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Oxidative Stability and in Vitro Digestibility of Fish Oil-in-Water Emulsions Containing Multilayered Membranes

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The oxidative stability and lipid digestibility of fish oil-in-water emulsions (d_{43}; 5.26−5.71 μm) laminated by primary, secondary, and/or tertiary layers of interfacial membranes have been investigated. The primary emulsion (5 and 0.5% wt % of fish oil and Citrem in acetate buffer) was produced through a membrane homogenizer. The second and tertiary emulsions were prepared by electrostatic deposition of chitosan and sodium alginate on the surfaces of the oil droplets, respectively. The lamination of biopolymers was measured by zeta potential. The lipid oxidative stability was assessed with peroxide value, thiobabituric acid reactive substances, and headspace aldehydes of the emulsions stored at 20 °C for 40 days. The positively charged secondary emulsions (+56.27 ± 2.5 mV) were more stable to lipid oxidation compared to negatively charged primary (−45.13 ± 1.7 mV) and tertiary emulsions (−24.8 ± 1.2 mV). An in vitro digestion model was used to study the impact of different layers on the digestibility of oil droplets. Lipid digestion was decreased with multilayer coating, and chitosan coating further reduced the digestion. These findings have implications for the design of structured emulsions to achieve better oxidative stability with more controlled digestibility of lipids.

KEYWORDS: Citrem; chitosan; sodium alginate; oxidative stability; lipid digestibility

1. INTRODUCTION

There is a considerable interest within the food and pharmaceutical industries in the creation of delivery systems that can encapsulate, protect, and deliver ω-3 fatty acids because of their potential health benefits in decreasing the risk of coronary heart disease, immune response disorders, ulcerative colitis and Crohn's disease, and mental illnesses (*1*−*6*). However, incorporation of these highly unsaturated fatty acids into food products is problematic because they are highly susceptible to oxidative degradation resulting in rancid off-flavors, which has greatly limited their more widespread usage (*7*). An electrostatic layer-by-layer (LbL) deposition method has previously been used to form nanolaminated biopolymer coatings around lipid droplets, and the method has been shown to yield physically stable emulsions (*8*−*10*). The LbL method involves repeated deposition of oppositely charged polyelectrolytes onto the surfaces of charged lipid particles. The LbL method enables one to systematically control coating characteristics, such as composition, net electrical charge, layer thickness, permeability, and environmental responsiveness (*11*−*19*). Although a great deal of research has been focused on the physical stability of multilayered emulsions, very little research has focused on the oxidative stability and in vitro digestibility or release of encapsulated lipophilic bioactive compounds of these emulsions.

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Conventional emulsification devices such as high pressure valve homogenizers generate emulsions with relatively small droplet sizes but wide particle size distributions. Further, homogenization often results in considerable temperature elevation which may not be suitable for thermolabile and oxidatively susceptible bioactive compounds. Membrane emulsification is a relatively new emulsification technology, aimed at achieving precise control of the particle size distribution over a wide range of mean droplet sizes (*20*). This technique could be particularly useful for producing multiple emulsions (*21*) because of its effectiveness in preparing droplets with very narrow particle size distributions.

The utilization of multilayered emulsions to control the bioavailability of emulsified lipids within the human digestive tract depends on a detailed understanding of the physicochemical and physiological processes that occur during lipid digestion. Before these lipids can be absorbed by the human body, they must first be converted into diacylglycerols, monoacylglycerols, and free fatty acids, through the action of digestive enzymes such as gastric and pancreatic lipase (*22*). The digestion of emulsified lipids can therefore be controlled by altering structure and integrity of the interfacial coatings surrounding them, because this can interfere with the ability of the digestive enzymes to interact with the lipids (*23*−*26*). It is therefore important to understand how multilayer emulsion droplets behave under the complex physicochemical conditions present in the gastrointestinal tract. In vitro digestion models can be used to provide mechanistic insights into

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the physicochemical processes that affect the digestibility of multilayer fish oil emulsion droplets.

In this study, we aimed to prepare monodispersed fish oil-in-water emulsions containing large droplets coated by multilayers using the LbL method to study their oxidative stability and lipase digestibility. Three natural food grade ingredients viz., anionic Citrem (citric acid ester of a mono- and diglyceride), cationic chitosan, and anionic sodium alginate were selected to produce interfacial membrane layers. Chitosan has been widely used as a structural component in delivery systems that are designed for utilization within the pharmaceutical, personal care, and food industries because of its nontoxicity, biodegradability, and biocompatibility (*27*−*32*). In addition, chitosan is one of the few cationic biopolymers available, and so it is often used to form electrostatic complexes with anionic biopolymers. Therefore, chitosan was used as a building block to assemble multilayered emulsions based on electrostatic interactions. Alginates are a group of acidic polysaccharides that occur naturally as the major structural polysaccharides of brown marine algae (*Phaeophyceae*) and as extracellular mucilages (*33*). These are linear polymers which have 1−4′ linked-β-D-mannuronic acid and α-L-guluronic acid residues arranged as blocks of either type of unit or as a random distribution of each type (*34*).

