**Biopolymer-stabilized conjugated linoleic acid (CLA) oil-in-water emulsions:
Impact of electrostatic interactions on formation and stability of
pectin-caseinate-coated lipid droplets**

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Submitted: August 8, 2016

Revised: September 21, 2016

Journal: Colloids and Surfaces A.

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Abstract

Conjugated linoleic acid (CLA) may be used as a nutraceutical, supplement or pharmaceutical due to its potential health benefits. In this study, CLA oil-in-water emulsions were fabricated using biopolymers as stabilizers: sodium caseinate as an emulsifier and pectin as a coating material. The effect of electrostatic interactions on the formation of these mixed biopolymer coatings, as well as on the stability of the resulting emulsions, was determined by systematically varying pH and ionic strength. Caseinate-coated CLA droplets were stable from pH 7 to 5 due to the strong electrostatic repulsion between them, but aggregated from pH 5 to 3 due to weakening of the electrostatic repulsion near the protein's isoelectric point. Pectin addition greatly improved emulsion aggregation stability, particularly at levels sufficient to saturate the caseinate-coated oil droplet surfaces. Indeed, emulsions with pectin-to-caseinate ratios $> 1:1$ were stable under acidic solution conditions (pH 5 to 3). Ionic strength had a pronounced impact on droplet aggregation, which was pH dependent. At pectin-to-caseinate ratios of 2:1, emulsions were stable against NaCl addition (0 to 200 mM) at pH 3.5 and 4.0 due to strong electrostatic attraction between protein and polysaccharide molecules in the interfacial complexes, but they were unstable to NaCl addition at pH 3.0, 4.5, and 5.0 due to a weaker electrostatic attraction between the biopolymers. Mixed biopolymer-coated emulsions were stable against thermal processing (90 °C, 20 min). These results have important implications for the development of natural CLA delivery systems for utilization in foods and other products.

Keywords: Emulsion; Sodium caseinate; Pectin; Conjugated linoleic acid; environmental stresses; Stability

1. Introduction

Conjugated linoleic acids (CLA) are a class of positional and geometric isomers of octadecadienoic acid (18:2) that naturally occur in dairy products and ruminant tissue [1, 2]. There are eight potential isomers of CLA, but only the *cis*-9, *trans*-11 (C18:2*c*9, *t*11) isomer is commonly found in foods. A great deal of research has been carried out on CLA over the past two decades due to its potentially beneficial biological activities, including anticancer effects, prevention of cardiovascular diseases, body fat reduction, anti-inflammatory properties, improvement of bone mass, and immune related responses [3]. CLA has been approved as generally recognized as safe (GRAS) for certain food applications (milk-based fruit drinks, milk-based beverages, fruit juices, *etc.*) in the United States [1], which has led to growing interest in its utilization as a nutraceutical ingredient into functional food beverage products [4].

Like most other highly non-polar bioactive lipids, CLA cannot simply be dispersed into aqueous-based foods and beverages due to its extremely low water-solubility [5]. The oil phase would rapidly coalesce and separate through gravitational forces leading to a layer of oil on top of the product [6]. For this CLA is usually converted into an oil-in-water emulsion by homogenizing it with an aqueous solution containing a suitable emulsifier [7-9]. The emulsion must be carefully formulated to obtain desirable physicochemical, sensory, and biological attributes in the final product [4]. The selection of an appropriate emulsifier is to avoid emulsion instability, such as gravitational separation, flocculation, coalescence, and phase separation, during processing, storage, and utilization [10]. Many of the emulsifiers commonly used by the food and beverage industry to stabilize emulsions are synthetic small-molecule surfactants (such as Tweens, Spans, DATEM, CITREM, monoglycerides, diglycerides, and sucrose esters) due to their low cost and high efficiency [11]. However, there has recently been considerable interest from food consumers and manufacturers in replacing synthetic emulsifiers with natural alternatives, such as surface-active proteins, polysaccharides, or

phospholipids [5, 12-15].

