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Research paper

Controlling the functional performance of emulsion-based delivery systems using multi-component biopolymer coatings

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ABSTRACT

The digestion and release of bioactive lipophilic components encapsulated within emulsion-based delivery systems can be controlled by coating the lipid droplets with biopolymer coatings. In this study, multi-component biopolymer coatings were formed around lipid droplets using an interfacial electrostatic deposition approach. These coatings consisted of an inner layer of globular protein (β-lactoglobulin), an intermediate layer of cationic polysaccharide (chitosan), and an outer layer of anionic polysaccharide (pectin or alginate). In the absence of the outer anionic polysaccharide layer, the protein–chitosan-coated droplets were highly unstable to aggregation at high pH values (pH > 6), due to loss of chitosan charge. In the presence of the outer layer, the droplets had good stability to aggregation from pH 7 to 4, but aggregated at lower pH due to loss of pectin or alginate charge. An *in vitro* lipid digestion model (pH stat) indicated that polysaccharide coatings reduced the rate of lipid digestibility. These findings have important implications for the design of delivery systems for bioactive lipophilic components in the pharmaceutical, biopharmaceutical, and food industries.

1. Introduction

Delivery systems designed to control the digestion, release, and absorption of encapsulated lipophilic components within the gastrointestinal tract are being developed for a variety of applications within the pharmaceutical, biopharmaceutical, and food industries [1–4]. They can be used to control the release of drugs and other bioactive components at specific locations within the GI tract, such as the mouth, stomach, small intestine, or colon. For example, delivery systems can be designed so that they retain and protect bioactive components that would normally be absorbed or degraded within the upper GI tract, but release them within the lower GI tract, *e.g.,* lipophilic components that inhibit colon cancer [5]. Alternatively, delivery systems may be designed to increase the amount of undigested food reaching the ileum, thereby stimulating the ileal brake mechanism that modulates hunger, satiety, and satiation [6–8]. Functional or medical foods containing lipids that are digested at a slower rate, and therefore that reach the ileum at a higher concentration, could be developed to control appetite, thereby reducing energy intake and the incidence of obesity.

A number of emulsion-based delivery systems have been developed to control the digestion and release of bioactive lipophilic components within the GI tract, including microemulsions, emulsions, solid lipid nanoparticles, filled hydrogel particles, and multiple emulsions [9–15]. Each of these systems has its own advantages and disadvantages for encapsulation and delivery applications [1]. In the present study, we focus on the development of *multilayer emulsions* that consist of lipid droplets surrounded by multi-component biopolymer coatings [16–18]. These biopolymer coatings can be designed to improve the stability of the delivery systems to environmental stresses [19–23], to protect encapsulated components from chemical degradation [24,25], and to release encapsulated components in response to specific environmental triggers [18]. A potential benefit of using multilayer emulsions as delivery systems is that they can be fabricated entirely from natural food grade (GRAS) ingredients (lipids, proteins, polysaccharides) using simple processing operations (homogenization, mixing). They could therefore be utilized in the development of pharmaceutical products or functional foods designed to combat diet-related diseases, such as obesity, heart disease, cancer, and hypertension.

Multilayer emulsions are formed by electrostatic deposition of ionic biopolymers onto oppositely charged lipid droplets [19,25–32]. The charge on the droplets usually reverses in sign after the deposition of each biopolymer layer, so that multiple-layered biopolymer coatings can be formed by sequential deposition

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of two or more oppositely charged biopolymers [33]. The nature of the biopolymer coatings formed using the electrostatic deposition method are affected by biopolymer molecular characteristics such as charge density and molecular weight [34,35], as well as by solution conditions such as pH [36,37] and ionic composition [38,39]. The functional properties of these coatings can be controlled by varying the number of layers deposited, the type of biopolymers used, the sequence of biopolymer layers, and the solution conditions used during deposition.

The overall characteristics of droplets surrounded by biopolymer coatings are believed to be largely determined by the properties of the outer biopolymer layer [16]. The main purpose of the present study was therefore to examine the influence of the outer biopolymer layer on the electrical properties and physical stability of biopolymer-coated lipid droplets. Previously, we have shown that O/W emulsions containing cationic droplets coated with β-lactoglobulin–chitosan could be prepared using the electrostatic deposition technique [40]. β-Lactoglobulin is a globular protein typically isolated from bovine milk [41], whereas chitosan is a cationic polysaccharide usually isolated from crustacean shells [42]. Lipid droplets surrounded by β-lactoglobulin–chitosan coatings had better stability to droplet aggregation than those surrounded by β-lactoglobulin coatings around the isoelectric point (pI ∼ 4.5–5.0) of the adsorbed protein, which was attributed to increased electrostatic and steric repulsion between droplets [40]. Nevertheless, chitosan loses its positive charge at relatively high pH values (>6.5) and becomes insoluble in aqueous solutions, which limits its application in food or pharmaceutical products at neutral pH.

We therefore aimed to determine whether it was possible to improve the functional properties of these two-layer emulsions by adding anionic polysaccharides to create a third biopolymer layer. Two negatively charged natural polysaccharides, alginate and pectin, were selected to further coat the β-Lg–chitosan-coated droplets. Alginates are a linear polymer having 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 [43]. Pectins are predominantly linear polymers of mainly α-(1-4)-linked D-galacturonic acid residues interrupted by 1,2-linked L-rhamnose residues [44]. Hence, these two types of polysaccharide tend to be negatively charged across a wide range of pH values. We hypothesized that forming an additional layer of anionic polysaccharides around β-lactoglobulin–chitosan-coated lipid droplets would improve their physical stability, as well as alter the rate of lipid digestion by restricting the access of lipase to the emulsified triacylglycerols. These emulsion-based delivery systems may therefore prove useful for controlling the digestion and release of encapsulated lipophilic components within the GI tract.

2. Materials and methods

2.1. Materials

Powdered lactoglobulin (β-Lg) was obtained from Davisco Foods International (Lot # JE 002-8-415, Le Sueur, MN). Medium molecular weight chitosan powder, pectin extracted from citrus fruit (Lot # 91K1420), and alginic acid (sodium salt) were purchased from the Sigma Chemical Company (St. Louis, MO). Glyceryl tributyrate (tributyrin) (98%) was obtained from Sigma. Corn oil was purchased from a local supermarket and used without further purification (Mazola, ACH Food Companies, Inc., Memphis, TN). The manufacturer reported that the corn oil contained approximately 14.3, 28.6, and 57.1 wt.% of saturated, monounsaturated, and polyunsaturated fats, respectively. Analytical grade hydrochloric acid (HCl) and sodium hydroxide (NaOH) were purchased from Sigma. Purified water from a Nanopure water system (Nanopure Infinity, Barnstead International, Dubuque, IA) was used for the preparation of all solutions.