2. MATERIALS AND METHODS

2.1. Materials. Citrem (Grindsted LR 10 Extra) was obtained from Danisco. Powdered chitosan (dry matter content, 92.5%; ash content, < 0.5%; degree of deacetylation, 89%; medium molecular weight) was procured from Primex (Siglufjordur, Iceland). Sodium alginate (20−40 cps) was obtained from Sigma-Aldrich (St Louis, MO). Porcine pancreatin and bile extract (porcine) were obtained from Sigma. Pancreatin contains many enzymes, including pancreatic lipase, amylase, trypsin, lipase, ribonuclease, and protease. According to the supplier, the lipase in pancreatin releases not less than two microequivalents of acid per min per mg of pancreatin from olive oil at pH 9.0 and 37.0 °C (Sigma-Aldrich, St. Louis, MO). The composition of bile extract has previously been analyzed: total bile salt content = 49 wt %; with 10−15% glycodeoxycholic acid, 3−9% taurodeoxycholic acid, 0.5−7% deoxycholic acid; 5 wt % phosphatidylcholine (*35*). Fish oil (Ropufa 30 *n*-3 food oil) was obtained from DSM Nutritional Products Ltd. (Basel, Switzerland). The oil contained 101 mg of EPA/g of oil, 148 mg of DHA/g oil, and a total ω-3 PUFA of 312 mg/g of oil. Calcium chloride ($CaCl_2 \cdot 2H_2O$) was obtained from Fisher Scientific. Analytical-grade hydrochloric acid (HCl) and sodium hydroxide (NaOH) were purchased from Sigma-Aldrich (St. Louis, MO). Water from a water purification system (Nanopure Infinity, Barnstead International) was used for preparation of all solutions.

2.2. Solution Preparation. A stock buffer solution was prepared by dispersing 100 mM acetic acid in water and then adjusting the pH to 3.5 using 1 N NaOH. Chitosan (0.2 wt %) and alginate (0.4 wt %) solutions were prepared by dispersing the polysaccharides into the stock buffer solution.

2.3. Emulsion Preparation. A primary emulsion was prepared using 5.0 wt % fish oil, 0.5 wt % Citrem, and 94.5% aqueous phase containing acetate buffer (100 mM, pH 3.5). Oil-in-water coarse emulsions were made by blending the lipid and aqueous phases for 2 min using a hand-held homogenizer (M 133/1281-Biospec Products, Inc. Bartlesville, UK). The coarse emulsions were passed through a membrane homogenizer (external pressure-type micro kit, MG-20-5, Kiyomoto Iron Works Ltd., Japan). The pressure vessel was filled up with 100 mL of the coarse emulsion, and the required driving pressure was built up with compressed air using a precision pressure regulator. The operating pressure was measured with an accuracy of ±1 kPa using a digital pressure gauge. The fine emulsion that had exited the membrane tube was collected in a beaker. The membrane used was an SPG membrane (8.5 mm inner diameter, 0.8 mm wall thickness) supplied from SPG Technology Co., Ltd. (Sadowara, Japan). The mean pore size of the membrane was 8.0 μm, the effective membrane length was 12 mm, and the effective cross-sectional area was 3.75 cm^2. The membrane was cleaned after use by immersing it for 2 days in ethanol plus 2 days in toluene, followed by heating at 500 °C for 30 min in an electric muffle furnace. The inherent membrane permeability to pure water was completely restored by this treatment.

Secondary emulsions were formed by diluting the primary emulsions (1 wt % fish oil) with chitosan solutions to produce a series of emulsions with different chitosan concentrations: 0.5 wt % fish oil, 0.05 wt % Citrem, 0−0.01 wt % chitosan, and 100 mM acetic acid (pH 3.5). These systems were stirred for 1 h using a magnetic stirrer at ambient temperature. Tertiary emulsions were formed by diluting the secondary emulsion (final concentration of chitosan: 0.006 wt %) with aqueous sodium alginate solutions to produce a series of emulsions with different alginate concentrations: 0.25 wt % fish oil, 0.025 wt % Citrem, 0.003 wt % chitosan, 100 mM acetic acid, and 0−0.05 wt % sodium alginate (pH 3.5). These systems were stirred for 1 h using a magnetic stirrer at ambient temperature. The emulsions were stored at room temperature for 24 h before they were analyzed.

