarities to human salivary mucin MUC5B (Offner, Nunes, Keates, Afdhal, & Troxler, 1998; Van Klinken, Einerhand, Buller, & Dekker, 1998).

Sodium caseinate was obtained from MP Biomedicals LLC. (Solon, OH). As stated by the supplier, the protein and moisture content of the caseinate were 91.4% and 5.0% respectively. Fish oil (Ropufa 30 n-3 food oil) was obtained from DSM Nutritional Products Ltd. (Basel, Switzerland). The oil contained 101mg of EPA/g of oil, 148mg of DHA/g oil, and a total ω -3 PUFA of 312 mg/g of oil. Other chemicals were analytical grade and purchased from Sigma-Aldrich (St. Louis, MO). Deionized water was used for the preparation of all solutions.

2.2. Solution preparation

A stock buffer solution was prepared by dispersing 5 mM sodium phosphate monobasic and 0.02 wt% sodium azide in water and then adjusting to pH 7.0 using sodium hydroxide. Emulsifier solutions were prepared by dispersing 2 wt% caseinate or 2 wt% Tween 80 in the stock buffer, and stirring for 3 h to ensure complete dissolution. Mucin solution at 5 wt% was prepared by dispersing mucin into the stock buffer and stirring overnight.

2.3. Emulsion preparation

Stock emulsions (final composition: 20 wt% fish oil, and 1 wt% caseinate or Tween 80) were prepared by homogenizing 20 wt% fish oil, 50 wt% emulsifier solution, and 30 wt% stock buffer using a high-speed blender (M133/1281-0, Biospec Products, Inc. ESGC, Switzerland) for 2 min at 10000 rpm, followed by six passes at 12 kpsi through a high pressure homogenizer (Microfluidizer M-110L, Microfluidics, Newton, MA). The volume-weighted mean diameter (d_{43}) measured by static light scattering (Section 2.5) of the prepared caseinate and Tween 80 emulsions were 323 nm and 313 nm, respectively.

For investigating the influence of pH, emulsions with 2 wt% fish oil, 0.1 wt% caseinate or Tween 80, and 0.05 wt% mucin were prepared by mixing stock emulsion, mucin solution, and stock buffer solution together. The emulsions were stirred for 10 min using a magnetic stirrer at ambient temperature, and their pH was adjusted to final values ranging from 2.0 to 7.0 by using HCl or NaOH solutions.

For determining the influence of emulsion and mucin concentration, the stock emulsion was diluted with different ratios of mucin solution and stock buffer to prepare

samples with a final composition of 0.5 to 5.0 wt% fish oil and 0 to 2.0 wt% mucin. After 10 min stirring, the emulsions were adjusted to pH 6.8 or 2.5 using HCl solution.

The emulsions were then stored for 24 h at room temperature prior to all measurements.

2.4. Creaming stability

Five milliliters of emulsion were transferred into a test tube (10 mm internal diameter and 100 mm height), and tightly sealed with a plastic cap. After 24 h storage, some of the emulsions had an optically opaque “cream layer” at the top and a transparent or turbid “serum layer” at the bottom. The height of the cream layer (H_C) and the total height of the emulsion (H_E) were measured. The extent of creaming was depicted by a creaming index $= (H_C/H_E) \times 100\%$. Photos were taken using a digital camera.

2.5. Particle size

The particle size distribution of emulsions was measured using a static light scattering instrument (Mastersizer 2000, Malvern Instruments, Worcestershire, United Kingdom). Samples were diluted in appropriate buffer solutions (same pH as sample) by adding small aliquots into a measurement chamber with stirring at 1200 rpm (Hydro 2000SM, Malvern Instruments, Worcestershire, United Kingdom). A refractive index of 1.330 was used for the phosphate buffer (continuous phase) and 1.481 for the fish oil (dispersed phase). The mean particle size was reported as the volume-weighted mean diameter: $d_{43} = \sum n_i d_i^4 / \sum n_i d_i^3$, where n_i is the number of particles with diameter d_i .