2.2. Solution preparation

Emulsifier solution containing 1.0 wt.% protein was prepared by dispersing powdered β-Lg into 100 mM acetate buffer (pH 3.0), stirring for at least 2 h, then storing overnight at 4 °C to ensure complete hydration. A chitosan solution (0.5 wt.%) was prepared by dispersing weighed amounts of powdered ingredient into 100 mM acetate buffer (pH 3.0) and storing overnight to ensure complete hydration. The chitosan solution was centrifuged and filtered before use to remove any extraneous matter. The pH of protein and chitosan solutions was readjusted to pH 3.0 if necessary. Pectin and alginate solutions were prepared by dispersing weighed amounts of powdered materials into 5 mM phosphate buffer (0.5 wt.%, pH 7.0) and stirring for at least 2 h to ensure complete hydration. The pH of pectin and alginate solutions was then adjusted to pH 5.5 using HCl and NaOH solutions.

2.3. Emulsion preparation

A primary emulsion was prepared by homogenizing 10 wt.% oil (composing of 50 wt.% corn oil and 50 wt.% tributyrin) with 90 wt.% aqueous emulsifier solution (1.0 wt.% β-Lg, 100 mM acetate buffer, pH 3.0) with a high-speed blender for 2 min (M133/1281-0, Biospec Products, Inc., ESGC, Switzerland) followed by five passes at 9 K psi (62 MPa) through a high pressure homogenizer (Microfluidics M-110Y, F20Y 75 μm interaction chamber, Newton, MA). This emulsion was referred to as the stock emulsion. A series of emulsions containing the same oil (1 wt.% oil) and protein (0.1 wt.% β-Lg) contents but different chitosan contents (0–0.1 wt.% chitosan) were prepared by mixing different ratios of stock emulsion (10 wt.% oil, 1 wt.% β-Lg), chitosan solution (0.5 wt.% chitosan), and buffer solution (100 mM acetate buffer, pH 3.0). The emulsion was adjusted from pH 3.0 to 5.5 to promote the adsorption of cationic chitosan onto the anionic lipid droplet surfaces through electrostatic attraction. Emulsions containing no chitosan were referred to as "primary emulsions", whereas emulsions containing chitosan were referred to as "secondary emulsions". The pH of the primary and secondary emulsions was then adjusted to values ranging from 3.0 to 7.0 by addition of HCl or NaOH. The resulting emulsions were stirred for 30 min and then stored at room temperature overnight before measurement. The secondary emulsion (1 wt.% oil, 0.1 wt.% β-Lg and 0.05 wt.% chitosan, pH 5.5) was treated with sonication to disrupt any flocculated droplets formed during its preparation. Sonication was conducted for 2 min at a frequency of 40 kHz, amplitude of 40%, and duty of cycle of 0.5 s (Model 500, Sonic Disembrator, Fisher Scientific, Pittsburgh, PA).

Tertiary emulsions were prepared by mixing a secondary emulsion, with different ratios of an anionic polysaccharide solution (0.5 wt.% pectin or alginate at pH 5.5) and a buffer solution (pH 5.5). The final composition of the tertiary emulsions was as follows: 0.5 wt.% oil, 0.05 wt.% β-Lg, 0.025% chitosan, and 0–0.25 wt.% pectin or alginate. These emulsions were adjusted to a range of pH values (3.0–7.0) by addition of HCl or NaOH. The resulting emulsions were stirred for 30 min and then stored at room temperature overnight before measurement.

2.4. Droplet charge measurements

The ζ-potential of emulsions was determined using a particle electrophoresis instrument (ZEM5003, Zetamaster, Malvern

Instruments, Worcestershire, UK). The ζ-potential is determined by measuring the direction and velocity of droplet movement in a well-defined electric field. Emulsions were diluted to a droplet concentration of approximately 0.001 wt.% using buffer solutions of the appropriate pH to avoid multiple scattering effects, *i.e.,* the diluting buffer had the same pH as the aqueous phase of the sample being tested.

2.5. Particle size measurements

Particle size distributions of emulsions were measured using a laser diffraction instrument (Mastersizer, Malvern Instruments, MA). This instrument measures the angular dependence of the intensity of laser light (λ = 632.8 nm) scattered by a dilute emulsion and then finds the particle size distribution that gives the best agreement between theoretical predictions (Mie theory) and experimental measurements. To avoid multiple scattering effects, emulsions were diluted to a droplet concentration of approximately 0.005 wt.% using buffer solution at the pH as the sample. A refractive index ratio of 1.08 was used in the calculations of the particle size distribution. The particle size measurements are reported as the surface-weighted mean diameter $d_{32} = \sum n_i d_i^3 / \sum n_i d_i^2$, where n_i is the number of droplets of diameter d_i.

2.6. Creaming stability measurements

Ten grams of emulsion was transferred into a test tube (internal diameter 15 mm, height 125 mm), tightly sealed with a plastic cap, and then stored at ambient temperature (25–27 °C) for approximately 24 h. After storage, several emulsions separated into an opaque layer at the top, a turbid layer in the middle, and a transparent layer at the bottom. We defined the "serum layer" to be the sum of the turbid and transparent layers. The total height of the emulsion (H_E) and the height of the serum layer (H_S) were measured using a ruler. The extent of creaming was then characterized by a creaming index: CI = 100 × (H_S/H_E). In relatively dilute emulsions, where the particles are well separated, droplet aggregation promotes creaming by increasing the size of the particles in the system, so an increase in creaming index is associated with an increase in droplet aggregation.

2.7. In vitro digestion model

The *in vitro* digestion model used in this study was a modification of those described previously [45,46]. The basic procedure used was as follows:

- A volume of 30.0 mL of emulsion containing 100 mg oil was placed into a glass beaker that was placed in a water bath at 37.0 °C for 10 min, and then adjusted to pH 7 using NaOH or HCl solutions.
- A volume of 5.0 mL of preheated bile extract solution (187.5 mg bile extract dissolved in phosphate buffer, pH 7.0) and 1.0 mL of preheated $CaCl_2$ solution (750 mM $CaCl_2$ in double distilled water) were added to the emulsion under stirring and then the system was adjusted back to pH 7 if required.
- A volume of 1.5 mL of freshly prepared lipase suspension (60 mg lipase powder dispersed in phosphate buffer, pH 7) was added to the above mixture. The final composition was 100 mg lipid, 5 mg/mL bile extract, 1.6 mg/mL lipase, and 5 or 20 mM $CaCl_2$.
- A pH-stat automatic titration unit (Metrohm, USA Inc.) was then used to automatically monitor the pH and maintain it at pH 7.0 by titrating 0.1 M NaOH solution into the reaction cell.

2.8. Optical microscopy

The microstructures of selected emulsions were observed using an optical microscope (Nikon Eclipse E400, Nikon Corp., Japan). Emulsion samples were thoroughly mixed in a glass test tube before analysis. A drop of emulsion was then placed on a microscope slide, covered by a cover slip. An image of the sample was acquired using digital image processing software (Micro Video Instruments Inc., Avon, MA) and stored on a personal computer.

A Nikon Confocal Microscope (C1 Digital Eclipse, Tokyo, Japan) with a 60× oil immersion objective lens was used to capture the confocal images. Nile red (a fat soluble fluorescent dye) was excited with 488 nm argon laser line. A drop of Nile red was added into the samples (~2 mL) and then mixed well. 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. The lipid phase was stained green.

