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Influence of Tripolyphosphate Cross-Linking on the Physical Stability and Lipase Digestibility of Chitosan-Coated Lipid Droplets

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The impact of cross-linking chitosan with tripolyphosphate (TPP) on the physical stability and lipase digestibility of chitosan-coated lipid droplets was investigated. Relatively, high TPP levels ($\geq$0.004 wt %) promoted droplet aggregation and gravitational separation, which was attributed to charge neutralization and interdroplet cross-linking. Cross-linked chitosan-coated lipid droplets were formed at lower TPP levels that were relatively small ($d \approx 450$ nm), cationic ($\zeta \approx +60$ mV), and stable to particle aggregation and gravitational separation (pH 3, 21 days). However, these droplets were highly unstable at pH 7 because of a reduction in net particle charge and weakened electrostatic repulsion. An in vitro lipid digestion model was used to study the impact of the chitosan coating on the digestibility of lipid droplets by pancreatic lipase (pH 7, bile salts, pancreatic lipase, and 5.0 mM $CaCl_2$). The rate of lipid digestion decreased when the lipid droplets were coated with chitosan and decreased further when the chitosan coating was cross-linked with TPP. Indeed, both cross-linked and noncross-linked chitosan coatings were able to prevent lipid digestion under conditions simulating the small intestine. This study has important implications for the design of structured emulsions with controlled lipid digestibility and for the targeted delivery of lipophilic functional components to specific regions within the gastrointestinal tract.

KEYWORDS: Chitosan; emulsions; lipase; lipids; cross-linking; TPP

1. INTRODUCTION

There is an increasing interest within the food and biomedical industries in the creation of delivery systems that can encapsulate, protect, and deliver lipophilic bioactive compounds, such as ω-3 fatty acids, carotenoids, phytosterol (*1−7*), and pharmacological drugs (*8*, *9*). The electrostatic layer-by-layer (LbL) deposition method has previously been used to form nanolaminated biopolymer coatings around lipid droplets that have been shown to alter lipid digestibility using in vitro studies (*10−13*). 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 (*14−22*), thus potentially affecting the in vitro digestibility or release of encapsulated lipophilic bioactive compounds. In this study, we examined the impact of noncovalent (physical) cross-linking of a chitosan coating formed around lipid droplets using the LbL method on their physical stability and lipase digestibility.

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 high natural abundance, biodegradability, and biocompatibility (*8*, *23−27*). In addition, chitosan is one of the few cationic biopolymers available, and therefore, it is often used to form electrostatic complexes with anionic biopolymers, such as alginate, carrageenan, xanthan, and pectin. Chitosan can therefore be used as a building block to assemble a variety of structures based on electrostatic interactions, e.g., multilayers, coacervates, and hydrogels. A number of studies have shown that the properties and integrity of chitosan-based structures can be altered using physical or chemical cross-linking agents, e.g., multivalent ions or aldehydes (*9*, *28*). Noncovalently cross-linked chitosan-based structures have attracted particular attention because they have better biocompatibility than covalently cross-linked ones (*29−36*). TPP is a polyvalent anion that can be used to noncovalently cross-link cationic chitosan molecules through electrostatic bridging. TPP carries five negatively charged ionizable groups with different pK_a values, and therefore, its net charge is highly pH-dependent: $pK_{a1} = 1$, $pK_{a2} = 2$, $pK_{a3} = 2.79$, $pK_{a4} = 6.47$, and $pK_{a5} = 9.24$ (*36*). However, chitosan has positively charged amine side groups along its backbone with pK_a values around pH 6.5 (*37*). Thus, TPP can interact strongly with chitosan at pH values around and below neutral since TPP is highly negatively charged, while chitosan is highly positively charged.