2.4. Particle Size Measurements. The particle size distribution of the emulsions was measured by static light scattering (Mastersizer X, Malvern Instruments Ltd., Malvern, U.K.). A few drops of samples were dispersed in approximately 125 mL of buffer in the same chamber with agitation until approximately 11−14% obscuration was obtained. Measurements were conducted at ambient temperature (22 °C). The computer software used to analyze the angular dependence of the scattered light intensity identified the particle size distribution that gave the best fit between the experimental measurements and the theoretical predictions made using the Mie theory. A refractive index ratio of 1.08 was assumed in the calculation of the particle size distributions. The particle size measurements are 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 droplets of diameter d_i, the volume surface mean diameter.

2.5. Zeta-Potential Measurements. Zeta-potential was determined using a commercial dynamic light scattering and microelectrophoresis device (Nano-ZS, Malvern Instruments, Worcestershire, UK). The samples were diluted 100 times in buffer solution at 22 °C before measurement. The particle charge data were reported as the zeta-potential which was calculated from the measurement of the electrophoretic mobility of particles in an applied oscillating electric field using laser Doppler velocimetry.

2.6. Oxidative Stability of Emulsions. To monitor lipid hydroperoxide formation during storage, emulsions were placed in lightly sealed screw-cap vials and allowed to oxidize at 20 °C in the dark.

Lipid hydroperoxides were measured by mixing 0.3 mL of emulsion with 1.5 mL of isooctane/2-propanol (3:1, v/v) by vortexing (10 s, three times) and isolation of the organic solvent phase by centrifugation at 1000*g* for 2 min. The organic solvent phase (200 μL) was added to 2.8 mL of methanol/1-butanol (2:1, v/v), followed by 15 μL of 3.97 M ammonium thiocyanate and 15 μL of ferrous iron solution (prepared by mixing 0.132 M $BaCl_2$ and 0.144 M $FeSO_4$). The absorbance of the solution was measured at 510 nm, 20 min after addition of the iron (*36*). Hydroperoxide concentrations were determined using a standard curve made from cumene hydroperoxide.

Thiobarbituric acid-reactive substances (TBARs) were determined according to an adapted method of McDonald and Hultin (*37*). The emulsion (1.0 mL) was combined with 2.0 mL of TBA solution (prepared by mixing 15 g of trichloroacetic acid, 0.375 g of thiobarbituric acid, 1.76 mL of 12 N HCl, and 82.9 mL of H_2O) in test tubes and placed in a boiling water bath for 15 min. The tubes were cooled to room temperature for 10 min and then centrifuged (2000*g*) for 15 min. The absorbance was measured at 532 nm. Concentrations of TBARs were calculated from a standard curve prepared with 1,1,3,3-tetraethoxypropane.

For headspace analysis of lipid oxidation products, emulsion samples (1 mL) were placed into 10 mL headspace vials and sealed with poly (tetrafluoroethylene) butyl rubber septa. Headspace propanal and hexanal concentrations were determined using a Shimadzu GC-2014 gas chromatograph equipped with a Shimadzu AOC-5000 headspace autoinjector (*38*). The headspace conditions were as follows: sample temperature, 50 °C; incubation time, 15 min; extraction time, 2 min; desorption time, 3 min. The aldehydes were separated isothermally at 65 °C on a Supelco (Equity-1) fused silica capillary column (30 m, 0.30 mm i.d., 1.0 μm film thickness). The splitless injector temperature was 250 °C, and the eluted compounds were detected with a flame ionization detector at 250 °C. Concentrations were determined from peak areas using a standard curve made from propanal and hexanal.

2.7. In Vitro Digestion Model. The dynamic in vitro digestion model used was a modification of those described by Mun et al. (*24*) and Zangenberg et al. (*35*). Emulsion (30.0 mL; pH 3.5) was placed in a beaker in a water bath at 37.0 °C for 10 min and adjusted to pH 7 with NaOH solution. Then 5.0 mL of bile extract solution containing 187.5 mg of bile extract dissolved in phosphate buffer (pH 7.0) and 1.0 mL of $CaCl_2$ solution containing 27.5 mg of $CaCl_2$ dissolved in phosphate buffer (pH 7) were added into the emulsion under stirring. The resultant mixture was then adjusted to pH 7. Finally, 1.5 mL of freshly prepared pancreatin suspension containing 60 mg of lipase dissolved in phosphate buffer (pH 7) was added to the mixture. At this point, the pH-stat measurements were started. The pH-stat (Metrohm, USA Inc.) was used to monitor and control the pH (at pH 7) of the digestion solution. The volume of added NaOH solution reflected the amount of free fatty acids generated by lipolysis of the fish oil triacylgycerols.