2.9. Statistical analysis

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

3. Results and discussion

3.1. Secondary emulsion formation

Initially, our objective was to determine the appropriate concentration of cationic biopolymer (chitosan) required to form a coating around protein-stabilized lipid droplets. The influence of chitosan concentration on the properties of protein-coated droplets was therefore investigated at pH 4.5, 5.0, and 5.5. These pH values were selected because they are close to the isoelectric point (pI ≈ 5) of the protein-coated droplets, which promotes chitosan adsorption (see Section 3.2). The emulsions used in this study consisted of 1 wt.% oil, 0.1 wt.% β-Lg, and 0 to 0.1 wt.% chitosan.

The influence of chitosan concentration on the ζ-potential of the droplets at pH 4.5, 5.0, and 5.5 was measured (Fig. 1a). At all pH values, the ζ-potential became increasingly positive as the chitosan concentration was increased until a relatively constant value was attained, indicating that cationic chitosan molecules adsorbed to the surfaces of the protein-stabilized droplets until they became saturated with chitosan. At pH 4.5, the droplets in the primary emulsions had an appreciable positive charge (~+20 mV); hence, positively charged chitosan molecules adsorbed to the surfaces of positively charged droplets. The electrical charge on the surfaces of protein molecules is known to be heterogeneously distributed [47,48], so that there are some patches of positive charge and some patches of negative charge. Hence, it seems likely that cationic chitosan molecules adsorbed to negative patches on the surfaces of the protein-coated droplets at pH 4.5, thereby increasing the net positive charge on them (by ~+15 mV). Cationic chitosan is also likely to have adsorbed to negatively charged groups on the protein-coated droplet surfaces at pH 5.0 and 5.5; however, there will have been more negative charges (*e.g.,* $-COO^-$) available and less positive charges (*e.g.,* $-NH_3^+$) present on the proteins as the pH increased. This would account for the fact that the amount of chitosan needed to reach a constant positive charge on the droplets increased slightly with increasing pH (Fig. 2a). It should be noted that chitosan also has some hydrophobic character and so hydrophobic attraction may also have contributed to its ability to adsorb to the protein-coated lipid droplet surfaces.

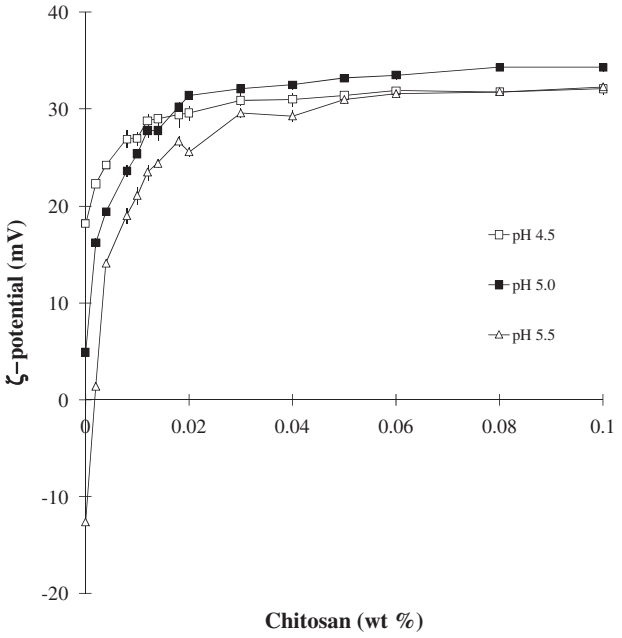

Fig. 1a. Electrical charge (ζ-potential) of biopolymer-coated lipid droplets in secondary emulsions as a function of chitosan concentration at pH 4.5, 5.0, or 5.5. The emulsions consisted of 1 wt.% oil, 0.1 wt.% β-Lg, and 0–0.1% chitosan.

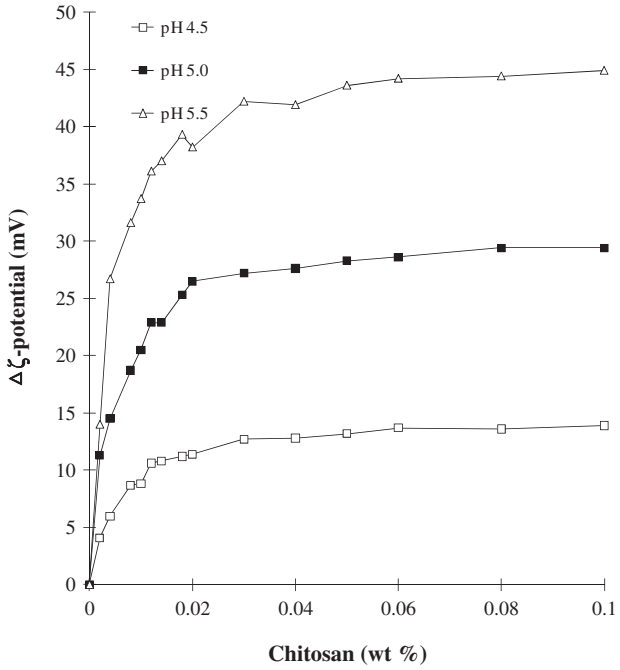

Fig. 1b. Change in ζ-potential of biopolymer-coated lipid droplets in secondary emulsions as a function of chitosan concentration at pH 4.5, 5.0, or 5.5. The emulsions consisted of 1 wt.% oil, 0.1 wt.% β-Lg, and 0–0.1% chitosan.

The polysaccharide concentration (c_{Sat}) where the droplet surfaces became saturated with chitosan was determined by modeling the *ζ-potential* versus polysaccharide concentration curves using an empirical equation developed earlier [39]:

$$\frac{\Delta\zeta(c)}{\Delta\zeta_{Sat}} \approx \exp\left(-\frac{c}{3c_{Sat}}\right) \quad (1)$$

where $\Delta\zeta(c)$ and $\Delta\zeta_{sat}$ are the differences in ζ-potential of the oil droplets from the value in the absence of polysaccharide at polysaccharide concentration c and when they are saturated with polysaccharide, respectively. The variable c_{Sat} provides an estimate of the minimum polysaccharide concentration required to cover the droplet surfaces. Values of c_{Sat} were calculated by fitting the above expression to the experimental ζ-potential data as described previously [39]: c_{Sat} = 0.025, 0.022 and 0.019 wt.% for pH 4.5, 5.0 and 5.5, respectively. The corresponding surface load at saturation can be calculated using the following expression:

$$\Gamma_{Sat} = \frac{c_{Sat} d_{32}}{6\phi} \quad (2)$$

Here, c_{Sat} is expressed as the mass of material adsorbed to the surface of the droplets per unit volume of emulsion (C_a/kg m^{-3}), d_{32} is the volume–surface mean droplet diameter, and ϕ is the droplet volume fraction. For this study, d_{32} = 0.22 μm, ϕ = 0.01 (1%) and $c_{Sat} \approx$ 0.25, 0.22 and 0.19 kg m^{-3}, hence Γ_{Sat} = 0.93, 0.80 and 0.70 mg m^{-2} for pH 4.5, 5.0 and 5.5, respectively. These values are in the range reported for the surface loads of other biopolymers adsorbed to the surfaces of lipid droplets [39,40], with differences being attributed due to differences in solution conditions (*e.g.,* pH, ionic strength, buffer type) and polysaccharide characteristics (*e.g.,* molecular weight, conformation, charge density). It therefore appears that a smaller amount of chitosan is required to saturate the droplet surfaces as the pH is increased and the protein-coated droplets become more negatively charged.