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The rational utilization of cross-linked chitosan coatings to control the bioavailability of emulsified lipids within the human digestive tract relies on a detailed understanding of the physicochemical and physiological processes that occur during lipid digestion. One of the most common classes of encapsulated lipids used in pharmaceutical, supplement, and food products is the highly water-insoluble triacylglycerols. Before these molecules 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 (*38*). The lipase-catalyzed lipid-digestion reaction occurs at the interface between the aqueous and oil phases. To function efficiently, these enzymes must adsorb to the interface of the lipid droplets so that they come into close physical proximity to the lipid substrate. The digestion of emulsified lipids can therefore be controlled by altering the composition, 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 (*10*−*14*). It is therefore important to understand how specific coatings behave under the complex physicochemical conditions present in the GI tract, e.g., acids, bases, minerals, proteases, lipases, colipases, bile salts, phospholipids, mucins, lipolytic products, and mechanical processes (*39*−*43*). We hypothesize that noncovalently cross-linking chitosan coatings using TPP may be a practical method of controlling the digestibility of encapsulated lipids within the GI tract since cross-linking should change the integrity and permeability of the coatings.

In vitro digestion models can be used to provide mechanistic insights into the physicochemical processes that affect the digestibility of emulsified lipids (*10*, *12*, *41*, *44*−*47*). In the present study, we used a relatively simple in vitro lipolysis model to test the impact of cross-linking chitosan coatings on lipid digestibility. In previous studies, we used the LbL method to prepare lipid droplets coated with lecithin−chitosan layers (*18*, *48*). In this study, we used a similar system but examined the impact of cross-linking the chitosan coating with TPP on the physical stability and lipase digestibility of the encapsulated lipids. The results of this research have important implications for the rational design of delivery systems for encapsulation, protection, and release of bioactive lipids.

2. MATERIALS AND METHODS

2.1. Materials. Powdered lysolecithin (Solec 8160, 97% pure, HLB 9) was obtained from The Solae Company (St. Louis, MO). Powdered chitosan (dry matter content, 92.5%; ash content, <0.5%; degree of deacetylation, 91.8%; viscosity of 1% solution, 45 mPa s; solubility, 99.2%; molecular weight, 200 kDa) was obtained from Primex (Siglufjordur, Iceland). Sodium tripolyphosphate (TPP, tech. 85%), was purchased from Sigma-Aldrich (St. Louis, MO). Lipase (from porcine pancreas pancreatin) and bile extract (porcine) were obtained from Sigma-Aldrich. Pancreatin is assumed to contain equivalent moles of colipase and lipase (*44*). Pancreatic lipase contains many enzymes, including amylase, trypsin, lipase, ribonuclease, and protease. According to the supplier, pancreatic lipase releases no less than two microequivalents of acid per min per mg 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, and 0.5−7% deoxycholic acid; and 5 wt % phosphatidylcholine (*44*). Corn oil was purchased from a local supermarket and used without further purification (100% Pure Mazola Corn oil). Corn oil contains ≥99% triacylglyceride, with proportions of approximately 58.7% polyunsaturated fatty acid, 28.7% monounsaturated fatty acid, and 12.6% saturated fatty acid (*49*). Calcium chloride ($CaCl_2 \cdot 2H_2O$) was obtained from Fisher Scientific. Reagent-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 3.0 using 1 M HCl. An emulsifier solution was prepared by dispersing 1.0 wt % lysolecithin powder into buffer solution. The emulsifier solution was stirred for at least 3 h, and the pH of the solution was adjusted to 3.0 using HCl. A chitosan solution was prepared by dispersing 0.2 wt % chitosan powder into stock buffer solution and stirring overnight to ensure complete dispersion. A stock sodium tripolyphosphate (TPP) solution was made by dissolving 0.01 wt % TPP into stock buffer solution and then adjusting to pH 3 using 1 M HCl. Different levels of working solution of TPP were prepared by diluting the stock TPP solution with stock buffer solution.

