**Low density lipoprotein/pectin complex nanogels as potential oral delivery vehicles for
curcumin**

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ABSTRACT

Development of delivery systems from natural protein/polysaccharide complexes is of particular interests for applications in food, pharmaceuticals and biomedicine. In this study, novel nanogels smaller than 60 nm were prepared from egg yolk low density lipoprotein (LDL) and pectin complexation via a pH- and heat-induced facile process. The nanostructure of egg yolk LDL was elucidated for the first time under different pH conditions and its complexation with pectin was comprehensively investigated. Under optimized condition, the prepared nanogels had a diameter of 58 nm and zeta potential of -41 mV, with spherical shape, smooth surface, and homogeneous size distribution, as evidenced by dynamic light scattering, scanning and transmission electron microscopes. The Fourier transform infra-red spectrum revealed that hydrophobic and electrostatic interactions were the driving forces to form nanogels. The innovative Nano Spray Drying technology was studied and optimized to obtain nano-size powder of nanogels which exhibited excellent re-dispersibility in water, overcoming the drying challenge (irreversible gelation) of egg yolk LDL and greatly expanding its practical applications. Curcumin was adopted as model compound to investigate the drug delivery potential of LDL/pectin nanogels. The mass ratio of curcumin/LDL played an essential role in determining the particle size and encapsulation efficiency. The LDL/pectin nanogels had excellent stability under simulated gastrointestinal conditions with the presence of digestive enzymes and enabled controlled release of curcumin. The novel LDL/pectin nanogels have promising features for oral delivery of nutrients and drugs.

Keywords: low density lipoprotein; egg yolk; pectin; nanogels; controlled release; curcumin.

1. INTRODUCTION

In recent decades, nanoscale oral delivery vehicles have received extensive research interests in the fields of food, pharmaceuticals, and biomedicine. Among various nanoscale vehicles, biopolymer polyelectrolyte nanocomplex (PEC) is of particularly interest due to their great biocompatibility and biodegradability, as well as excellent loading capacity (Jones & McClements, 2011; Luo & Wang, 2014a; Luo & Wang, 2014b). Protein-polysaccharide nanocomplex is among the most studied PEC delivery systems for drugs and nutrients. In such PEC vehicles, proteins contribute to extraordinary binding capability to bioactive compounds through hydrogen bonding and hydrophobic interactions, whereas polysaccharides serve as coating barrier to protect proteins from enzymatic degradation in stomach and ensure the controlled release in small intestine. In our previous work, we reported a promising potential oral delivery vehicle prepared from casein/pectin complexation under facile condition (Luo, Pan, & Zhong, 2015), and many other similar protein/polysaccharide PECs have been developed recently (Luo, Zhang, Whent, Yu, & Wang, 2011; Teng, Luo, & Wang, 2013; Yu, Hu, Pan, Yao, & Jiang, 2006). The continuous efforts are still being made to search for natural nano-structured materials for fabrication of nanoscale oral delivery systems.

Egg is a key ingredient of a variety of food products and egg industry is an important segment of the US and world food industries. Egg proteins have many desirable and unique functional properties, including emulsifying, gelling, and foaming characteristics (Kiosseoglou, 2003). Egg proteins generally include egg white (mainly ovalbumin) and yolk proteins. Ovalbumin as a food protein-based biomaterial has been widely studied for its potential to encapsulate and deliver bioactives (Elzoghby, Samy, & Elgindy, 2012), while yolk protein received very limited attention in this field. However, egg yolk as a natural biomaterial may