The final ζ-potential of the droplets in the secondary emulsions at saturation was fairly similar at pH 4.5, 5.0, and 5.5 (∼+30 mV) irrespective of the initial ζ-potential on the primary emulsion droplets (Fig. 1a). Studies of the adsorption of synthetic polyelectrolytes onto oppositely charged surfaces have also found that the final surface charge tends to have a maximum achievable value [42,49]. Once the surface charge reaches this critical value, there will be a strong electrostatic repulsion between the surface and similarly charged polyelectrolytes in the aqueous phase, which limits further adsorption of the polyelectrolyte. Thus, the final ζ-potential of the droplets is determined by this critical value rather than the initial droplet charge. On the other hand, the overall change in the ζ-potential ($\Delta\xi = \xi_2^\circ - \xi_1^\circ$) due to adsorption of the chitosan layer onto the protein-coated droplets increased appreciably with increasing pH (Fig. 1b), which can be attributed to the fact that more chitosan molecules are required to reach this critical charge level when more negative charges are initially present on the droplet surfaces.

Differences in the dependence of the mean particle diameter and creaming stability of the emulsions on chitosan concentration at the different pH values (Figs. 1c and d) clearly show the impact of initial droplet charge on emulsion stability. At pH 4.5, the addition of chitosan did not promote any increase in particle aggregation or creaming, except at the highest chitosan level, which can be attributed to depletion flocculation caused by the high level of non-absorbed polymer in the continuous phase [50]. The good aggregation stability of the droplets at lower chitosan levels at pH 4.5 can be attributed to the fact that the ζ-potential is high (>+15 mV) even in the absence of added chitosan, and therefore the electrostatic repulsion between the droplets is strong, which prevents them from coming into close proximity. At pH 5.0 and 5.5, appreciable droplet aggregation and creaming were observed at relatively low chitosan concentrations (<0.05 and 0.02 wt.%, respectively), which can be attributed to the low net charge on the droplets, as well as some bridging flocculation that occurred when the droplet surfaces were not saturated with chitosan. Interestingly, more chitosan was required to prevent droplet aggregation at pH 5.0 than at pH 5.5, even though there should be more negative charges on the droplet surfaces at higher pH value. One possible explanation for this phenomenon is that the attractive force between the chitosan molecules and the droplet surfaces is

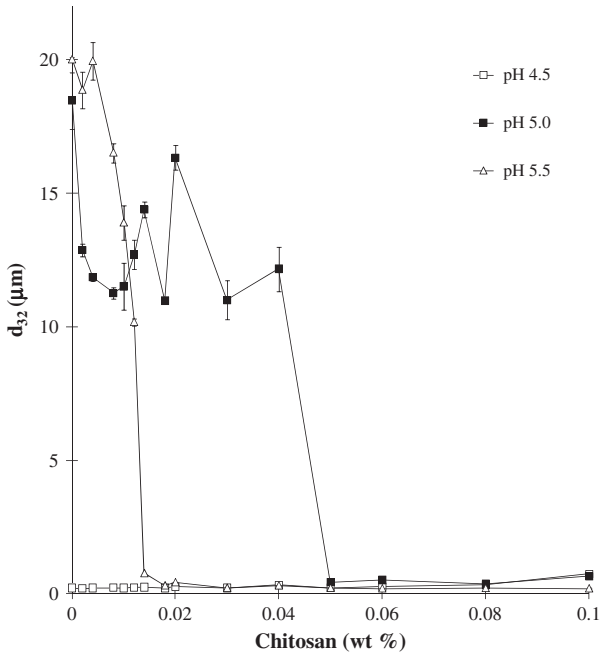

Fig. 1c. Mean particle diameter (d_{32}) of biopolymer-coated lipid droplets in secondary emulsions as a function of chitosan concentration at pH 4.5, 5.0, or 5.5. The emulsions consisted of 1 wt.% oil, 0.1 wt.% β-Lg, and 0–0.1% chitosan.

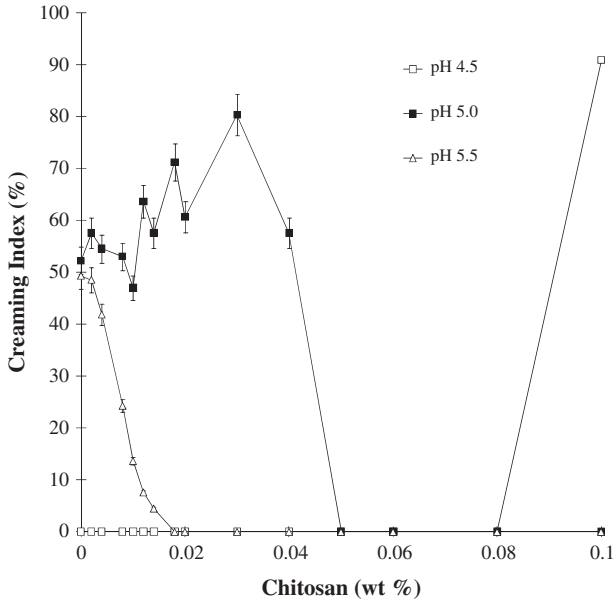

Fig. 1d. Creaming index of biopolymer-coated lipid droplets in secondary emulsions as a function of chitosan concentration at pH 4.5, 5.0, or 5.5. The emulsions consisted of 1 wt.% oil, 0.1 wt.% β-Lg, and 0–0.1%.

weaker at pH 5.0 so that bridging flocculation can occur at higher chitosan levels, *e.g.,* part of an adsorbed chitosan molecule can detach from one droplet and adsorb to another one.

3.2. Secondary emulsion properties

In this series of experiments, the pH stability of protein-coated lipid droplets (primary emulsions) and protein–chitosan-coated lipid droplets (secondary emulsions) was compared. The influence of pH on the ζ-potential, mean particle diameter, and creaming stability of the primary and secondary emulsions was measured (Figs. 2a and b). The emulsions used in this study consisted of 1 wt.% oil, 0.1 wt.% β-Lg, and either 0 wt.% (primary) or 0.04 wt.% (secondary) chitosan. This level of chitosan was used to prepare the secondary emulsions since it had previously been found to be sufficient to saturate the droplet surfaces without promoting extensive droplet flocculation (Figs. 1a–d).