Surface-active proteins from a variety of sources can be used to form and stabilize emulsions, including those derived from milk, egg, animal tissue, and plants [16]. However, protein-stabilized emulsions are usually more sensitive to environmental stresses (such as alterations in pH, ionic strength, and temperature) than surfactant-stabilized ones [17]. In particular, protein-coated droplets are primarily stabilized against flocculation by electrostatic repulsion, and so they are highly to droplet aggregation near the isoelectric point of the adsorbed proteins [18]. This problem can often be overcome by covering the protein-coated lipid droplets with a layer of ionic polysaccharides, such as gum arabic, pectin, dextran sulphate, carboxymethyl cellulose, fucoidan, alginate, or carrageenan [19-26]. The charged groups on the polysaccharides bind to oppositely charged groups on the adsorbed proteins through electrostatic attraction. The resulting polysaccharide-protein coatings increase the electrostatic and steric repulsion between the droplets, while also reducing the van der Waals attraction, thereby improving the stability of the emulsion to aggregation. Nevertheless, the interfacial complexes have to be designed to avoid bridging or depletion flocculation from occurring in the emulsions [24].

In this study, sodium caseinate (NaCas) was used as an emulsifier to form CLA oil-in-water emulsions and pectin was used to form a protective coating around the droplets. Sodium caseinate ingredients contain a mixture of water-dispersible surface-active proteins that can form and stabilize emulsions [27, 28]. Pectin is a complex polysaccharide that has an anionic backbone with neutral side chains protruding at certain locations [29].

Previous studies have shown that the formation of pectin-caseinate complexes is highly pH-dependent because pH modulates the electrostatic interactions between two biopolymers [30]. Electrostatic complexes are typically formed at pH values 3.0 to 5.5 where the pectin is anionic and the casein has an appreciable number of cationic groups. At higher pH values, there is a strong electrostatic repulsion between the two biopolymers that inhibits complex formation [19, 20, 30-32]. The fact that

protein and polysaccharide molecules are held together by electrostatic attraction under acidic pH conditions means that they are also sensitive to ionic strength, since mineral ions screen electrostatic interactions.

The objective of this study was to investigate the impact of electrostatic interactions on the formation and stability of pectin-caseinate-coated CLA lipid droplets. We hypothesized that physically stable CLA delivery systems could be fabricated from natural stabilizers under certain conditions. This knowledge facilitate the incorporation of CLA into commercial functional food and beverage products.

2. Materials and methods

2.1. Materials

Conjugated linoleic acid triglycerides (CLA oil, Tonalin[®] TG 80) were provided by BASF Corp. (Florham Park, NJ). As stated by the supplier, the CLA was from safflower oil and contained 75-80% conjugated linoleic acid with a 50:50 ratio of active conjugated linoleic acid isomers (C18:2 c9, t11 and C18:2 t10, c12). Moreover, it was reported that the lipids were in the form of approximately 80% triglycerides, 20% diglycerides, and < 1% monoglycerides. High methoxyl pectin (DE) was obtained from the Sigma Chemical Company (Lot #91K1420, St Louis, MO). Spray-dried sodium caseinate (ALANATE 180) was provided by NZMP # 0034-W5166, Lemoyne, PA). The supplier stated that this product contained 92.7% protein, 4.2% moisture, 3.5% ash, 0.8% fat, and 0.1% lactose. Fluorescein isothiocyanate (FITC) isomer I, Nile red, hydrochloric acid (HCl), sodium hydroxide (NaOH), citrate acid anhydrous, trisodium citrate dehydration, sodium azide (NaN₃), and all other chemicals were analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO). Deionized water was used for the preparation of all solutions.

2.2. Solution preparation

Citrate buffer solutions (5 or 10 mM) were prepared by dispersing weighed amounts of trisodium citrate and citrate acid into deionized water and then adjusting

the pH to 5.0, 4.5, 4.0, 3.5, or 3.0. An emulsifier solution containing 1.0 wt% sodium caseinate was prepared by dispersing a weighed amount of powdered protein into deionized water, followed by stirring overnight at room temperature to ensure complete dissolution, and then adjustment to pH 7.0 using 0.1M HCl. A stock 2 wt% pectin solution was prepared by dispersing weighed powdered pectin into citrate buffer solution (pH 5.0, 10 mM), and then stirring overnight at room temperature to ensure complete dissolution before adjusting back to pH 5.0. This stock pectin solution was then diluted with 10 mM citrate buffer solution (pH 5.0) to obtain a series of pectin solutions of varying concentration (0.25, 0.375, 0.5, 1, and 1.5 wt%).