61 possess a greater potential than egg white, given the fact that egg yolk itself is a natural carrier
62 for many bioactives present in egg, such as unsaturated fatty acid, lipophilic vitamins, and
63 carotenoids. Low density lipoprotein (LDL), the major constituent in egg yolk protein
64 responsible for nutrient transport, has been shown to have a micellar structure with diameter of
65 20-60 nm, consisting of a lipid core surrounded by phospholipids and proteins (Anton, et al.,
66 2003). The extraction and purification of LDL from egg yolk has been well established, and it
67 has been extensively studied for emulsifying properties and widely used in food products to
68 prepare stable emulsions, such as mayonnaises, salad dressings and creams. Recent evidence
69 indicates that daily consumption of eggs significantly increases plasma lutein and zeaxanthin in
70 various populations (Ballesteros, Cabrera, Saucedo, & Fernandez, 2013; Blesso, Andersen,
71 Bolling, & Fernandez, 2013). It has been postulated that the consumption of eggs may result in a
72 better transport and delivery of these carotenoids to the blood circulation, which may be
73 attributed to the LDL nanoparticles in egg yolk. Nevertheless, there is little research on the
74 nanostructure of egg yolk LDL and its potential for nano-delivery applications.

75 Several challenges are still yet to be resolved for applications of egg yolk LDL nanoparticles.
76 Although egg yolk LDL exist as nanoparticles in nature, many processing conditions, including
77 heating and freezing/thawing cycle, can easily induce irreversible gelation by triggering
78 unfolding and covalent disulfide bonds among lipoproteins (Cordobés, Partal, & Guerrero, 2004;
79 Telis & Kieckbusch, 1997), which will significantly destroy the nanostructure of LDL. Heating
80 may also trigger the release of lipids from the core of LDL followed by large aggregation due to
81 disruption of micellar structures. Thus, the ideal situation for developing LDL-based nano-
82 delivery vehicles is to induce disulfide bonds among LDL lipoprotein fractions to form a nanogel
83 structure by partial denaturation while maintaining its intact physical structure to avoid release of

core lipids and extensive gelation. To achieve this goal, a secondary coating will be needed. By coating a polysaccharide onto LDL surface during heating, the release of lipids from core and extensive gelation can be controlled. By far, several proteins, mainly β -lactoglobulin and ovalbumin, have been extensively studied to form nanoparticles with polysaccharides, such as pectin, gum Arabic, chitosan (Jones, et al., 2011; Schmitt & Turgeon, 2011). One recent study reported the complexation between egg yolk LDL and different polysaccharides, with a focus on separation of LDL from egg yolk using polysaccharides rather than the nutrient delivery potentials (Navidghasemizad, Temelli, & Wu, 2015).

Another big challenge for practical applications is how to transform the LDL-based nanogels dispersion into dry powder, as freeze drying and traditional spray drying will certainly destroy nanostructure and induce severe aggregation or agglomeration (Kiosseoglou, 2003). Recently, Büchi® has introduced a new generation of spray drying technology, Nano Spray Drying, enabling the production of nano-sized solid powders from liquid samples. The Nano Spray Dryer B-90 utilizes an innovative vibration mesh spray technology, creating tiny droplets (before evaporation) in a size range of a smaller order of magnitude than in traditional spray dryers. This novel technology has been recently evaluated for its capability to produce powder particles in submicron size by different scientists for drug delivery purpose (Lee, Heng, Ng, Chan, & Tan, 2011; Li, Anton, Arpagaus, Belleiteix, & Vandamme, 2010). Thus, this technology might be a potential solution to dry LDL-based nanoformulations and obtain nanoscale dry powders.

The first objective of the present work was to systematically characterize the nanostructure of egg yolk LDL. The physicochemical properties of freshly extracted LDL were analyzed at different pH conditions to understand its ultrastructure. The second objective was to develop LDL-based nanogels by investigating the complexation behavior with polysaccharides using

pectin as a model polysaccharide. The encapsulation and delivery potentials of nanogels were then explored, including particle size, zeta potential, encapsulation efficiency and controlled release in simulated gastrointestinal conditions, using curcumin as a model compound. Thirdly, the Nano Spray Drying technique was studied as a novel strategy to obtain nano-size solid powders of LDL-based nanogels.