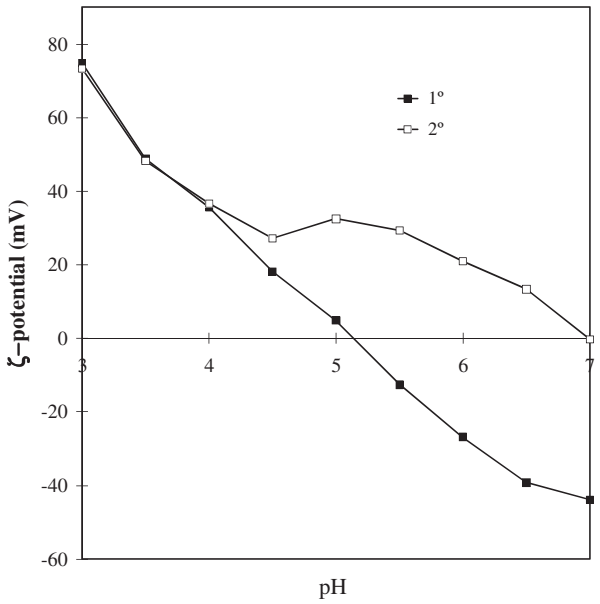

Fig. 2a. The pH dependence of ζ-potential of primary emulsions (1 wt.% oil, 0.1 wt.% β-Lg) and secondary emulsions (1 wt.% oil, 0.1 wt.% β-Lg, 0.04 wt.% chitosan).

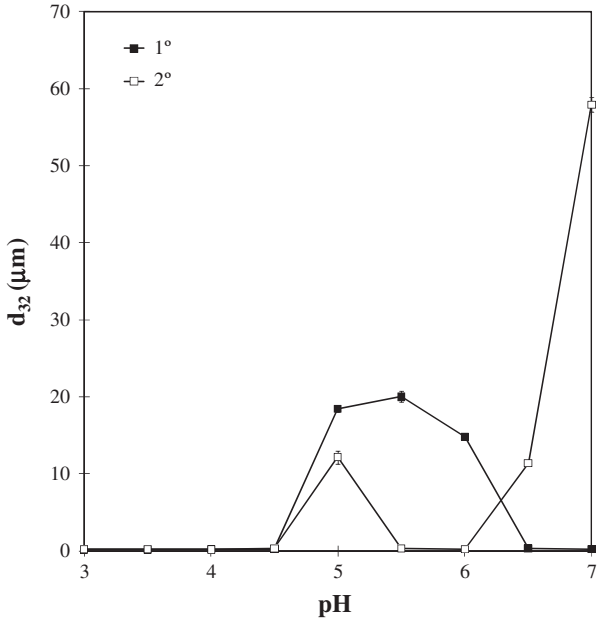

Fig. 2b. The pH dependence of mean particle diameter (d_{32}) of primary emulsions (1 wt.% oil, 0.1 wt.% β-Lg) and secondary emulsions (1 wt.% oil, 0.1 wt.% β-Lg, 0.04 wt.% chitosan).

The droplet ζ-potential in the primary emulsions changed from highly positive to highly negative when the solution was adjusted from pH 3 to 7, with a point of zero charge around pH 5 (Fig. 2a). The pH dependence of the ζ-potential of this type of primary emulsion is well established in the literature, where it has been attributed to the fact that the isoelectric point of adsorbed β-Lg

molecules is around pH 5 [39]. The ζ-potential of the secondary emulsions varied from highly positive at pH 3 to close to zero at pH 7. From pH 3 to 4, the ζ-potentials of both emulsions were fairly similar, which can be attributed to the electrostatic repulsion between the cationic chitosan molecules and cationic protein-coated droplets inhibiting chitosan adsorption. Above pH 4, the ζ-potential of the secondary emulsion was appreciably more positive than that of the primary emulsion, indicating that cationic chitosan molecules adsorbed to the surface of the protein-coated droplets. At pH 5, the protein-coated droplets had a small net positive charge, but there would be some negatively charged patches on the droplet surfaces where the positively charged chitosan molecules could adsorb [51]. From pH 5 to 7, the cationic chitosan molecules would adsorb strongly to the negatively charged protein-coated droplets, thus making the droplets more positive (Fig. 2a). The pK_a values of the charged groups on polyelectrolytes may change considerably when they are present within densely packed multilayer coatings rather than free in solution [48], and so it is possible that the chitosan altered the acid–base behavior of some of the charged groups on the underlying protein layer.

At relatively low pH (⩽4.5) and high pH (⩾6.5) the primary emulsions were relatively stable to droplet aggregation (Fig. 2b) and creaming (no evidence of phase separation), which can be attributed to the relatively strong electrostatic repulsion between the highly charged droplets [51]. On the other hand, at intermediate pH values (pH 5–6), there was a large increase in mean particle diameter (Fig. 2b), visible creaming (data not shown), and evidence of droplet flocculation in the microscopy images (data not shown). These effects can be attributed to weakening of the electrostatic repulsion between the droplets in the primary emulsion near the adsorbed protein's pI, leading to droplet flocculation [51].

The droplets in the secondary emulsions were relatively stable to droplet aggregation and creaming from pH 3 to 6 (Figs. 2a and b). The improved stability of the secondary emulsions compared to the primary emulsions around the isoelectric point of β-lactoglobulin (pI ∼ 5.0) can be attributed to the fact that chitosan molecules adsorbed to the protein-coated droplets (Fig. 2a), thereby increasing the net repulsion between them. Previously, it has been proposed that an adsorbed secondary polysaccharide layer increases the stability of lipid droplets to flocculation by increasing the electrostatic and steric repulsion, as well as decreasing the van der Waals attraction [51]. At higher pH values (pH > 6), extensive droplet aggregation was observed in the secondary emulsions, as demonstrated by an increase in mean particle diameter (Fig. 2b), rapid creaming, and presence of large flocs in optical microscopy images (data not shown). This instability at higher pH values can be attributed to the relatively low ζ-potential on the droplets in this pH range (Fig. 2a), so that the electrostatic repulsion between them was insufficient to prevent them from coming into close proximity [52]. Lipid droplets with biopolymer coatings that consist of an outer layer of cationic chitosan, may therefore have limited application in systems that must operate around neutral pH due to the large degree of droplet aggregation that occurs under these conditions.

3.3. Tertiary emulsion formation

In this section, we examined the influence of adding an outer anionic biopolymer layer onto the surfaces of the protein–chitosan-coated droplets, in order to improve their stability to aggregation at higher pH values. Initially, our objective was to determine the amount of two different anionic polysaccharides, pectin and alginate, required to form stable tertiary emulsions. The electrical charge, mean particle diameter, creaming stability, and microstructure of emulsions (0.5 wt.% oil, 0.05 wt.% β-Lg, 0.025 wt.% chitosan, and 5 mM phosphate buffer, pH 5.5) containing different amounts of the anionic polysaccharides (0–0.25 wt.%) were therefore measured.