2.8. Assessment of Microstructure of Emulsions. The microstructures of selected emulsions were also measured using a laser scanning confocal microscope (C1 Digital Eclipse, Nikon, Tokyo, Japan) with a 60× oil immersion objective lens. The oil phase of O/W emulsions was stained with an oil-specific fluorescent dye (Nile Red). The Nile red was excited with a 488 nm argon laser line. The fluorescence emitted from the sample was monitored using a fluorescence detector (515/30) with a pinhole size of 150 μm. The resulting images consisted of 512 × 512 pixels, with a pixel size of 414 nm, and a pixel dwell time of 61.44 μs. Images were recorded using image analysis software (Scion Image, Frederick, MD, USA). At least three pictures were taken of each sample.

2.9. Statistics. All analysis was performed on triplicate samples and was repeated twice. Data are expressed as means ± standard deviations. Statistical differences were defined as $P \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. Preparation of Multilayer Emulsions. In order to produce monodispersed emulsion, membrane homogenization was carried out by following a "premix membrane emulsification" approach (*39*, *40*), in which coarse emulsions (fish oil 5 wt %, Citrem, 0.5 wt %,100 mM acetic acid, pH 3.5) were forced through the membrane, and the droplets (d_{43} = 5.26−5.71 μm) were formed by reducing the size of the large droplets in premix emulsions. The other available membrane homogenization method is "direct membrane emulsification", where a pure liquid (the disperse phase) is forced through the membrane pores into another immiscible liquid (the continuous phase), and the small droplets are formed in situ at the membrane-continuous phase interface (*41*−*43*). The major advantages of the "premix membrane emulsification" method are that emulsions with higher droplet concentrations can easily be produced, and higher trans membrane fluxes can be achieved (*44*, *45*).

Optimization of Chitosan Concentration on Droplet Characteristics. By following the LbL approach, chitosan was added to the primary Citrem-stabilized emulsion where it adsorbed to the droplet surface and produced a two-layer, secondary emulsion. An optimization experiment was conducted to determine the amount of chitosan necessary based on the physical characteristics and stability of the secondary emulsions. Initially, the electric charge on the lipid droplets in primary emulsions containing different concentrations of chitosan (0−0.01 wt %) was measured (**Figure 1**). In the absence of chitosan, the electric charge on the Citrem-stabilized emulsion droplets was −45.3 ± 1.7 mV at pH 3.5. With increasing chitosan concentration, the net charge on the droplets became progressively less negative, neutral, and then positive, indicating that cationic chitosan adsorbed onto the surfaces of the anionic lipid droplets. The positive charge on the droplets reached a fairly constant value when the chitosan concentration exceeded 0.002−0.003 wt %, suggesting that the droplet surfaces had become saturated with chitosan. The requirement for chitosan to saturate the droplet surface in the present study was found to be less compared to 0.02−0.03 wt %

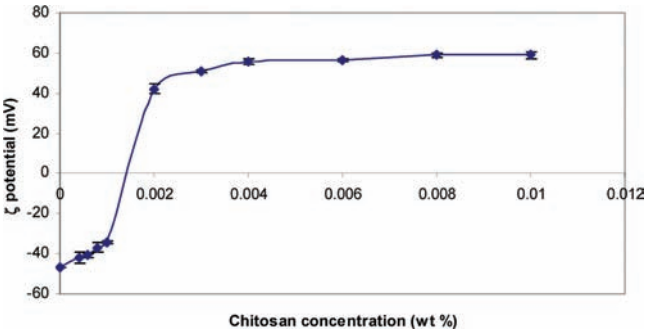

Figure 1. Changes in electrical charge of emulsion droplets as cationic chitosan is added to the anionic primary emulsion (1.0 wt % fish oil, 0.1 wt % Citrem, and 100 mM acetic acid, pH 3.5).

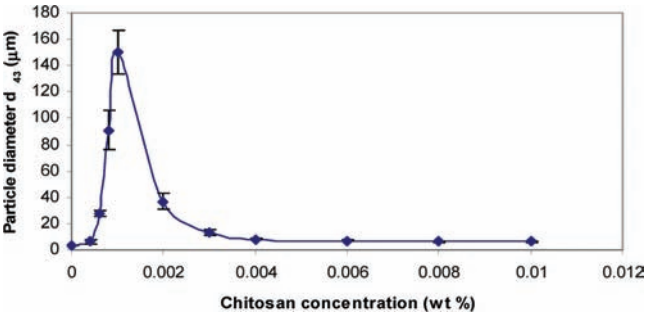

Figure 2. Changes in particle diameter (d_{43}) as chitosan is added to primary emulsion (1.0 wt % fish oil, 0.1 wt % Citrem, and 100 mM acetic acid, pH 3.5).

observed in our earlier studies where lecithin and chitosan have been used to prepare multilayer emulsions using the LbL approach (*46*). This may be due to the large particle size of the emulsions and thus less surface area in the present study.