2.6. Electrical charge

The ζ -potential was determined using a particle micro-electrophoresis device (Nano-ZS, Malvern Instruments, Worcestershire, UK). Emulsions were diluted to a droplet

concentration of approximately 0.05 wt% using buffer solutions with the same pH as the sample being analyzed (prepared from the stock buffer by addition of HCl or NaOH solution). The ζ -potential of the mucin solution was determined at a concentration of 0.5 wt% mucin so as to obtain a light scattering signal strong enough to measure.

2.7. Microstructure

The microstructure of the samples was characterized using confocal scanning laser microscopy. A Nikon D-Eclipse C1 80i microscope (Nikon, Melville, NY) equipped with a 60 $\times$ oil immersion objective lens was utilized. Before analysis, 2 mL samples were mixed with 0.1 mL Nile Red solution (1 mg/mL in ethanol) and 0.1 mL fluorescein isothiocyanate (FITC) solution (1 mg/mL in dimethyl sulfoxide) to stain the oil and protein, respectively. The excitation and emission spectrum for Nile Red were 543 nm and 650 nm, and for FITC were 488 nm and 515 nm. Images were captured and analyzed using instrumental software (NIS-Elements, Nikon, Melville, NY).

2.7. HPSEC-MALLS-RI analysis

The weight-average molecular weight (M_w) and radius of gyration (R_g) of mucin were determined using high performance size exclusion chromatography combined with laser light scattering, and differential refractive index detectors (HPSEC-MALLS-RI). The HPSEC-MALLS-RI system consisted of an HPLC unit (Agilent 1200, Agilent Technologies, USA, Santa Clara, CA), a size exclusion column (Shodex OHPAK SB-806 HQ, 8.0 mm $\times$ 300 mm, Shodex, Japan), a Dawn Heleos-II multi-angle laser light scattering instrument (Wyatt Technology Co., Santa Barbara, CA), and a Opti-lab T-rEX differential refractive index detector (Wyatt Technology Co., Santa Barbara, CA). A 5 mM phosphate buffer solution (pH 7.0) containing 0.15 M NaCl and 0.02 % (w/v) NaN₃

was employed as the mobile phase. The flow rate was set to 0.4 mL/min, and the temperature of column and detectors was set at 25 °C. Mucin was fully dispersed in the mobile phase at a concentration of 5 mg/mL and filtered through a 0.2 µm syringe membrane. A 20 µL sample was loaded into the column for analysis. Data were collected and processed using instrumental software (ASTRA, Version 6.1.2.84, Wyatt Technology Co., Santa Barbara, CA). The curve fitting for calculating M_w was based on the first-order Zimm method. A dn/dc of 0.165 ml/g was used according to previous reports (Henderson, et al., 2014; Jumel, Fiebrig, & Harding, 1996). It should be noted that passing the mucin solution through a 0.2 µm syringe membrane prior to analysis may remove some large self-aggregated structures that may have been present in the original sample. Nevertheless, this procedure had to be carried out to avoid blocking the size exclusion column.

2.8. Statistical Analysis

All experiments were performed twice, and means and standard deviations were calculated from these measurements.

3. Results and discussion

3.1. Characterization of mucin

The mucin used in this study was found to be composed of several fractions with different molar masses and sizes as indicated in the HPSEC chromatogram (Fig. 1). As expected from the principles of size exclusion chromatography, fractions with molecular weights (M_w) ranging from high to low were successively eluted from the column. The calculated M_w of the two early eluted major fractions were around 4684 ± 27 kDa

(designated as “A” in Fig. 1) and 1169 ± 9 kDa (designated as “B”), and their radius of gyration (R_g) were 92.2 ± 0.3 nm and 50.8 ± 0.7 nm, respectively. The average values for M_w and R_g of these two fractions were 2981 ± 21 kDa and 75.1 ± 0.4 nm, which were in agreement with the values reported previously for porcine gastric mucin (Jumel, et al., 1996; Sarkar, et al., 2010; Vingerhoeds, et al., 2005). The last major positive peak and the subsequent negative peak found at the end of the HPSEC-RI chromatograms might be attributed to ions and impurities in the mucin product.