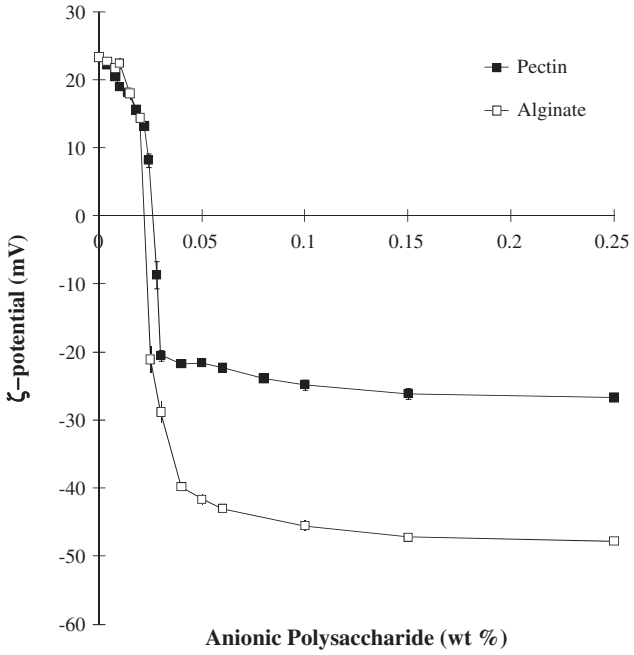

Fig. 3a. Electrical charge (ζ-potential) of biopolymer-coated lipid droplets in tertiary emulsions as a function of pectin or alginate concentration at pH 5.5. Emulsions consisted of 0.5 wt.% oil, 0.05 wt.% β-Lg, 0.025% chitosan, and 0–0.25 wt.% pectin or alginate.

In the absence of anionic polysaccharides, the net charge on the droplets in the secondary emulsions was about +24 mV at pH 5.5 (Fig. 3a), which can be attributed to the cationic nature of the chitosan outer layer in the biopolymer coating. The electrical charge on the droplets became increasingly less positive and eventually changed from positive to negative when the anionic polysaccharide concentration in the emulsions was increased (Fig. 3a). The polysaccharide concentration where there was no net charge on the droplets was fairly similar for both alginate and pectin, being around 0.02–0.03 wt.%. Presumably at this concentration, there were sufficient anionic groups on the adsorbed anionic polysaccharides to neutralize the cationic groups on the adsorbed chitosan. The negative charge on the droplets reached a relatively constant level when the anionic polysaccharide concentration exceeded ∼0.1 wt.% for alginate and pectin. These measurements indicated that negatively charged alginate and pectin molecules adsorbed to the surfaces of positively charged β-Lg–chitosan stabilized lipid droplets. The negative charge on the droplets fully coated with alginate (≈−48 mV) was appreciably higher than that on the droplets coated with pectin (≈−27 mV), which can be attributed to higher charge density of alginate [43]. It was not possible to calculate the surface load of the pectin or alginate on the droplet surfaces for these systems because the dependence of the ζ-potential on polysaccharide concentration did not follow the exponential dependence given by Eq. (1). This may have been because there was a sudden alteration in the structure of the interfacial layer above a critical polysaccharide concentration, which caused a sharp drop in ζ-potential. Previous studies have shown that the growth of polyelectrolyte multilayers at surfaces may be either exponential or linear depending on the strength of the attractive interactions involved [53,54]. The first few polyelectrolyte layers deposited on a surface are often fairly open in structure, but the later ones are more densely packed [54].

The mean particle diameter, creaming stability, and microstructure of tertiary emulsions were measured to determine their

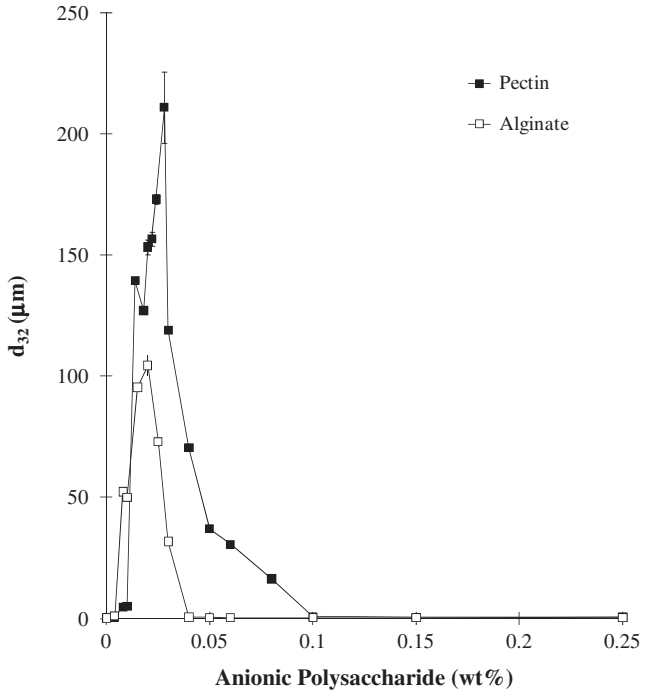

Fig. 3b. Mean particle diameter (d_{32}) of biopolymer-coated lipid droplets in tertiary emulsions as a function of pectin or alginate concentration at pH 5.5. Emulsions consisted of 0.5 wt.% oil, 0.05 wt.% β-Lg, 0.025% chitosan, and 0–0.25 wt.% pectin or alginate.

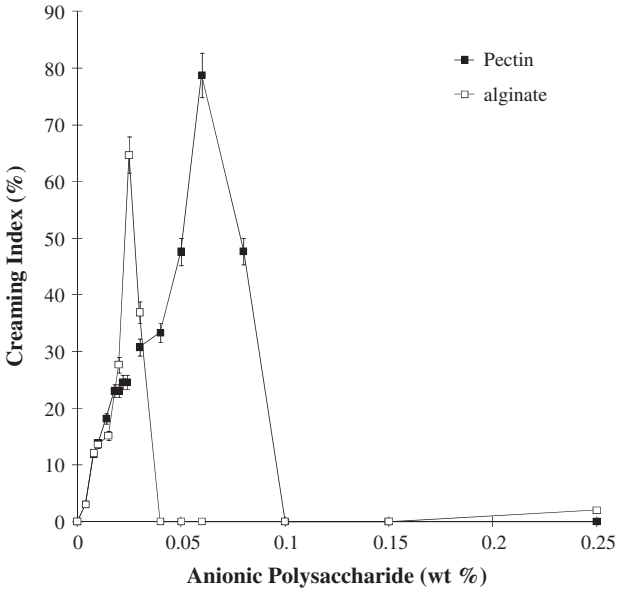

Fig. 3c. Creaming stability (creaming index) of biopolymer-coated lipid droplets in tertiary emulsions as a function of pectin or alginate concentration at pH 5.5. Emulsions consisted of 0.5 wt.% oil, 0.05 wt.% β-Lg, 0.025% chitosan, and 0–0.25 wt.% pectin or alginate.