2. EXPERIMENTAL SECTION

2.1 Materials

Fresh hen eggs were purchased from Uconn Dairy Bar at University of Connecticut and stored at refrigerator. All eggs were used within two weeks. Citrus peel pectin (galacturonic acid content of >74%) was purchased from Sigma-Aldrich Corp. (St Louis, MO). Unless noted otherwise, other chemicals were of analytical grade and purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA).

2.2 Extraction of LDL

LDL was extracted from fresh hen egg yolk according to a method previously described (Moussa, Marinet, Trimeche, Tainturier, & Anton, 2002), with minor modifications. Briefly, eggs were carefully broken and albumen was eliminated by a commercial egg separator. Yolk was slowly rolled on a filter paper until it was fully dried. The vitellin membrane was penetrated by a pipet tip and the flowing yolk was collected in a beaker cooled in ice water. An equal volume of saline solution (0.17 M NaCl) was added into yolk and stirred for 1 h in iced water. The mixture was centrifuged twice at 10,000 g for 45 min at 4 °C to remove granules. Then, ammonium sulfate (40%) was added to the supernatant and stirred for another 1 h at 4 °C. The resulting viscous solution was centrifuged at 10,000 g for 30 min at 4 °C and the yellow

supernatant was dialyzed using 10 kDa cut-off membrane against deionized water overnight to eliminate ammonium sulfate, changing bath hourly in the first four hours. The desalted solution was then centrifuged at 10,000 *g* for 5 h at 4 °C to fully remove free lipids. The supernatant after centrifugation was rich in LDL and its concentration was determined by Lowry assay using bovine serum albumen as standard protein, based on the average protein content in egg yolk being 12% (Anton, et al., 2003).

2.3 Physicochemical properties of LDL

The physicochemical properties of freshly prepared LDL were analyzed at different pH conditions. The pH of LDL was adjusted to 3 – 12 using either NaOH or HCl. Then, particle size, polydispersity index (PDI), and zeta potential of LDL at different pH conditions were measured using a Nano Zetasizer ZS model (Malvern Instruments, Ltd., Worcestershire, UK). Particle size, expressed as apparent Z-average hydrodynamic diameter, was measured at a scattering angle of 173° at 25 °C, and zeta potential was calculated from the electrophoretic mobility of the sample. The morphology of LDL was observed under transmission electron microscopy (TEM), the detailed procedures were described in the Section 2.5.

2.4 Preparation of LDL/pectin complex nanogels

LDL/pectin nanogels were prepared based on heat-induced complexation between lipoprotein and polysaccharide. The detailed procedures were modified from a previously described method for LDL/carboxymethyl cellulose nanogels (He, et al., 2015). LDL and pectin were separately prepared in distilled water at 2 mg/mL as stock solutions. LDL was kept as 2 mg/mL for all samples, while pectin stock solution was diluted appropriately to the working solutions to achieve different mass ratios of LDL/pectin mixture. To prepare nanogels, LDL and

pectin solutions were respectively adjusted to pH 4 and 7, and then 2.5 mL of pectin solution was dropwise added to 10 mL of LDL solution. After gently stirred for 30 min at 25 °C, the above mixture was adjusted to pH 7.0 and heated at 70 °C for 45 min, and then cooled down to room temperature. The affecting factors were systematically optimized, including the mass ratio of LDL/pectin, initial pH of pectin, final pH of the mixture (prior to heating), and such obtained nanogels were characterized for physicochemical properties as described in the next section.

2.5 Characterization of nanogels

Particle size, PDI, and zeta potential were determined as described in the Section 2.3.

For Fourier transform infrared (FT-IR) spectrum analysis, samples were cast-dried on aluminum pan and then stored in a vacuum desiccator for complete removal of moisture, and then mounted onto ATR crystal directly for measurement using a Nicolet iS5 FT-IR spectrometer (Thermo Scientific, Waltham, MA, USA). The infrared spectra were collected from the wavenumber of 500 to 4000 cm^{-1} at a resolution of 4 cm^{-1} and analyzed using OMNIC software version 8.0.