The mucin was negatively charged at neutral pH (Fig. 2), and its ζ -potential was -15.4 mV at pH 7. The electrical characteristics of mucin are reported to be determined by structural elements in both the polypeptide backbones (*e.g.*, aspartic and glutamic acid residues, $pK_a \approx 4$) and in the oligosaccharide side chains (*e.g.*, sialic acid residues, $pK_a \approx 2.6$; and, sulfate groups, $pK_a \approx 1$) (Cao, et al., 1999; Maleki, Lafitte, Kjoniksen, Thuresson, & Nystrom, 2008; Waigh, et al., 2002). The ζ -potential of mucin decreased from negative to close to zero (-0.9 mV) when the pH was reduced from 7 to 2. This result was in accordance with previous studies that have shown that porcine gastric mucin loses much of its charge around pH 2 or 3 (Lee, Muller, Rezwan, & Spencer, 2005; Ritzoulis, et al., 2012).

3.2. Influence of mucin on the caseinate emulsion

3.2.1 The role of pH

It has been widely reported that pH is a major factor determining the stability of emulsions containing protein-coated lipid droplets due to its impact on their electrical properties and their colloidal interactions (McClements, 2004). The ζ -potential of the caseinate-coated lipid droplets in the fish oil-in-water emulsions changed from negative

(-41.9 mV) at pH 7 to positive (+28.1 mV) at pH 2 (Fig. 2). This change is a result of the net electrical charge of the caseinate molecules adsorbed to the surfaces of the lipid droplets changing from negative to positive when the pH was adjusted from above to below its isoelectric point. The ζ -potential of the caseinate emulsion reached 0 mV at a pH somewhere between 4 to 5, which was in line with the reported isoelectric point of caseinate, *i.e.*, $pI \approx 4.6$ (Khaldi & Shields, 2011).

The caseinate emulsion was physically stable at pH values sufficiently far from the protein's isoelectric point (*i.e.*, pH 7 to 6 and pH 3 to 2), as demonstrated by the high creaming stability (Figs. 3A and S1A) and small mean particle size (Fig. 3B). This good stability can be attributed to a combination of electrostatic and steric repulsion between the protein-coated droplets. Extensive droplet aggregation was observed in the emulsions at pH 4 and 5, as demonstrated by an increase in creaming index (Figs. 3A and S1A) and particle size (Fig. 3B). This type of isoelectric flocculation can be attributed to the relatively low magnitude of ζ -potential on the protein-coated lipid droplets (Fig. 2), which results in an electrostatic repulsion that is not strong enough to overcome the van der Waals attraction and thereby prevent droplet aggregation (McClements, 2004).

The pH also exerted a significant influence on the stability of the caseinate emulsions containing mucin. At pH values below 5, these emulsions were highly unstable to creaming and contained large aggregates (Figs. 3A and S1B). Although the emulsions with and without mucin were both unstable at pH 5 and 4, their appearances were distinctly different (Fig. S1). For instance, in the presence of mucin a turbid serum layer was observed in the emulsions at pH 5, rather than the clear serum layer observed in the absence of mucin. In the pH range from 3 to 2, the emulsions containing mucin were

highly unstable to droplet aggregation and creaming, whereas those containing no mucin were stable. The mean particle diameter (Fig. 3B) decreased as the pH was reduced from 4 to 2. This effect may have occurred because of a weakening of the attractive interactions between the protein-coated lipid droplets and mucin at lower pH values due to the reduction in negative charge on the mucin.