The mean particle diameters (d_{43}) of the lipid droplets increased as the chitosan concentration in the emulsions increased from 0.00 to 0.001% (**Figure 2**), which can be attributed to charge neutralization and polymer bridging flocculation effects (*46*). At higher chitosan concentrations, where the droplet surfaces were saturated with chitosan, the lipid droplets were relatively stable to aggregation due to the increased electrostatic and steric repulsion between them. We therefore used the secondary emulsions with chitosan concentration of 0.006% in the remainder of these studies since the droplet surfaces were saturated, and there was little droplet aggregation.

Optimization of Sodium Alginate Concentration on Droplet Characteristics. Similarly, the required amount of sodium alginate was optimized by adding different concentrations of polysaccharide to the secondary emulsion. In the absence of sodium alginate, the electrical charge on the emulsion droplets was +56.27 ± 4.5 mV (**Figure 3**). The electrical charge on the droplets decreased gradually and became negative as the anionic alginate concentration in the emulsion was increased. The negative charge on the droplets reached a relatively constant level (−24.8 ± 1.2 mV) when the anionic polysaccharide concentration exceeded ~0.01 wt %. These measurements indicated that negatively charged alginate molecules adsorbed to the surfaces of positively charged Citrem−chitosan stabilized secondary emulsion forming a third layer.

Changes in particle size distribution of emulsion droplets were shown in **Figure 4** when sodium alginate was added to secondary emulsions. The mean particle diameters increased with an increase in concentration of alginate up to 0.01 wt % indicating

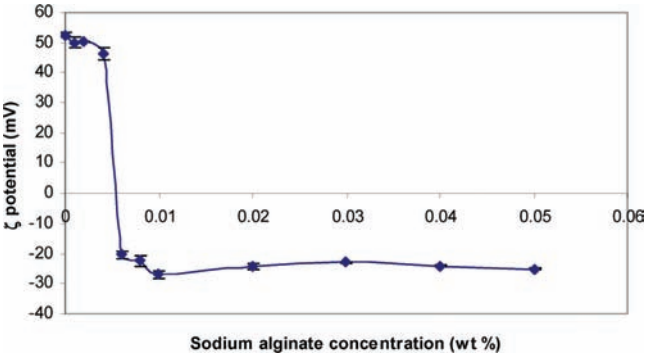

Figure 3. Changes in electrical charge of emulsion droplets as anionic sodium alginate is added to the Citrem−chitosan secondary emulsion (0.5 wt % fish oil, 0.05 wt % Citrem, 0.003 wt % chitosan, and 100 mM acetic acid, pH 3.5).

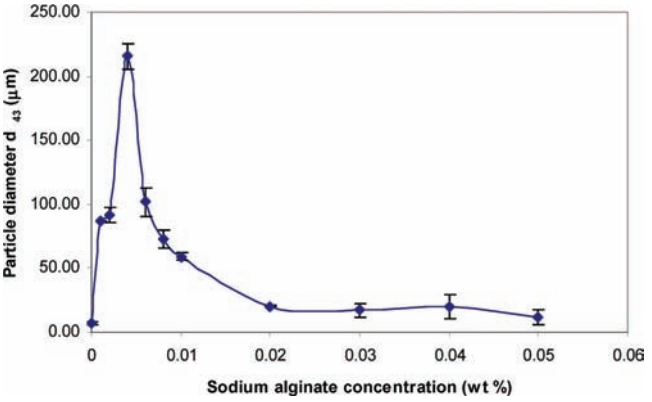

Figure 4. Changes in particle diameter (d_{43}) as sodium alginate is added to secondary emulsion (0.5 wt % fish oil, 0.05 wt % Citrem, 0.003 chitosan, and 100 mM acetic acid, pH 3.5).

charge neutralization and/or bridging flocculation effects by the biopolymer. Further increase in the alginate concentration yielded relatively stable emulsions. In the remainder of this study, we prepared tertiary emulsions having sodium alginate concentration of 0.02 wt % because the droplets had a negative zeta-potential and there would be the minimum amount of free alginate present in the continuous phase as the droplets appeared to be saturated with alginate at this concentration. A common problem of droplet aggregation while using the LbL technique to prepare multilayer emulsions was overcome by controlling solution composition and preparation conditions. The care was taken to provide a sufficient amount of polyelectrolyte to saturate all of the droplet surfaces and an excess amount of polyelectrolyte was avoided in order to prevent depletion flocculation.