2.3. Emulsion Preparation. A primary emulsion was made by homogenizing a 5 wt % corn oil with a 95 wt % aqueous emulsifier (lysolecithin) solution with a high-speed blender (Tissue Tearer, Biospec Products, Inc., Bartlesville, OK) for 2 min, followed by 6 passes at 11,000 psi through a microfluidizer (Model 1101, Microfluidics, Newton, Massachusetts). Aliquots of primary emulsion were added to aqueous buffered solutions containing different amounts of chitosan to form secondary emulsions with a final concentration of 1% corn oil, 0.2 wt % lysolecithin, 0.00−0.08 wt % chitosan, and 100 mM acetic acid (pH 3.0). Any flocs formed in these emulsions were disrupted by sonicating it for 2 min at a frequency of 20 kHz, an amplitude of 40%, and a duty cycle of 0.5 s (Model 500, Sonic Dismembrator, Fisher Scientific, Pittsburgh, PA, USA). Ionic cross-linking of chitosan with TPP at the interface of lysolecithin−chitosan coated lipid droplets was conducted by adding the secondary emulsion into TPP solutions at ambient temperature (20 °C) and stirring for 30 min. This gave a sample with a final concentration of 0.5 wt % corn oil, 0.1 wt % lysolecithin, 0.00−0.04 wt % chitosan, 0.00−0.01 wt % TPP, and 100 mM acetic acid at pH 3.

2.4. Particle Size and ζ-Potential Measurements. Particle size and ζ-potential were 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 size data were reported as the Z-average mean diameter, while the particle charge data were reported as the ζ-potential. The Z-average diameter of particles was calculated from their Brownian motion via the Stokes−Einstein equation taking corrected aqueous phase viscosities into account. The ζ-potential was calculated from the measurement of the electrophoretic mobility of particles in an applied oscillating electric field using laser Doppler velocimetry. The particle size distribution (PSD) of the samples before and after adjusting pH to 7 were measured using a static light scattering instrument (Mastersizer S, Malvern Instruments). 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).

2.5. In Vitro Digestion Model. The dynamic in vitro digestion model used was a modification of those described by Mun et al. (*11*) and Zanenberg et al. (*44*). The procedure was as follows: Emulsion (30.0 mL) (pH 3.0) was placed in a clean beaker in a water bath at 37.0 °C for 10 min and adjusted to pH 7 with NaOH solution (0.1 or 1 M). 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) was added into the 30 mL 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 was 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 (50 mM) reflected the amount of free fatty acids generated by lipolysis of the initial triacylgycerols. Blank experiments were performed in the absence of corn oil to account for the fatty acids present or released from the lysolecithin and other components.

2.6. Data Analysis. Differences among the treatments were evaluated by one-way ANOVA with Post Hoc mean ranking test using Tukey by SPSS v 16.0 (SPSS Inc., Chicago, IL). Mean values with statistical difference of $P < 0.05$ were considered as significant.