2.3. Emulsion preparation

A primary emulsion containing 10 wt% CLA oil and 0.5 wt% sodium caseinate was prepared by blending 10 wt% CLA oil, 50 wt% emulsifier solution, and 40 wt% deionized water with a high-speed blender (M133/1281-0, Biospec Products, Inc. ESGC, Switzerland) at 10,000 rpm for 2 min. The resulting coarse emulsion was then passed six times through a high-pressure homogenizer (Microfluidizer M-110L, Microfluidics, Newton, MA) at 12 kpsi to reduce the particle size. Secondary emulsions containing 1% CLA oil and different mass ratios of pectin-to-caseinate ($R = 1:2, 3:4, 1:1, 2:1$ and $3:1$) were prepared at pH 5.0, 4.5, 4.0, 3.5 and 3.0. This was achieved by slowly pouring 50 wt% of primary emulsion (10% CLA oil and 0.5% sodium caseinate) into 50 wt% of pectin solution (0.25, 0.375, 0.5, 1, or 1.5%, pH 5.0, 10 mM) with continuous magnetic stirring (300 rpm) at ambient temperature. All the primary emulsion was added into the pectin solution within 5 minutes, and then the resulting emulsions were stirred for another 15 minutes. It should be noted that the method of mixing the emulsion and biopolymer solution together can impact the nature and performance of the interfacial coatings formed, and should therefore be carefully controlled [33]. The pH of the emulsions was then adjusted to 5.0, 4.5, 4.0, 3.5 or 3.0, using 0.1 or 1 M HCl or NaOH solutions. Each emulsion was then diluted with a citrate buffer solution (5 mM) with the corresponding pH value, to obtain secondary emulsions that finally contained 1% CLA oil. This concentration

was utilized to simulate the fat droplet content in nutritional beverages that could be used as suitable delivery systems for this type of bioactive lipid. In order to prevent microbial growth, sodium azide (0.02% w/v) was included in all the secondary emulsions.

To investigate the effects of ionic strength, known amounts of sodium chloride were dissolved in the citrate buffer solutions used to dilute the emulsions. The impact of thermal processing on selected samples (2:1 pectin-to-caseinate ratio) was studied by placing the emulsions in glass test tubes and holding them from 0 to 30 min at 90 °C. The samples were then cooled to ambient temperature before analysis.

2.4. Particle size determination

The size of the particles in the emulsions was determined using static light scattering (Mastersizer 2000, Malvern Instruments, Worcestershire, United Kingdom). An aliquot of sample was injected into the measurement chamber where it was diluted with buffer solution (same pH as sample) to avoid multiple scattering effects. 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 .

2.5. ζ -potential determination

The ζ -potential of the particles was determined using a micro-electrophoresis device that measures the direction and velocity of particle movement (Nano-ZS, Malvern Instruments, Worcestershire, UK). Emulsions were diluted using 5 mM citrate buffer solutions (same pH as sample) prior to analysis to keep the instrument attenuation value between 5 and 10.

2.6. Confocal scanning laser microscopy

The microstructure of the emulsions was examined using confocal scanning laser microscopy with a 60× oil immersion objective lens (Nikon D-Eclipse C1 80i, Nikon, Melville, NY, U.S.). Before each measurement, 2 mL samples were mixed with 10 μ L Nile Red solution (1 mg/mL in ethanol) to dye the oil and 10 μ L FITC

solution (1 mg/mL in dimethyl sulfoxide) to dye the proteins. The excitation and emission wavelength used for FITC were 488 nm and 515 nm, and the excitation and emission wavelength for Nile red were 543 nm and 605 nm, respectively. An aliquot of sample was placed on a microscope slide, covered by a cover slip, and then microstructure images were acquired using specialized image analysis software (NIS-Elements, Nikon, Melville, NY).