3.2. Oxidative Stability of Multilayered Emulsions. The physical properties that can influence lipid oxidation rates in oil-in-water emulsions include particle size, which influences surface area; emulsion droplet charge, which causes either attraction or repulsion of transition metals; and thickness of the layer at interfacial region of the emulsion droplet that can impact interactions between lipids and aqueous phase prooxidants. The emulsion droplet size can influence lipid oxidation rates because smaller particle sizes result in a larger surface area and thus a greater possibility for lipid−aqueous phase prooxidant interactions. It has been reported that increasing surface area in bovine serum albumin stabilized emulsions increased lipid oxidation as measured by oxygen consumption and formation of conjugated dienes (*47*).

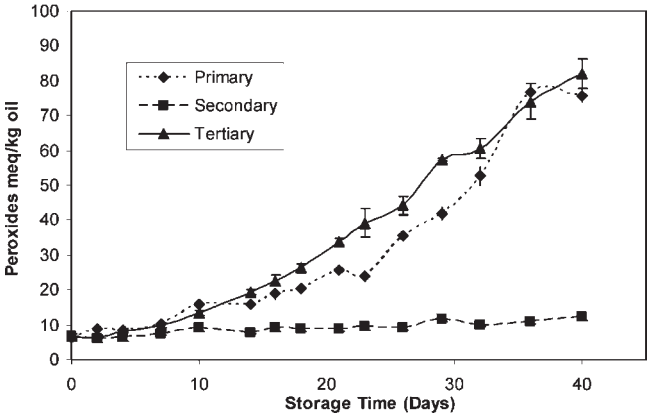

Figure 5. Formation of lipid hydroperoxides in multilayered emulsions (fish oil 0.25 wt %) stored at 20 °C.

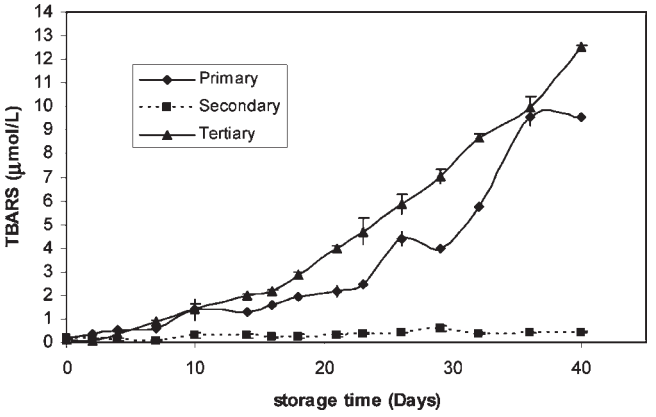

Figure 6. Formation of TBARS in multilayered emulsions (fish oil 0.25 wt %) stored at 20 °C.

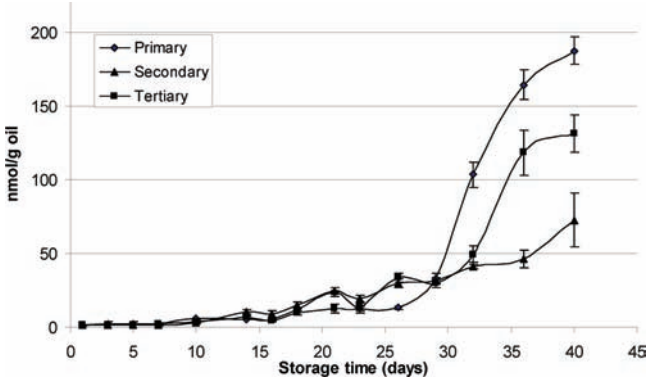

Figure 7. Formation of headspace propanal in multilayered emulsions (fish oil 0.25 wt %) stored at 20 °C.

All the three emulsions were stored at 20 °C for 40 days in order to determine differences in the oxidative stability of the samples. The lipid oxidation rates in primary (Citrem), secondary (Citrem−chitosan), and tertiary (Citrem−chitosan−alginate) emulsions containing fish oil were compared by measuring lipid hydroperoxides, TBARS and headspace propanal and hexanal by GC-SPME (**Figures 5**−**8**). Lipid hydroperoxides and TBARS formation was much faster in the primary and tertiary emulsions than in the secondary emulsions where almost no hydroperoxide accumulation was observed for up to 40 days of storage. Lipid hydroperoxides and TBARS formation in the primary and tertiary emulsions were similar with oxidation proceeding rapidly