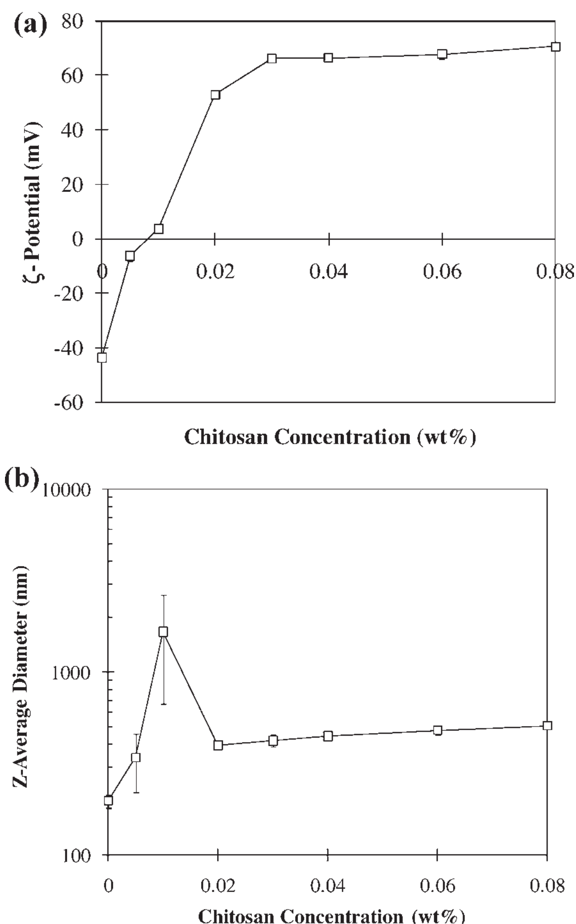

Figure 1. (a) ζ-Potential and (b) Z-average particle diameter of primary emulsions in the presence of different chitosan concentrations (1% corn oil, 0.2% lysolecithin, 100 mM acetic acid, and pH 3).

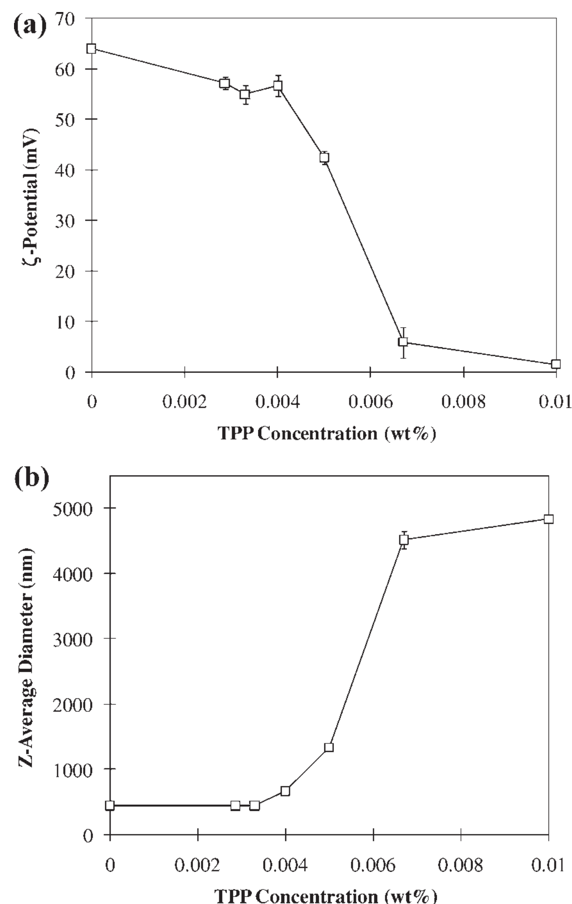

Figure 2. (a) ζ-potential and (b) Z-average particle diameter of secondary emulsions in the presence of different TPP concentrations (0.5% corn oil, 0.1% lysolecithin, 0.02% chitosan, 100 mM acetic acid, and pH 3).

3. RESULTS AND DISCUSSION

3.1. Influence of Chitosan Concentration on Droplet Characteristics. Initially, the electric charge on the lipid droplets in primary emulsions containing different concentrations of chitosan (0 to 0.08 wt %) was measured (**Figure 1a**). In the absence of chitosan, the electric charge on the emulsion droplets was around −43 mV at pH 3, which was due to the fact that the pH of aqueous phase was greater than the pK_a of the anionic groups on the adsorbed lysolecithin layer. With increasing chitosan concentration, the net charge on the droplets became progressively less negative, neutral, and then positive, indicating that cationic chitosan adsorbed to the surfaces of the anionic lipid droplets. The positive charge on the droplets reached a fairly constant value when the chitosan concentration exceeded 0.02−0.03%, suggesting that the droplet surfaces had become saturated with chitosan. These results are in close agreement with previous studies where lecithin and chitosan have been used to prepare multilayer emulsions using the LbL approach (*48*).