The ζ -potential of caseinate emulsions *versus* pH in presence of mucin was also determined (Fig. 2). Since the mucin molecules scattered light much more weakly than the emulsion droplets (determined in a separate preliminary experiment), the measured ζ -potential of droplet-mucin mixtures was assumed to be that of the droplets. It was noted that at pH 7 to 6, the ζ -potential of the particles in the emulsions with or without mucin was fairly similar. Since the caseinate-coated lipid droplets and mucin were both negatively charged in this pH range, it was supposed that there was a relatively strong electrostatic repulsion between them that kept the droplets and mucin isolated. Consequently, the electrical characteristics of the caseinate-coated lipid droplets were similar in this pH range. Nevertheless, the influence of mucin on the electrical properties of the emulsions became evident at pH 4 and below, which was in good agreement with the critical pH where changes in the creaming stability and droplet size occurred. From pH 4 to 2, the ζ -potential of the particles in the emulsions containing mucin was between those of the plain emulsion and mucin, suggesting that mucin adsorbed to the surfaces of the lipid droplets in this pH range. This effect can be attributed to electrostatic attraction between the anionic mucin molecules and cationic patches on the surfaces of the protein-coated lipid droplets (Guzey & McClements, 2006). The high amount of droplet aggregation observed in the caseinate emulsions containing mucin at low pH values may

be attributed to the reduction in the net particle charge after mucin adsorption (charge neutralization) or due to the ability of mucin molecules to bind to more than one droplet (bridging flocculation). A similar phenomenon has been reported in the interactions of other anionic polyelectrolytes (*e.g.*, fucoidan or pectin) with caseinate-coated lipid droplets (Chang & McClements, 2015; Surh, Decker, & McClements, 2006).

To gain further insight into the role of mucin on the stability and properties of the emulsions, additional experiments were carried out under conditions where there should be an electrostatic repulsion between the mucin and protein-coated droplets (pH 6.8), and under conditions where there should be a strong electrostatic attraction (pH 2.5). These two pH conditions were selected to simulate the pH conditions in the mouth and stomach respectively (Gal, Fovet, & Adib-Yadzi, 2001). In these experiments, the concentrations of both the mucin and lipid droplets were varied to simulate the conditions that might occur within the GIT when different foods are consumed.

3.2.2 Impact of mucin and droplet levels in the presence of electrostatic repulsion

At pH 6.8, the caseinate-coated lipid droplets and mucin molecules are both highly negatively charged and so there should be a strong electrostatic repulsion between them (Fig. 2). Under these conditions, the emulsions containing 0.5 wt% lipid droplets were stable to creaming at all mucin concentrations studied (Figs. 4 and S2). The emulsions containing 1, 2 and 5 wt% lipid droplets were stable at low levels of mucin (0.01-0.1 wt%), but not at levels ≥ 0.2 wt% (Figs. 4 and S2). The rapid creaming observed at these higher mucin levels could be attributed to an increase in particle size caused by depletion flocculation (Vingerhoeds, et al., 2005). Depletion flocculation occurs when there is a relatively high concentration of non-adsorbing biopolymers present in the aqueous phase

surrounding the lipid droplets (McClements, 2015). The biopolymer molecules cannot get closer to the lipid droplet surfaces than their radius of hydration, and therefore there is an “exclusion zone” surrounding each droplet of about these dimensions.

The particle size of the diluted emulsions was not altered by mucin addition under conditions where the emulsions were stable to creaming, *i.e.*, low mucin levels. However, an appreciable increase in particle size was observed in the diluted flocculated emulsions *e.g.*, 3.3 μm for the emulsions containing 1 wt% lipid droplets and 1 wt% mucin. Dilution of emulsions containing droplets flocculated by a depletion mechanism usually causes the flocs to become dissociated because of a weakening of the osmotic driving force. However, droplet coalescence is often promoted when the droplets are brought into close proximity for extended periods due to depletion flocculation (Klinkesorn, Sophanodora, Chinachoti, & McClements, 2004; Ye, Hemar, & Singh, 2004). Consequently, the observed increase in droplet size above the critical flocculation concentration may be attributed to droplet coalescence. Observation of the plain caseinate emulsions using confocal microscopy (Fig. 5A) showed that the plain caseinate emulsions consisted of fine droplets that were homogeneously distributed through the sample and rapidly moved around due to Brownian motion. Conversely, the emulsions containing relatively high levels of mucin (*e.g.*, 1 wt% lipid droplets and 1 wt% mucin) appeared to be composed of a mixture of individual lipid droplets and small flocs (Fig. 5B). After 10-times dilution with stock buffer (pH 6.8), the flocs disappeared (Fig. 5C), which can be attributed to a reduction in the osmotic pressure between the droplets, and therefore a reduction in the tendency for depletion flocculation to occur (Klinkesorn, et al., 2004).