stability to droplet aggregation. The emulsions were stable to droplet aggregation and creaming before the addition of anionic polysaccharide due to the large electrostatic repulsion between them at pH 5.5 (Figs. 3a–c). Extensive droplet aggregation and creaming were observed in the emulsions at pectin concentrations from 0.004 to 0.1 wt.%, and at alginate concentrations from 0.004 to 0.04 wt.% (Figs. 3b and c). These concentration ranges were close to the region where the droplets had relatively low net electrical charges (Fig. 3a), and hence aggregation can be attributed to charge neutralization and bridging flocculation effects. It is interesting to note that the alginate system was unstable to droplet aggregation over an appreciably narrower range of polymer concentrations than the pectin system. This effect may be attributed to the fact that the droplets coated by alginate had higher ζ-potentials and therefore generated a stronger electrostatic repulsion between them, or because the pectin molecules somehow promoted more bridging flocculation. At higher anionic polysaccharide concentrations, both types of emulsion contained highly negatively charged particles that were stable to droplet aggregation and creaming. The concentration where the anionic polysaccharide-coated droplets first became stable to aggregation and creaming was considerably lower for alginate (0.04 wt.%) than for pectin (0.1 wt.%). In the subsequent experiments, we therefore used anionic polysaccharide concentrations above these critical levels to form tertiary emulsions, *i.e.,* 0.06 wt.% for alginate and 0.15 wt.% for pectin.

3.4. Tertiary emulsion properties

In this series of experiments, we compared the pH stability of tertiary emulsions coated with an outer layer of either anionic polysaccharide (alginate or pectin), with a secondary emulsion coated with an outer layer of cationic polysaccharide (chitosan). The electrical charge, mean particle diameter, creaming stability, and microstructure of the emulsions were measured (0.5 wt.% oil, 0.05 wt.% β-Lg, 0.025 wt.% chitosan, 0.06 wt.% alginate, or 0.15 wt.% pectin).

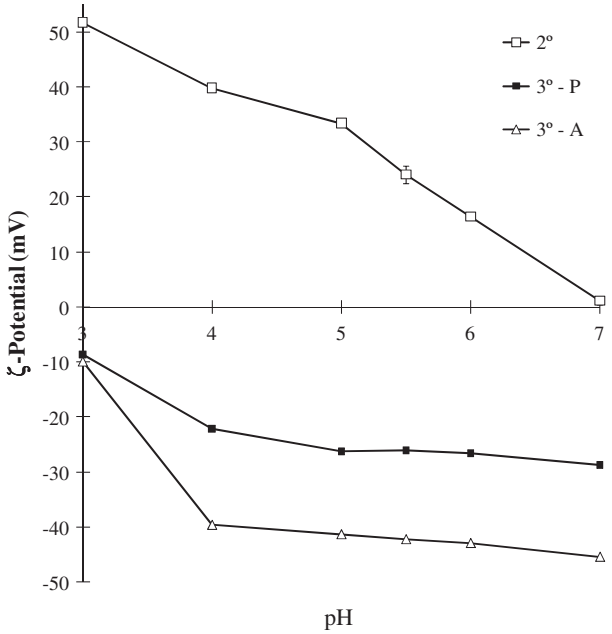

Fig. 4a. The pH dependence of ζ-potential of secondary emulsions (0.5 wt.% oil, 0.05 wt.% β-Lg, 0.025% chitosan) and tertiary emulsions (0.5 wt.% oil, 0.05 wt.% β-Lg, 0.025 wt.% chitosan, 0.06 wt.% alginate or 0.15 wt.% pectin).

The ζ-potential of the secondary emulsion went from highly positive to close to zero as the pH was increased from 3 to 7 for reasons discussed above (Fig. 4a). On the other hand, the ζ-potential of both tertiary emulsions remained negative at all pH values, indicating that the anionic polysaccharides had formed a coating around the lipid droplets. At similar pH values, the ζ-potential of the droplets coated with alginate was more highly negative than those coated with pectin, which can be attributed to the higher charge density of alginate [43]. The ζ-potential of both tertiary emulsions decreased slightly when the pH was decreased from 7

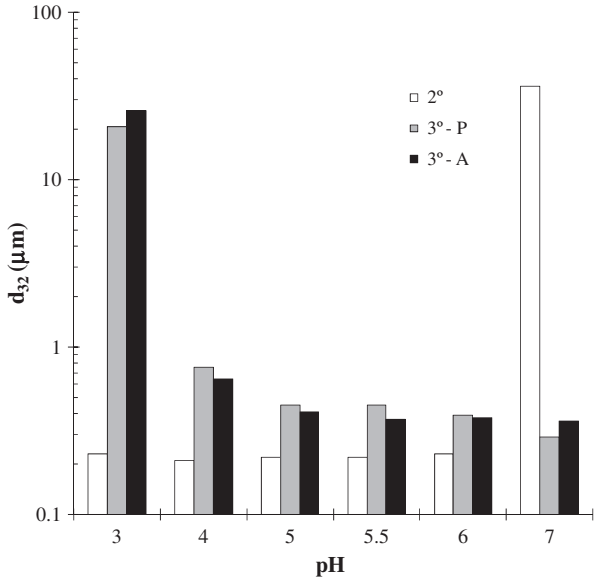

Fig. 4b. The pH dependence of mean particle diameter (d_{32}) of secondary emulsions (0.5 wt.% oil, 0.05 wt.% β-Lg, 0.025% chitosan) and tertiary emulsions (0.5 wt.% oil, 0.05 wt.% β-Lg, 0.025 wt.% chitosan, 0.06 wt.% alginate, or 0.15 wt.% pectin).

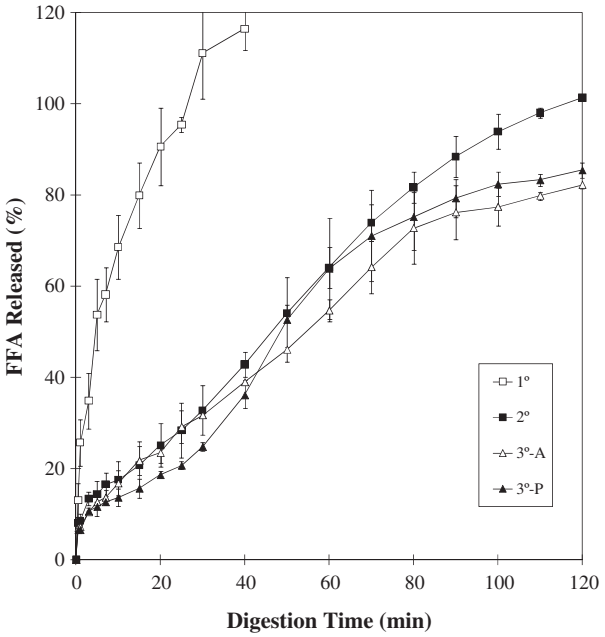

Fig. 5. Percentage of free fatty acids released versus time for primary, secondary, and tertiary corn oil-in-water emulsions determined using a pH-stat method. Emulsion compositions are stated in the text.

to 4, but decreased much more steeply when the pH was further reduced from 4 to 3 (Fig. 4a). The large reduction in negative charge at acidic pH values can be attributed to the fact that the anionic groups on pectin and alginate are carboxylic acids ($COOH \Longleftrightarrow COO^- + H^+$) with reported p$K_a$ values around pH 3.5 [55].