The mean particle diameters of the lipid droplets increased as the chitosan concentration in the emulsions increased from 0.00 to 0.01% (**Figure 1b**), which can be attributed to charge neutralization and polymer bridging effects associated with incomplete surface coverage (*48*). At higher chitosan concentrations, where the droplet surfaces were saturated with chitosan, the lipid droplets were relatively stable to aggregation because of the increased electrostatic and steric repulsions between them. We therefore used a concentration of 0.03% chitosan to prepare the secondary emulsions in the remainder of these studies since the droplet surfaces were saturated, and there was little droplet aggregation.

3.2. Influence of TPP Concentration on Droplet Characteristics. The objective of this series of experiments was to examine the impact of tripolyphosphate (TPP) addition on the electrical properties and physical stability of chitosan-coated lipid droplets. Different levels of TPP were incorporated into 1% corn oil-in-water emulsions (0.2% lysolecithin, 0.03% chitosan, 100 mM acetic acid, and pH 3). A pH of 3 was selected because previous studies have shown that the ionic cross-linking of chitosan by TPP is pH-dependent and that a controlled homogeneous cross-linking reaction occurs at this pH (*50*, *51*). In the absence of TPP, the electrical charge on the chitosan-coated lipid droplets was highly cationic (+ 64 mV, **Figure 2a**). The electric charge on the droplets became less positive, moving from around +64 to +1.5 mV, as the TPP concentration was increased from 0 to 0.01% (corresponding to mass ratios of TPP-to-chitosan of 0:1 to 0.5:1). On the basis of previous studies, it is postulated that the TPP anions diffused from the aqueous solution into the chitosan layers and noncovalently cross-linked the protonated amine groups (*50*, *51*). Interestingly, the ζ-potential only decreased slightly from 0 to 0.004 wt % TPP but then decreased sharply when TPP levels increased from 0.004 to 0.0067% (**Figure 2a**). A possible explanation for this effect is that the chitosan layer around the droplets collapsed above a critical TPP level, possibly due to extensive cross-linking between the polymer molecules within the layers.

The mean particle diameter of the chitosan-coated lipid droplets was around 450 nm in the absence of TPP (**Figure 2b**).

From 0 to 0.004 wt % TPP, the mean particle size remained relatively constant indicating that droplets were stable to aggregation, but at higher TPP levels, extensive particle aggregation occurred. The creaming stability of the emulsions was also measured after 1 and 21 days of storage (data not shown). After 1 day of storage, some creaming was visible in the emulsions containing 0.0067 and 0.01 wt % TPP (i.e., a whiter cream layer was observed at the top of the tubes, and a watery serum layer was observed at the bottom), but at lower TPP concentrations, they appeared homogeneous. After 21 days of storage, some creaming was observed in all of the emulsions, but the most extensive creaming occurred in the emulsions with the higher TPP concentrations. The high degree of particle aggregation and rapid creaming observed above 0.004 wt % TPP may be attributed to a number of reasons: (i) the relatively low ζ-potential (**Figure 2a**) would reduce the electrostatic repulsion among the droplets; (ii) the high levels of TPP may have acted as ionic bridges between chitosan molecules adsorbed to different droplets; and (iii) if the chitosan layer collapsed because of the presence of TPP, then the steric repulsion between the droplets would have been reduced.

Our objective was to produce a delivery system that was relatively stable to droplet aggregation, and therefore, we selected a TPP level of 0.004 wt % to cross-link the chitosan coatings in the remainder of the experiments.