It should be noted the mucin concentration in human saliva is often relatively low, *i.e.*, about 0.02 wt% (Vingerhoeds, et al., 2005). In addition, the concentrations of the two major types of mucin in human saliva (MUC5B and MUC7) have also been reported to be relatively low (80-500 $\mu\text{g/mL}$ and 10-200 $\mu\text{g/mL}$, respectively) depending on the stimulus (Huq, et al., 2007). Consequently, the mucin levels in real human saliva may be below the levels required to promote depletion flocculation in emulsions.

3.2.3 Impact of mucin and droplet levels in the presence of electrostatic attraction

At pH 2.5, the caseinate-coated lipid droplets are positively charged while the mucin is negatively charged and so there should be an electrostatic attraction between them (Fig. 2). The stability of the caseinate emulsions exhibited a complex dependence on lipid droplet and mucin concentration as represented by a stability map (Fig. 6). For instance, the emulsions containing 1 wt% lipid droplets were stable to creaming at 0 wt% mucin, unstable and showing an opaque serum layer at 0.01, 0.5 and 1 wt% mucin, and unstable and showing a clear or slightly turbid serum layer at 0.02, 0.05, 0.1 and 0.2 wt% mucin (Figs. 6 and S3). The creaming index initially increased and subsequently decreased with the addition of mucin (Fig. 7A). Although the creaming was assessed after 24 h storage for a sufficient phase separation, visible aggregation actually occurred within a few seconds of mixing.

The mean particle diameter (d_{43}) followed a similar pattern with increasing mucin concentration as the creaming index (Fig. 7B). For example, the d_{43} of the emulsions containing 1 wt% lipid droplets, increased from 0.40 μm at 0 wt% mucin to 131 μm at 0.1 wt% mucin, and then slightly decreased to 45 μm at 1.0 wt% mucin. More detailed information about the change in particle size was revealed in the particle size distribution

profiles (inserted picture in Fig. 7B). The emulsions had a monomodal distribution at low droplet size in the absence of mucin, which is indicative of individual lipid droplets. After the addition of a small amount of mucin (0.01 wt% mucin), a population of larger sized particles was observed, which is indicative of aggregation. The mean particle size increased as the mucin concentration was increased further (from 0.01 to 0.1 wt%), but then decreased slightly at higher mucin levels.

The microstructure of the emulsions also changed appreciably depending on the amount of mucin present. Taking the caseinate emulsion containing 1 wt% lipid droplets as an example, the emulsion without mucin consisted of small lipid droplets that were homogeneously distributed throughout the sample (Fig. 8). The addition of mucin clearly promoted droplet flocculation, and the size of the flocs increased with increasing mucin concentration from 0 to 0.1 wt%, which was in good accordance with the particle size measurements made by light scattering (Fig. 7B). At 0.1 and 0.2 wt% mucin, almost all the lipid droplets appeared to be densely packed into large clumps. At 0.5 and 1 wt% mucin, the emulsions also contained large aggregates, but the size of the individual lipid droplets within them was appreciably larger than the original lipid droplets, indicating that coalescence had occurred. Coalescence might have occurred at high mucin levels because the lipid droplets were forced into close contact through a bridging mechanism.