The morphology of samples was observed under both scanning (SEM) and transmission (TEM) electron microscopy. For SEM, samples were first cast-dried overnight on an aluminum pan and then cut into appropriate size and mounted onto specimen stub. Samples were coated with a thin layer of gold by a sputter coater before observed under SEM (JSM-6330F, JEOL Ltd., Tokyo, Japan). For TEM, samples were mixed with equal volume of 2% phosphotungstate and then one drop of stained sample was deposited on a carbon film on 400-mesh copper grid for 10 min. Samples were imaged with a TEM (Tecnai T12, FEI, Hillsboro, Oregon, USA) at 80 kV.

To investigate the stability of nanogels in simulated gastric and intestinal conditions, 1 mL of freshly prepared nanogels was added to 9 mL of simulated gastric fluid (pH 2 or 4, with 1 mg/mL pepsin) or 9 mL of simulated intestinal fluid (pH 7.5 with 10 mg/mL pancreatin), followed by incubation at 37 °C for 2 h or 4 h, respectively. After each incubation, nanogels were checked for particle size, PDI and zeta potential.

2.6 Drug delivery applications

Curcumin was used as a model drug to study the encapsulation and delivery applications of LDL nanogels. Prior to preparation of nanogels, curcumin was first loaded into LDL nanoparticles using a recently developed novel pH-driven technique (Pan, Luo, Gan, Baek, & Zhong, 2014) without using organic solvent. Before using this technique, the degradation of curcumin at pH 12 with the presence of LDL nanoparticles was monitored for 1 h to ensure its stability. For encapsulation process, different amount of curcumin powder was directly added into LDL (2 mg/mL) solution at pH 12 to induce deprotonation and solubilization of curcumin under alkaline condition. After mildly stirred for 45 min, the mixture was adjusted to pH 4 and then centrifuged (8,000 g for 20 min) to remove any excess curcumin. The supernatant was collected and used to prepare nanogels following the same procedure as detailed in the Section 2.4. The excess curcumin in the precipitate was dissolved in ethanol and measured by a UV-Vis spectrophotometer (Evolution 201, Thermo Scientific, Waltham, MA, USA) at 400 nm with an established curcumin standard curve ($R^2 \geq 0.999$). Four curcumin/LDL mass ratios were studied, i.e. 1%, 2.5%, 5%, and 7.5%. The encapsulation efficiency (EE) and loading capacity (LC) were calculated according to the equation 1 and 2, respectively.

$$EE (\%) = \frac{Total\ Cur - precipitated\ Cur}{Total\ Cur}$$

Eq. 1

$$LC (\%) = \frac{Total\ Cur - precipitated\ Cur}{Total\ mass\ of\ nanogels} \quad Eq. 2$$

where “Cur” stands for curcumin, and “Total mass of nanogels” include the mass of LDL, pectin, and curcumin in the final nanogels formulation.

The pH dependent release kinetics of curcumin from nanogels was determined using a dialysis method (He, et al., 2015; Luo, Zhang, Cheng, & Wang, 2010). The release was carried out in the simulated gastric and intestinal fluids premixed with equal volume of ethanol to create a sink condition (50% ethanol) for curcumin (Kakkar, Singh, Singla, & Kaur, 2011; Shaikh, Ankola, Beniwal, Singh, & Kumar, 2009). Briefly, 3 mL of curcumin-loaded nanogels was placed into dialysis bag and immersed into 60 mL of simulated gastric at pH 2 (fast condition) and pH 4 (fed condition), and intestinal (pH 7.5) fluids at 37 °C with gently stirring. At pre-determined time intervals, 1 mL of release medium was withdrawn to measure curcumin concentration and 1 mL of fresh medium was replenished.

2.7 Nano-spray drying of nanogels

Nanogels and LDL were dried by a Nano Spray Drier B-90 (Büchi Labortechnik AG, Flawil, Switzerland). The spray drying conditions, including inlet temperature (70, 100, and 120 °C), flow rate (120 L/min), sample concentration (0.5 and 2 mg/mL) and pH (6.5 and 7.0) were optimized. The outlet temperature for condition was in the range of 50 – 60 °C. The morphology of spray-dried powders was observed under SEM. The re-dispersibility of spray-dried nanogels was evaluated by dispersing the powder into water at 1 mg/mL and then characterized for particle size, PDI and zeta potential.