3.3. Influence of Chitosan Cross-Linking on the Physical Stability of Lipid Droplets. In this series of experiments, we examined the impact of a cross-linked and a noncross-linked chitosan coating on the physicochemical properties of encapsulated lipids using a simple in vitro digestion model. The particle size distributions and ζ-potentials of primary (1°, lysolecithin coated), secondary (2°, lysolecithin−chitosan coated), and cross-linked secondary (2°-X, lysolecithin−chitosan-TPP coated) emulsions were measured at pH 3 and 7. The three different systems all had essentially monomodal particle size distributions at pH 3 (**Figure 3a**), with surface-weighted mean particle diameters (d_{32}) of 0.22, 0.32, and 0.30 μm for the 1°, 2°, and 2°-X emulsions, respectively. All three emulsions exhibited monomodal size distributions at pH 7; however, the particles in the 1° emulsion remained relatively small with a mean particle diameter similar to that at pH 3 ($d_{3,2}$ = 0.22 μm), whereas the particles in the 2° ($d_{3,2}$ = 61 μm) and 2°-X ($d_{3,2}$ = 51 μm) emulsions were much larger than those at pH 3 (**Figure 3b**). There was no visible evidence of gravitational separation in any of the emulsions stored for 3 days at pH 3 at ambient temperature (**Figure 4**). These results indicated that all of the emulsions were relatively stable to droplet aggregation and creaming at pH 3. When the same emulsions were adjusted to pH 7, there were appreciable changes in their physical stability. The lipid droplets in the 1° emulsion were relatively stable to gravitational separation, whereas extensive creaming was observed in the 2° and 2°-X emulsions (**Figure 4**).

The good stability of the 1° emulsions to droplet aggregation and creaming at pH 7 can be attributed to the fact that the droplets were highly negatively charged (**Figure 5**); therefore, there would be a strong electrostatic repulsion preventing them from coming into close proximity. However, the poor stability of the 2° and 2°-X emulsions at pH 7 can be attributed to the fact that the net electrical charge on the droplets was close to zero (**Figure 5**); hence, the electrostatic repulsion among the droplets was insufficient to overcome the attractive van der Waals interactions. The low electrical charge on the secondary emulsion droplets at pH 7 was due to the fact that the pK_a value of chitosan is around 6.5; therefore, the amine groups lose their cationic charge at higher pH values ($-NH_3^+ \rightarrow -NH_2$). In principle, the chitosan coating could become detached from the lipid droplets at high pH values because the electrostatic attraction between the cationic chitosan and anionic lecithin would be decreased. However, the large difference in the measured ζ-potentials at pH 7 for the primary (1°) and the two secondary (2° and 2°-X) emulsions indicates that the chitosan layer remained attached to the lipid droplet surfaces. These observations are consistent with previously reported results on lipid droplets coated with lecithin−chitosan layers (*48*).

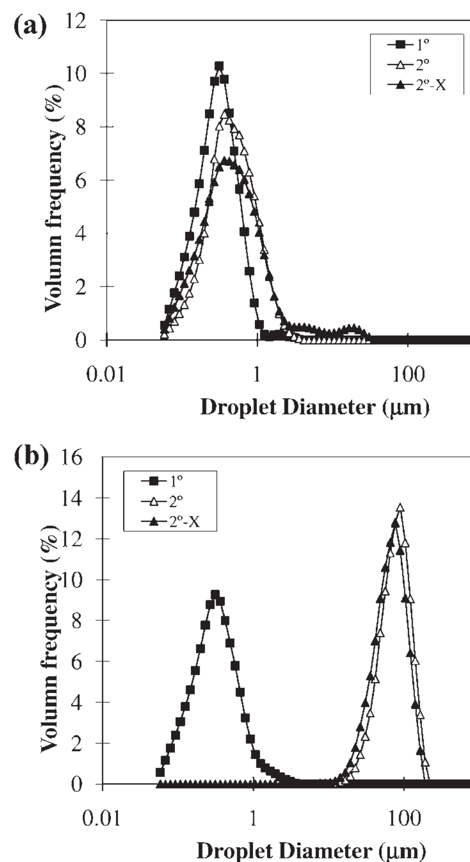

Figure 3. Particle size distributions of oil-in-water emulsions at (**a**) pH 3 and (**b**) pH 7: 1°, coated with lysolecithin; 2°, coated with lysolecithin−chitosan; 2°-X, coated with lysolecithin−chitosan-TPP.