With increasing mucin concentration, the ζ -potential of the caseinate emulsion gradually decreased from around +42 mV to 0 mV and then remained relatively constant (Fig. 9). This charge neutralization effect can be associated with electrostatic binding of negatively charged mucin molecules to the surfaces of the positively charged protein-coated lipid droplets. It is well established that electrostatic attraction between oppositely

charged biopolymers and colloidal particles may lead to bridging flocculation (Dickinson, 2003). This effect occurs because a charged biopolymer molecule is shared between two or more oppositely charged droplets (McClements, 2005).

Lipid droplet concentration also played an important role in the stability and physicochemical properties of the caseinate emulsions containing mucin. Generally, emulsions with higher droplet concentrations showed a slower response to the change in mucin concentration. For instance, for the caseinate emulsions containing 0.5, 1, 2 and 5 wt% droplets, the turbid serum layer turned to a clear one at 0.01, 0.02, 0.05 and 0.1 wt% mucin (Fig. 6), the maximum creaming index was achieved at 0.02, 0.05, 0.1 and 0.2 wt% mucin (Fig. 7A), and the electrical charge reached 0 mV at 0.02, 0.05, 0.1, 0.2 wt% mucin (Fig. 9), respectively. The main reason for this effect can be attributed to the increase in droplet surface area with increasing lipid droplet concentration. Consequently, more mucin molecules are required to saturate the droplet surfaces at high lipid concentration levels.

Special attention should be paid to the emulsions containing low droplet levels and high mucin levels, and vice versa. For instance, in the emulsion containing 0.5 wt% droplets and 1 wt% mucin light scattering indicated that the d_{43} was 33 μm , confocal microscopy indicated the presence of large aggregates containing coalesced oil droplets (Fig. S4B), and visual observation indicated that sedimentation had occurred (Fig. S4A). Sedimentation may have occurred in this system because the aggregates had a high mucin-to-oil ratio, and mucin is more dense than water, whereas oil is less dense. Conversely, the emulsion containing 5 wt% lipid droplets and 0.01 wt% mucin was visibly stable, and its d_{43} value (0.55 μm) was only slightly higher than that of the

emulsion without mucin (0.32 μm), which suggested that there may not have been enough mucin present to promote extensive bridging flocculation at these high lipid levels.

It has been reported in many previous studies that caseinate-coated lipid droplets and anionic polyelectrolytes (*e.g.*, fucoidan, alginate, pectin) can form stable multilayer emulsions when their concentrations are both in appropriate ranges (Chang & McClements, 2015; Pallandre, Decker, & McClements, 2007; Surh, et al., 2006). To form stable polyelectrolyte/caseinate-coated lipid droplets, the concentration of polyelectrolyte should be high enough to saturate the surfaces of the lipid droplets and therefore avoid bridging flocculation, but it should also not be high enough to induce depletion flocculation (McClements, 2005). The concentration range of polyelectrolyte for fabricating stable multilayer emulsion depends on its molecular characteristics (*e.g.*, molecular weight, conformation, and electrical charge), the droplet characteristics (*e.g.*, size and concentration), and the solution environment (*e.g.* pH and ionic strength). In the current study, we did not observe the formation of stable polyelectrolyte/caseinate-coated lipid droplets in the presence of mucin (Figure 7A and 7B). Instead, extensive droplet aggregation and creaming was observed at all mucin levels used, which suggests that the mucin molecules promoted instability through bridging flocculation. Presumably, the large mucin molecules could not adsorb to the droplet surfaces fast enough to prevent bridging flocculation (McClements, 2005).

3.3. Influence of mucin on the Tween 80 emulsion

A wide range of different emulsifiers is available to stabilize food emulsions, which lead to lipid droplets with different interfacial characteristics. We therefore examined the