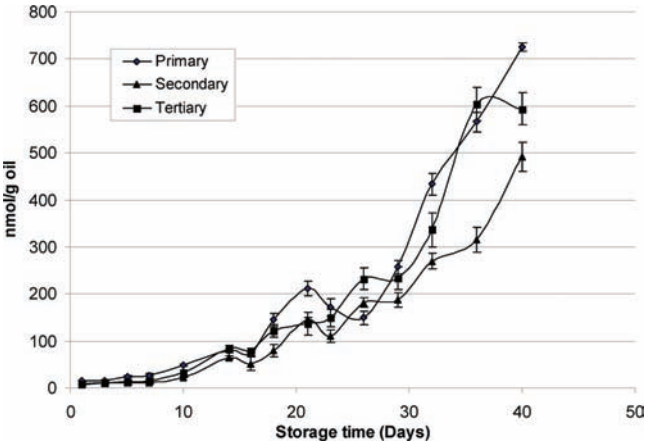

Figure 8. Formation of headspace hexanal in multilayered emulsions (fish oil 0.25 wt %) stored at 20 °C.

after 7 days of storage. Formation of propanal and hexanal which are considered lipid oxidation markers for ω-3 and ω-6 fatty acids respectively (*48*) also exhibited similar trends (**Figures 7** and **8**) indicating secondary emulsions had the lowest level of lipid oxidation after 37 days of storage.

One of the major mechanisms of oxidation of emulsified lipids is the iron-promoted degradation of lipid hydroperoxides into free radicals that can oxidize unsaturated fatty acids (*7*). Thus, factors that impact iron−hydroperoxide interaction can have a dramatic effect on lipid oxidation rates. The greater stability of the secondary emulsions can be attributed to the fact that the Citrem−chitosan coated droplets are highly positively charged (+56 mV), and therefore electrostatically repel prooxidative transition metals. Earlier experiments conducted in our laboratory have also shown that tuna oil-in-water emulsion droplets coated by lecithin and chitosan were more oxidatively stable than emulsions coated by lecithin alone (*49*), demonstrating a cationic emulsion droplet interfacial membrane can be an effective method to control lipid oxidation.

The observed instability of the primary and tertiary emulsions to lipid oxidation can be attributed to the ability of positively charged prooxidative transition metal ions to adsorb to the surface of the negatively charged Citrem or Citrem + alginate coated droplets, where they come into close proximity to the unsaturated lipids within the oil droplets. This is unlike a previous study where emulsions prepared with a multilayer system consisting of β-lactoglobulin and citrus pectin were more stable than emulsions stabilized with β-lactoglobulin alone (*50*). The improved oxidative stability of an anionic secondary emulsions compared to the anionic tertiary emulsion in this study could be due to a thicker interfacial membrane produced by β-lactoglobulin and citrus pectin which would be more effective at inhibiting metal−lipid interactions. The β-lactoglobulin and citrus pectin interfacial layer would be thicker because β-lactoglobulin would produce a thicker layer than Citrem and pectin is a branched biopolymer and may have more steric effects compared to alginate which is a linear polymer. These data suggest that the oxidative stability of oil-in-water emulsions stabilized by small molecule surfactants and polysaccharides could be improved by the use of multilayer emulsion systems containing a cationic outer layer.

3.3. Influence of Multilayered Coatings on in Vitro Digestibility of Lipid Droplets. In vitro digestion model simulating human intestinal conditions was used to examine the influence of multilayered coatings on the digestibility of emulsified fish oil by pancreatic lipase. The fatty acids released from the encapsulated

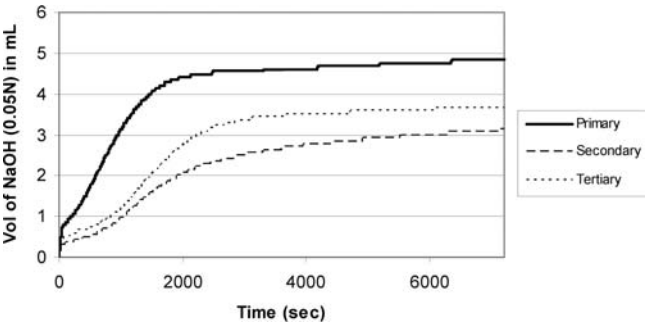

Figure 9. Rates of lipid digestion of multilayered emulsions measured using an in vitro digestion model at pH 7 and 37 °C.