3.4. Influence of Chitosan Cross-Linking on in Vitro Digestibility of Lipid Droplets. The purpose of these experiments was to examine the influence of cross-linked and noncross-linked chitosan coatings on the in vitro digestibility of emulsified corn oil by pancreatic lipase. The fatty acids released from the encapsulated triacylglycerides from corn oil because of the action of pancreatic lipase were measured by pH-stat titration at pH 7.0 (**Figure 6a**). The rate of fatty acid formation due to lipase digestion decreased in the following order: 1° > 2° > 2°-X. This is clearly seen when the rate of fatty acid formation during the first 5 min of digestion is compared for the three emulsions, 1° > 2° and 2°-X ($P < 0.05$, **Figure 6b**). These results clearly show that a chitosan coating can retard the digestion of emulsified lipids and that cross-linking the chitosan coating with TPP can further retard it. There are a number of physicochemical phenomena that may account for this result. The lipid droplets in the 1° emulsions are only coated with a layer of lysolecithin, which is a small molecule anionic surface-active lipid that can easily be displaced by bile salts and lipase, thereby facilitating the access of lipase to the encapsulated lipids. However, the lipid droplets in the 2° emulsions have an outer coating that consists of a cationic chitosan layer. The chitosan coating may inhibit lipid digestion by restricting the ability of lipases from reaching the lipid droplet surfaces, e.g., through a

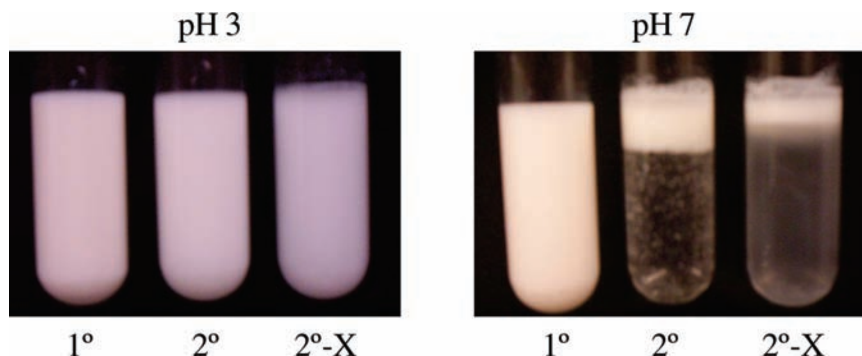

Figure 4. Digital photographs of oil-in-water emulsions after 3 days of storage at pH 3 and pH 7: 1°, coated with lysolecithin; 2°, coated with lysolecithin−chitosan; 2°-X, coated with lysolecithin−chitosan-TPP.

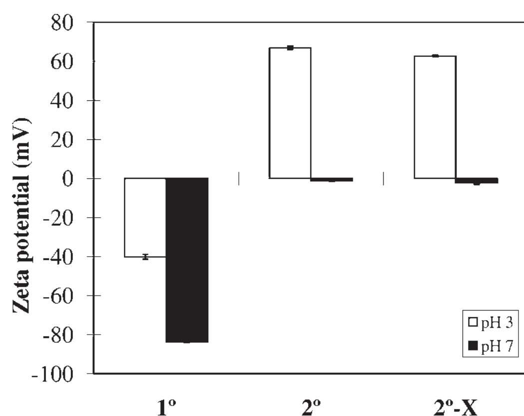

Figure 5. pH dependence of the ζ-potential of three oil-in-water emulsions: 1°, coated with lysolecithin; 2°, coated with lysolecithin−chitosan; 2°-X, coated with lysolecithin−chitosan-TPP.