2.7. Statistical analysis

All experiments were performed three times and averages and standard deviations were calculated from these measurements.

3. Results and discussion

3.1. Impact of pH and biopolymer ratio on properties of CLA emulsions

There are a number of ways to prepare emulsions stabilized by interfacial polysaccharide–protein complexes [18]. In particular, the complexes can either be formed before or after homogenization of the oil and water phases. In the current study, interfacial complexes were formed by electrostatic deposition of anionic pectin molecules onto cationic patches on the surfaces of preformed caseinate-coated oil droplets. A previous study using sodium caseinate and pectin reported that stable emulsions could be formed using this approach at pH 5 [32], and so similar preparation conditions were used in the present study.

In the absence of pectin, the caseinate-coated droplets were highly unstable to droplet flocculation around pH 5 (**Fig. 1**), which is because this pH is close to the isoelectric point of the adsorbed proteins ($pI \approx 4.6$). In the presence of pectin, all the emulsions were stable against droplet flocculation at pH 5, with the droplets remaining evenly distributed throughout the microscopy images (**Fig. 1**) and the mean particle diameter remaining relatively low (**Fig. 2**). The fact that no droplet aggregation was observed in any of the emulsions suggests that even the lowest level of pectin used was sufficient to saturate the droplet surfaces [31]. Previous research suggested that the layer of pectin molecules surrounding the

caseinate-coated oil droplets prevents flocculation by increasing the electrostatic and steric repulsion between them, while reducing the van der Waals attraction [24]. The mean particle diameter (d_{43}) of the emulsions decreased slightly, from around 0.34 to 0.31 μm , as the ratio of pectin-to-caseinate (R) increased from 1:2 to 3:1 (**Fig. 2**), which may have been due to differences in the packing of the pectin molecules at the oil droplet surfaces. The measured particle diameters in the secondary emulsions were all slightly larger than those in the primary emulsions ($d_{43}=0.30 \mu\text{m}$), which is likely due to the presence of the adsorbed polysaccharide layer. The interaction between the pectin molecules and caseinate-coated droplets observed at pH values slightly above the isoelectric point of the adsorbed protein layer has been reported previously, despite the fact that both the pectin and caseinate molecules have a net negative charge [19, 30-32]. In this pH range, negatively charged groups on the pectin molecules bind to positively charged groups on the protein surfaces, while the electrostatic repulsion between the pectin molecules and droplet surfaces is not large enough to prevent them from coming into proximity.

The size of the particles in many of the emulsions increased appreciably when the pH was decreased (**Figs. 1 and 2**), which can be attributed to droplet flocculation caused by charge neutralization or bridging effects. The ζ -potential of all the pectin-caseinate-coated oil droplets became less negative as the pH was lowered (**Fig. 3**) due to the increase in positive charge on the adsorbed protein molecules ($pI \approx 4.6$) and the decrease in the negative charge on the pectin molecules in the outer coatings ($pK_a \approx 3.5$). At low pH values, the ζ -potential became more negative as the pectin-to-caseinate ratio increased because there were more anionic pectin molecules at the droplet surfaces to neutralize the positive charge arising from the protein-coated droplets. These observations may account for the fact that the critical pH where extensive droplet flocculation was first observed (pH_c) increased as the pectin-to-caseinate ratio decreased, *i.e.*, $pH_c = 4.5$ for $R = 1:2$; $pH_c = 3.5$ for $R = 3:4$; and $pH_c < 3.0$ for $R = 1:1, 2:1$, and $3:1$ (**Figs. 1 and 2**). Interestingly, there was a slight increase in the mean particle diameter of the emulsions containing high levels of pectin when the pH was reduced from 5.0 to 3.5 and 4.0 (**Fig. 2**). This

effect may have been due to an increase in the thickness of the adsorbed pectin layer at low pH values due to a weakening of the electrostatic attraction between the pectin molecules and the caseinate-coated oil droplets. This weakening may have occurred because the carboxylic acid groups on the pectin molecules lost some of their negative charge at low pH values ($-\text{CO}_2^- \Rightarrow -\text{COOH}$; $\text{pK}_a \approx 3.5$).