influence of mucin on the behavior of emulsions stabilized by a food-grade non-ionic surfactant (Tween 80), so as to compare the results with those found for caseinate. In the absence of mucin, Tween 80 emulsions (2 wt% lipid droplets) were stable to creaming from pH 7 to 2 (data not shown), and showed no change in mean particle diameter ($d_{43} = 0.31$ to $0.32\ \mu\text{m}$). The presence of 0.1 wt% mucin did not change the creaming stability or particle size of the Tween 80 emulsions, and the microstructure of emulsions containing this level of mucin were similar at different pH values (Fig. S5). The Tween 80-coated lipid droplets had a moderate negative charge ($-17.8\ \text{mV}$) at pH 7 (Fig. 10). Many studies have reported that lipid droplets coated by non-ionic surfactants may be negatively charged rather than be neutral, which could be attributed to the presence of anionic impurities (such as free fatty acids) or the preferential adsorption of OH^- ions from water (Hsu & Nacu, 2003; Mun, Decker, & McClements, 2007). The magnitude of Tween 80 emulsion decreased appreciably as the pH was decreased, reaching a value close to zero ($0.1\ \text{mV}$) at pH 2 (Fig. 10). Coincidentally, the ζ -potential *versus* pH plots of mucin and Tween-80 coated lipid droplets were very similar to each other, which meant that the mixed system also behaved similarly. Therefore, it was expected that the electrostatic attraction between emulsion droplets and mucin would not happen in the Tween 80 emulsion.

The influence of mucin concentration on the stability of the Tween 80 emulsions (1 wt% lipid droplets) was investigated at pH 6.8 and pH 2.5 to compare to the caseinate emulsions. At pH 2.5, the Tween 80 emulsions were stable at all mucin concentrations studied and no significant change in particle size was observed ($d_{43} = 0.31$ to $0.32\ \mu\text{m}$), which was distinctly different from the behavior of the caseinate emulsion at this pH. At

pH 6.8, some evidence of creaming (creaming index = 6.5%) and droplet coalescence (d_{43} = 2.46 μm) was observed in the sample with the highest mucin concentration (1 wt%). This tendency is similar to the depletion flocculation observed in the caseinate emulsions at the same pH. The confocal microscopy images also support the fact that this high level of mucin induced droplet aggregation in the emulsions (Fig. S6). These aggregates were disrupted when the emulsions containing mucin were diluted (Fig. S6), which again supports a depletion flocculation mechanism. Nevertheless, a higher concentration of mucin was required to induce depletion flocculation in the Tween 80 emulsions than in the caseinate emulsions. This suggests that the repulsive forces activity between the Tween 80-coated droplets were stronger than those operating between the caseinate-coated droplets, presumably because of a stronger steric repulsion associated with the long hydrophilic head groups of the non-ionic surfactant molecules.

The reason that depletion flocculation was observed at pH 6.8 in the Tween 80 emulsions, but not at pH 2.5, may have been due to changes in the dimensions of the mucin molecules. At pH 6.8, the molecules have a relatively high negative charge and therefore they should have a more extended conformation as similarly charged groups try to move apart from each other. In addition, there will be a stronger electrostatic repulsion between the anionic mucin molecules and anionic lipid droplets at pH 6.8, which will prevent them from coming into close proximity. These two effects will increase the thickness of the exclusion zone surrounding the lipid droplets from which the mucin molecules cannot enter. Consequently, there will be an increase in the strength of the osmotic pressure in the system at higher pH values, leading to a stronger tendency for depletion flocculation to occur. Presumably, this effect was larger than the stronger

electrostatic repulsion between the Tween 80-coated droplets that should have occurred at the higher pH values.

4. Conclusions

This study has shown that the stability of caseinate emulsions depended on pH, lipid droplet concentration, and mucin concentration. The anionic mucin molecules interacted with the cationic caseinate-coated lipid droplets at pH 4 and below through electrostatic attraction. At certain droplet and mucin levels, these interactions promoted extensive bridging flocculation and droplet coalescence. There was an electrostatic repulsion between the anionic mucin molecules and the anionic caseinate-coated lipid droplets at pH 5 and above. At pH 7, the caseinate emulsions were stable to droplet aggregation and creaming in the presence of low levels of mucin, but the droplets flocculated at higher levels. This effect was attributed to depletion flocculation associated with an increase in osmotic attraction induced by high levels of non-adsorbed mucin. These results suggest that caseinate-coated lipid droplets are susceptible to depletion flocculation in the mouth, but bridging flocculation in the stomach, with the extent of flocculation depending on lipid and mucin concentration. Tween 80 emulsions were much more stable to the presence of mucin than caseinate emulsions, which was attributed to the relatively strong steric repulsion between lipid droplets coated by this non-ionic surfactant. Overall, these results have important implications for understanding the role of emulsifier type on the potential gastrointestinal fate of emulsions, which is important when designing functional foods for improved health and wellness..