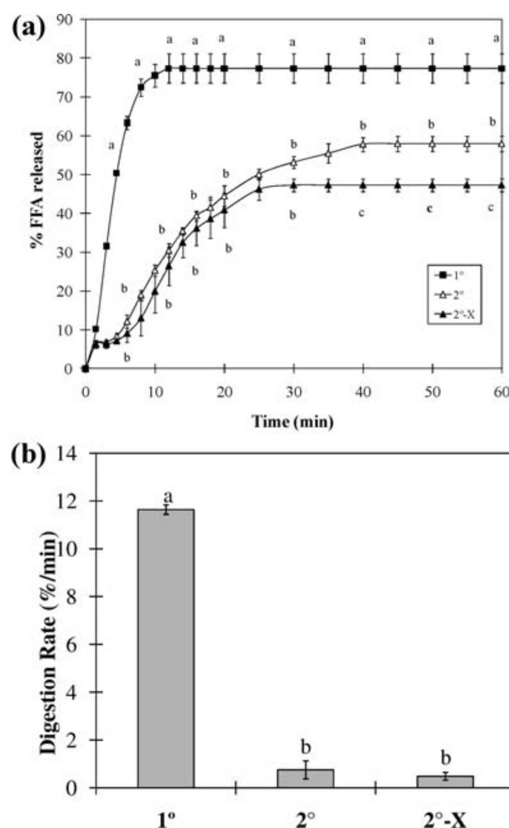

Figure 6. (**a**) Fatty acids released versus time and (**b**) digestion rate during the first 5 min for corn oil-in-water emulsions were determined using an in vitro digestion model at pH 7 and 37 °C: 1°, coated with lysolecithin; 2°, coated with lysolecithin−chitosan; 2°-X, coated with lysolecithin−chitosan-TPP. Different letters (a,b,c) represent samples that are significantly different at the 95% confidence level ($P = 0.05$).

steric hindrance effect or through specific binding effects. Alternatively, the high degree of droplet flocculation observed in the emulsions containing chitosan (**Figure 4**, at pH 7) may have reduced the lipid digestion rate because the lipase molecules would have to move through the interstices between the outer droplets in the flocs before they could reach the inner ones. Finally, chitosan itself is known to bind bile salts, which may inhibit lipid digestion by reducing the amount of bile salts available to adsorb to lipid droplet surfaces or to solubilize lipid digestion products (*46*, *52*). Cross-linking the chitosan coating may have further decreased the rate of lipid digestion by reducing the chitosan layer's susceptibility to displacement by bile salts and lipase, by decreasing the pore size of the adsorbed polymer coating or by changing the structure of the flocs formed. Additional research is required to identify the precise molecular mechanisms by which chitosan inhibits lipid digestion in these systems.

This study has shown that cross-linked and noncross-linked chitosan coatings surrounding lipid droplets affect their physical stability and lipid digestibility within in vitro digestion models. The initial rate of lipid digestion decreased when lipid droplets were coated with chitosan and further decreased when the cationic chitosan layer was noncovalently cross-linked using anionic tripolyphosphate (TPP). The ability of chitosan coatings to retard lipid digestion may be attributed to their ability to form relatively thick polymeric coatings around the lipid droplets or due to the fact that these systems are highly flocculated, which may reduce the lipase accessibility of lipid droplets within the interior of the flocs. Cross-linking the chitosan layer with TPP may have decreased its susceptibility to displacement by bile salts and lipase, reduced the pore size of the polymer coating, or changed the structure of the flocs formed. Indeed, both cross-linked and noncross-linked chitosan coatings were able to prevent lipid digestion under conditions simulating the small intestine. This research has implications for the design of structured emulsions with controlled lipid digestibility or for the delivery of lipophilic functional components to specific regions within the gastrointestinal tract. A biopolymer coating that decreases the rate of digestion of emulsified lipids may be useful for delivering bioactive components to the large intestine, e.g., components designed to combat colon cancer. However, reducing lipid digestion may have an adverse impact on individuals with health problems that inhibit their normal ability to absorb lipids, or it

may lead to the excretion of oil-soluble nutrients in the feces before they are absorbed (e.g., vitamin A or D).

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