3.2. Effect of thermal treatment on emulsion stability

To achieve a desirable shelf life, commercial beverages are often subjected to some form of thermal treatment, such as pasteurization or sterilization. For this reason, the impact of thermal treatment on the stability and physicochemical properties of the mixed biopolymer-coated droplets was determined. A pectin-to-caseinate ratio of 2:1 was used for these studies because it produced CLA emulsions that were stable against flocculation over a wide range of pH values at room temperature, without having too much excess polysaccharide present (**Section 3.1**). The influence of thermal treatment (0 to 30 min, 90 °C) on the mean particle diameter (**Fig. 4a**) and electrical potential (**Fig. 4b**) of the CLA emulsions was measured. At all pH values studied, there was no significant difference in the mean particle diameter between the unheated and heated samples. These results suggest that pectin-caseinate-stabilized emulsions were stable against thermal treatment from pH 3.0 to 5.0, which is important for their commercial application. The good thermal stability of oil droplets coated by polysaccharide-protein complexes can again be attributed to the strong electrostatic and steric repulsion between them [34, 35]. The ζ -potential measurements suggest that thermal processing did not cause any major changes in interfacial composition or structure at any of the pH values studied since there was no significant difference in the electrical characteristics of heated and unheated samples (**Fig.4b**).

3.3. Effect of salt on the stability of the emulsions

The role of electrostatic interactions on the stability of the mixed biopolymer-coated droplets was assessed by varying both the ionic strength and pH

of the surrounding aqueous phase. Again, 1 wt% CLA emulsions with a 2:1 pectin-to-caseinate ratio were used because they were stable against flocculation over a wide range of pH values in the absence of salt (**Section 3.1**). All emulsions were heated at 90 °C for 20 min and then immediately cooled in an ice water bath prior to adding the salt to simulate a thermal processing treatment. The ζ -potential, particle size, and morphology of the emulsions were measured after they had been stored at room temperature for either 1 or 14 days.

The addition of NaCl to the emulsions altered the electrical characteristics of the pectin-caseinate-coated oil droplets at all pH values tested (**Fig. 5**). There was a pronounced reduction in the magnitude of the ζ -potential on the droplets with increasing NaCl, which can be attributed to electrostatic screening of the surface charges by counter-ions in the added salt [36]. The stability of the emulsions to droplet aggregation was also highly dependent on pH and NaCl concentration (**Figs. 6 and 7**). At pH 5, extensive droplet aggregation and creaming occurred in the emulsions at 100 mM NaCl and higher. At pH 4.5, a higher level of salt was required to promote droplet aggregation and creaming, *i.e.*, ≥ 200 mM. At pH 3.5 and 4.0, the emulsions were relatively stable against droplet aggregation and creaming at all salt concentrations tested. At pH 3.0, the emulsions were again unstable to droplet aggregation at salt concentrations of 200 mM and higher. These effects can be attributed to changes in the strength of the electrostatic attraction between the pectin molecules and the caseinate-coated oil droplets with pH. At the higher pH values (pH 4.5 and 5.0), the electrostatic attraction is relatively weak because of the low positive charge on the caseinate-coated oil droplets ($pI \approx 4.6$). Consequently, a relatively low level of salt is required to weaken the electrostatic attraction, and cause segments of the pectin molecules to become detached from one droplet and attached to another droplet, thereby promoting bridging flocculation. At pH 3.0, the electrostatic attraction between the pectin molecules and caseinate-coated droplets is again relatively weak, but this time it is because the pectin molecules lose much of their negative charge ($pK_a \approx 3.5$). Conversely, at pH 3.5 and 4.0, there is a relatively strong electrostatic attraction between the pectin

molecules and caseinate-coated droplets because they have relatively strong negative and positive charges, respectively. Consequently, the pectin molecules remain strongly attached to the droplet surfaces, even at high salt levels.