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579

Fig. 1

Fig. 1. HPSEC chromatogram of mucin. The molar mass of eluate was shown by inserted lines (fraction A: long dash line; fraction B: short dash line).

Fig. 2

Fig. 2. Influence of pH on the electrical charge of mucin, caseinate emulsion (2 wt% fish oil, 0.1wt% caseinate), and emulsion with mucin (2 wt% fish oil, 0.1 wt% caseinate, 0.05 wt% mucin).

Fig. 3A

Fig. 3. Dependence of creaming index (A) and particle size d_{43} (B) of caseinate emulsions (2 wt% fish oil, 0.1wt% caseinate) in the absence or presence of mucin (0.05 wt% mucin) on pH.

Fig. 3B

Fig. 3. Dependence of creaming index (A) and particle size d_{43} (B) of caseinate emulsions (2 wt% fish oil, 0.1wt% caseinate) in the absence or presence of mucin (0.05 wt% mucin) on pH.

Fig. 4

Fig. 4. Creaming index of caseinate emulsions with different emulsion concentrations (0.5 wt%: circle; 1 wt%: diamond; 2 wt%: square; 5 wt%: triangle) and mucin concentrations at pH 6.8.

Fig. 5

Fig. 5. Confocal micrographs of 1 wt% caseinate emulsions containing 0 wt% (A) and 1 wt% (B) mucin at pH 6.8. The micrographs of 1 wt% emulsion with 1 wt% mucin after 10 times dilution was shown in (C).

Fig. 6

Fig. 6. Stability diagram of caseinate emulsions at different concentrations plotted against mucin concentration at pH 2.5. Diverse legends were used to show different stability states, *i.e.*, solid circle: stable and no visible phase separation; circle with strips inside: visible cream layer, opaque serum layer; hollow circle: visible cream layer, clear or slightly turbid serum layer; circle with dots inside: precipitation at the bottom.

Fig. 7A

Fig. 7. (A) Dependence of creaming index on caseinate emulsion concentration (0.5 wt%: circle; 1 wt%: diamond; 2 wt%: square; 5 wt%: triangle) and mucin concentration at pH 2.5. (B) Effect of mucin concentration on particle size d_{43} and size distribution (inserted picture) of 1 wt% caseinate emulsion, at pH 2.5. Plots of size distribution were overlaid with an increase of 5%.

Fig. 7B

Fig. 7. (A) Dependence of creaming index on caseinate emulsion concentration (0.5 wt%: circle; 1 wt%: diamond; 2 wt%: square; 5 wt%: triangle) and mucin concentration at pH 2.5. (B) Effect of mucin concentration on particle size d_{43} and size distribution (inserted picture) of 1 wt% caseinate emulsion, at pH 2.5. Plots of size distribution were overlaid with an increase of 5%.

Fig. 8

Fig. 8. The confocal micrographs of 1 wt% caseinate emulsions with different concentrations of mucin at pH 2.5.

Fig. 9

Fig. 9. ζ -potential of caseinate emulsions (0.5 wt%: circle; 1 wt%: diamond; 2 wt%: square; 5 wt%: triangle) with different concentrations of mucin at pH 2.5.

Fig. 10

Fig. 10. Influence of pH on the electrical charge of mucin, Tween 80 emulsion (2 wt% fish oil, 0.1wt% Tween 80), and emulsion with mucin (2 wt% fish oil, 0.1 wt% Tween 80, 0.05 wt% mucin).