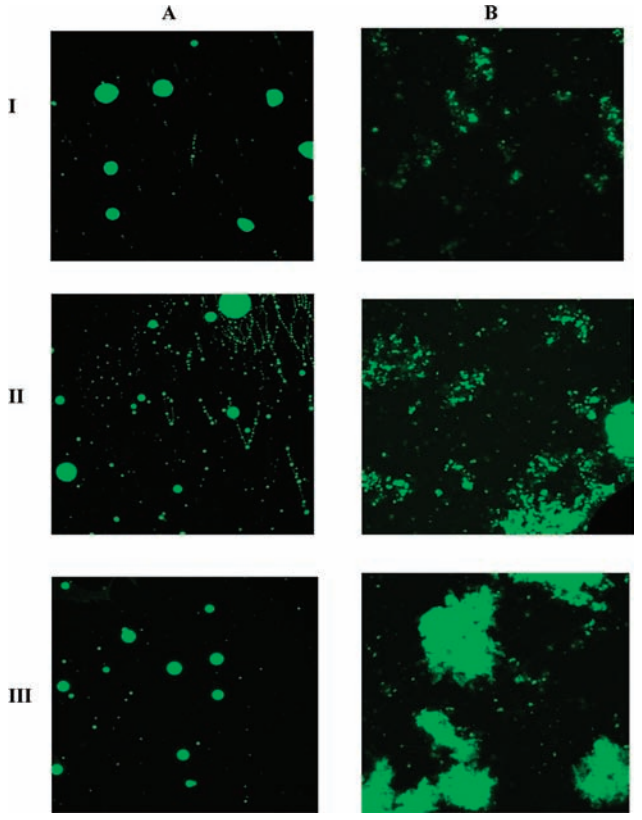

Figure 10. Confocal images of emulsions (**A**) before digestion; (**B**) after digestion; (I) primary; (II) secondary; (III) tertiary emulsions.

fish oil due to the action of pancreatic lipase were measured by pH-stat titration at pH 7.0. The rate of fatty acid release due to lipase digestion was in the order of primary > tertiary > secondary (**Figure 9**). The rate of lipid digestion was very rapid in the primary emulsion being almost complete after 40 min, whereas it was slower for the same amount of fish oil (0.25 wt %) in secondary and tertiary emulsions. The influence of the multilayer systems on digestion was also observed in emulsion microstructure over time using confocal microscopy (**Figure 10**). In the primary emulsions, there was a breakdown of the spherical shape of the emulsion droplet over time, which can be attributed to digestion and solubilization of the oil within mixed bile micelles. In the secondary and tertiary emulsion, the emulsions became highly aggregated through a mechanism that is not well understood at this time but could involve formation of complexes with the biopolymers, emulsion droplets, bile salts, and lipid hydrolysis products.

There are a number of physicochemical phenomena that may account for this result. The lipid droplets in the primary emulsions

are only coated with a layer of Citrem, which is a small anionic surface-active lipid that can easily be displaced by bile salts and lipase, thereby facilitating the access of lipase to the encapsulated lipids. On the other hand, the lipid droplets in the secondary and tertiary emulsions have outer coatings that consist of relatively thick, nondigestible polymeric coatings around the lipid droplets that decrease the ability of lipase access lipids in the emulsion droplet core (*24*). The slower lipid hydrolysis seen in the presence of the secondary chitosan-containing emulsion could be due to several factors. Chitosan is known to bind bile salts, and it may inhibit lipid digestion by reducing the amount of bile salts available to adsorb to lipid droplet surface. This could slow digestion since bile salts are needed to solubilize lipid digestion products out of the emulsion droplets to decrease the concentrations of end products below reaction equilibrium levels thus allowing for the continued activity of lipase (*51*). In the presence of alginate, the chitosan may not be as effective at binding either bile salts or lipase as it could be complexed with anionic alginate thus decreasing its positive charge.

On the basis of these results, it may be possible to create an emulsion delivery system that will pass through the stomach and small intestine, and then release its bioactive lipids into the lower portion of the gastrointestinal tract when the polysaccharides are digested via bacterial fermentations. This kind of delivery system would be useful for bioactive lipids that may be efficacious against colon cancer (*52*) and particularly applicable for ω-3 fatty acids which are known to have beneficial effect against inflammatory bowel disorders such as ulcerative colitis and Crohn's disease (*6*). In addition, these types of delivery systems may also be able to bring lipids into the lower intestinal tract where they could be more effective at inducing satiety.

It can be concluded from the present study that multilayered emulsions containing large droplets could be prepared with the LbL method using common food ingredients such as Citrem, chitosan, and sodium alginate. The remarkable oxidative stability achieved in the secondary emulsion prepared with the Citrem−chitosan interfacial membrane demonstrates the significance of positive charge on the outer layer of multilayered emulsions in inhibiting lipid oxidation reactions. In vitro digestion experiments revealed slower release of fatty acids from the multilayer emulsions indicating that these emulsion systems could be used to alter the location in the gastrointestinal tract where bioactive lipids are released. Overall, results of this study clearly indicate that multilayer encapsulation approach could have excellent promise in delivering bioactive lipids such as omega-3 fatty acids into functional foods.

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