4. Conclusion

In this study, CLA emulsions stabilized by mixed biopolymer coatings were formed by dispersing caseinate-coated droplets in pectin solutions at pH 5.0, so as to promote electrostatic attraction of the polysaccharide molecules to the droplet surfaces. The influence of electrostatic interactions on the stability of the CLA emulsions was then determined by varying the pH and ionic strength of the aqueous solution surrounding the oil droplets. The stability of the emulsion was highly dependent on the pectin-to-caseinate ratio. In general, the aggregation and creaming stability of the emulsions increased as the pectin-to-caseinate ratio increased, which was attributed to the formation of an interfacial complex that increased the steric and electrostatic repulsion between the droplets. At relatively high pectin-to-caseinate ratios (2:1), the emulsions were stable against thermal processing (90°C, 20 min), which may be important for many commercial products. The stability of the mixed biopolymer-coated droplets to salt addition was dependent on the strength of the electrostatic attraction between the pectin molecules and the adsorbed caseinate layer. When the attraction was relatively weak (pH 5.0, 4.5, and 3.0), the addition of NaCl promoted droplet aggregation and creaming, which was attributed to partial detachment of the pectin molecules from the droplet surfaces leading to bridging flocculation. Conversely, when the electrostatic attraction was relatively strong (pH 3.5 and 4.0), the emulsions were stable against NaCl addition because the pectin molecules remained strongly attached to the droplet surfaces. The biopolymer-coated oil droplets developed in this research may be useful for the development of natural delivery systems to incorporate CLA into commercial functional food and beverage products. In future studies it would be interesting to examine the impact of the biopolymer coatings on the chemical stability of the emulsions, since CLA is known to be susceptible to oxidation.

Acknowledgments

Wenjian Cheng greatly thanks Chinese Scholarship Council for support. This material was partly based upon work supported by the Cooperative State Research, Extension, Education Service, USDA, Massachusetts Agricultural Experiment Station (MAS00491) and USDA, NRI Grants (2013-03795).

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Fig. 1. Impact of pH on microstructure of 1 wt% CLA oil-in-water emulsions stabilized by sodium caseinate or different ratios of pectin/caseinate. Images were obtained using confocal fluorescence microscopy.

Fig. 2. Impact of pH on the mean particle diameter of 1 wt% CLA oil-in-water emulsions stabilized by different ratios of pectin-to-sodium caseinate.

Fig. 3. Influence of pH on the ζ -potential of pectin solution (“pectin”), sodium caseinate solution (“NaCas”) and 1 wt% CLA oil-in-water emulsions stabilized by different ratios of pectin-to-sodium caseinate.

Fig. 4. Effect of holding time at 90°C on (a) the mean particle diameter and (b) the ζ -potential of 1 wt% CLA oil-in-water emulsions stabilized by pectin-caseinate coatings ($R = 2:1$).

Fig. 5. Effect of NaCl concentration and pH on the ζ -potential of 1 wt% CLA oil-in-water emulsions stabilized by pectin-caseinate coatings (R = 2:1). The samples had previously been heated at 90°C for 20 min.

Fig. 6. Effect of NaCl concentration and pH on the mean particle diameter of 1 wt% CLA oil-in-water emulsions stabilized by pectin-caseinate coatings ($R = 2:1$) after (a) 1 day and (b) 14 days storage at room temperature. All samples had been thermally processed (90°C for 20 min) prior to salt addition.

Fig. 7. Appearance of 1 wt% CLA oil-in-water emulsions stabilized by pectin-caseinate coatings ($R = 2:1$) after (a) 1 day and (b)

14 days storage at room temperature. All samples had been thermally processed (90°C for 20 min) prior to salt addition.

**“Biopolymer-stabilized conjugated linoleic acid (CLA) oil-in-water emulsions:
Impact of electrostatic interactions on formation and stability of pectin-caseinate-
coated lipid droplets” by Cheng and McClements**

Graphical Abstract