Interestingly, the alginate or pectin layers did not appear to desorb from the droplet surfaces in the tertiary emulsions at high pH values, even though the net charge on the chitosan-coated droplets in the secondary emulsions was close to zero (Fig. 4a). This effect might be because the chitosan layer retained some positive charge at neutral pH, even though its pK_a value has been reported to be around 6.5. Indeed, previous research has shown that the pK_a values of charged groups on polyelectrolytes can change appreciably when they are sandwiched between two oppositely charged polyelectrolytes, thereby increasing the stability of the multilayer system to pH [56,57].

As discussed previously, the secondary emulsion was stable to droplet aggregation and creaming from pH 3 to 6, but exhibited extensive aggregation at pH 7 because of the reduction in net charge on the droplets. On the other hand, both tertiary emulsions were stable to droplet aggregation and creaming from pH 4 to 7, but became unstable at pH 3 (Fig. 4b). The better stability of the tertiary emulsions at pH 7 compared to the secondary emulsions can be attributed to the fact that the former had much higher net electrical charges at this pH (Fig. 4a), and therefore higher electrostatic repulsion between droplets. Conversely, the poor stability of the tertiary emulsions at pH 3 compared to the secondary emulsions can be attributed to the fact that the former had much lower net electrical charges, and therefore less electrostatic repulsion to stabilize droplets. In addition, there may have been some bridging flocculation between the negatively charged alginate or pectin molecules in the aqueous phase and the positively charged droplets. We did find that the droplets in the tertiary emulsions could be stabilized against droplet aggregation and creaming at pH 3 when higher concentrations of anionic polysaccharide were added, *e.g.,* 0.25 wt.% pectin suppressed aggregation entirely. These results indicated that emulsions with good stability against droplet aggregation from pH 4 to 7 can be produced using β-Lg–chitosan–alginate or β-Lg–chitosan–pectin coatings.

It should be noted that the formation and properties of the multilayer emulsions prepared in this work are likely to be strongly dependent on ionic strength, since this will affect the magnitude and range of the electrostatic interactions [58]. One would expect ionic strength effects to be complex because they affect polyelectrolyte conformation, as well as polyelectrolyte–droplet, polyelectrolyte–polyelectrolyte, and droplet–droplet interactions [39]. It would therefore be useful to systematically examine the influence of ionic strength and ion type on the formation and stability of this type of multilayer emulsion, particularly if they are going to be used as delivery systems in biological media that contain relatively high levels of monovalent and multivalent ions.

3.5. Influence of coating composition and structure on lipid digestibility

Finally, we examined the impact of the composition and structure of biopolymer coatings on the digestion rate and microstructure of emulsified lipids. The release of free fatty acids (FFA) from primary, secondary, and tertiary emulsions was measured using an *in vitro* digestion model (pH Stat), which clearly indicated that biopolymer coatings influenced lipid digestion (Fig. 5). The protein-coated lipid droplets in the primary emulsion were rapidly digested within the first 30 or 40 min, indicating that the lipase could adsorb to the droplet surfaces and promote triacylglycerol lipolysis. On the other hand, the digestion of the lipid droplets in the secondary and tertiary emulsions was considerably slower than that in the primary emulsions, with less than 40% of the lipids being digested within the first 30 min. We hypothesize that the polysaccharides formed a protective coating around the lipid droplets that restricted the access of the lipase to the emulsified triacylglycerols, thereby retarding the lipolysis reaction. In addition, confocal fluorescent microscopy images of the secondary and tertiary emulsions before and after digestion indicated that they were highly aggregated (Fig. 6). Lipase molecules would have to diffuse through the outer parts of these aggregates before they could reach the lipid droplets trapped inside, which would also have slowed down the rate of lipid digestion.

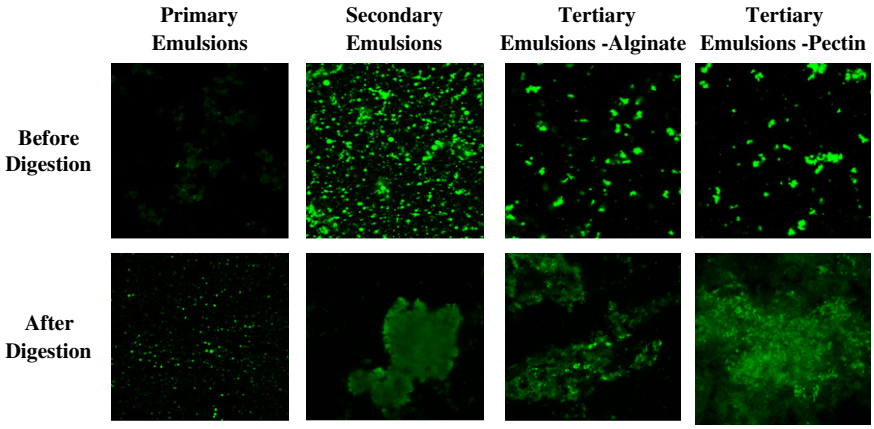

Fig. 6. Microstructure of primary, secondary, and tertiary emulsions before and after digestion determined by confocal microscopy. The green color represents the lipid phase in the emulsions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Conclusions

The objective of this study was to examine the influence of the electrical characteristics of the outer biopolymer layer on the aggregation stability and digestibility of lipid droplets stabilized by biopolymer coatings. The droplets in primary emulsions, which had an outer protein coating, changed from positive to negative when the pH was increased from 3 to 7. As a result these emulsions were unstable to droplet aggregation at intermediate pH values because of the low net charge on the droplets near the isoelectric point of the adsorbed proteins. The droplets in secondary emulsions, which had an outer chitosan coating, changed from highly positive to zero when the pH was increased from 3 to 7. These emulsions were stable to droplet aggregation from pH 3 to 6, but highly unstable at higher pH values because of the low net charge on the droplets at neutral pH. Tertiary emulsions, which had an outer alginate or pectin coating, changed from slightly negative to highly negative when the pH was increased from 3 to 7. These emulsions were unstable to droplet aggregation at pH 3 because of the low net negative charge on the droplets, but could be improved if higher anionic polysaccharide levels were used. Overall, it appeared that substantial droplet aggregation occurred when the charge on the droplets fell below some critical level ($|\zeta| < 20$ mV).

This study has shown that the physical stability of lipid droplets coated by multilayer biopolymer coatings is largely determined by the electrical characteristics of the outer biopolymer layer. This knowledge is useful for the rational design of encapsulation and delivery systems based on the layer-by-layer deposition method for different applications. For example, if a delivery system must remain stable under acid conditions but breakdown at neutral pH, then an outer coating of chitosan could be used, *i.e.,* a secondary emulsion. On the other hand, if a delivery system must remain stable under neutral conditions but breakdown at acid pH, then an outer coating of alginate or pectin could be used, *i.e.,* a tertiary emulsion. The presence of an outer coating of charged polysaccharides (dietary fibers) was shown to delay lipid digestibility, which has important consequences for the design of controlled delivery systems for nutraceuticals or pharmaceuticals.

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