2.8 Statistics analysis

All measurements were conducted in triplicate at least. The results were expressed as mean $\pm$ standard deviation. The one-way ANOVA was conducted with Tukey's multiple-comparison test to determine the significant difference among samples. The significance level (*P*) value was set at 0.05.

3. RESULTS AND DISCUSSION

3.1 Physicochemical properties of LDL

Lipoproteins are defined as spherical nanoparticles consisting of a hydrophobic core (cholesteryl esters and triacylglycerol) and an amphiphilic shell of phospholipids and apolipoproteins (Wasan & Cassidy, 1998). LDL belongs to the sub-categories of lipoproteins that is composed of low density fraction. Although LDL from human plasma is considered as naturally-occurring bionanoparticles that have been extensively studied as a delivery vehicle for therapeutic drugs to tumors (Harisa & Alanazi, 2014), the LDL from food sources have been rarely reported for such applications. Especially, when it comes to the delivery applications for nutrients or nutraceuticals for food and agricultural fields, food-derived bionanomaterials are always preferred. Egg yolk LDL has been well-documented for its excellent emulsifying and gelling properties which contribute to the development of many food products (Le Denmat, Anton, & Beaumal, 2000; Mine & Bergougnoux, 1998). Egg yolk LDL has been previously reported to exist as spherical nanoparticles with an average diameter of 35-40 nm (Anton, et al., 2003), however, the size distribution and zeta potential are unknown. In the present study, the physicochemical properties of egg yolk LDL, including particle size, polydispersity, and zeta potential, were systematically characterized under different pH conditions for the first time (Table 1). The freshly extracted LDL (pH ~5.2) showed a normal size distribution (20-110 nm). The diameter of most particles was in the range of 20-40 nm (Fig. 1), which agreed with

previous literature (Anton, et al., 2003). As shown in Table 1, LDL has an isoelectric point (pI) between pH 6.0 and 7.0, where the zeta potential crossed from positive to negative values. The particle size of LDL initially increased with increasing pH up to 7, where visible aggregation with a size close to 2 μm was observed due to the vicinity to its pI. Then the particle size dramatically decreased as the pH continued to increase to 8 and stabilized at ~ 45 nm up to pH 12. Coincidentally, a similar trend was observed for PDI which depicted the distribution of particle size, showing that the particles were more homogeneous at the pHs away from its pI. Thus, the size of ~ 45 nm (acidic or basic pHs) may represent individual LDL particles without aggregation owing to the strong repulsive forces (high absolute value of zeta potential), while the size at pH 6-7 may represent aggregated particles due to the least repulsive forces and the extensive hydrophobic interactions.

The ultrastructure of egg yolk LDL was also visualized under electron microscope. The TEM image (Fig. 1B) demonstrated that LDL had spherical shape with diameter of 40-60 nm, which confirmed the DLS results shown in Table 1. Several large or merged LDL particles with 300-500 nm were also observed, which was probably caused by the aggregation during staining and drying due to the poor stability, which was also noticed by others (Anton, et al., 2003).

3.2 Formation and characterization of nanogels

The formation of protein/polysaccharide complex nanoparticles can be induced by partial denaturation of amphiphilic globular protein molecules and the electrostatic interactions under specific conditions. In our recent work, we successfully prepared complex nanoparticles from sodium caseinate (milk protein) and pectin by heating-induced complexation process and demonstrated that such nanocomplex particles hold a promising potential to be an oral delivery vehicle. As shown in Scheme 1, in this study, the pH of LDL was first adjusted to 4 to ensure it

carried sufficient positive surface charge (Table 1) while pectin was adjusted to pH 7 such that it was negatively charged. After mixing the two polymers together, the partial denaturation occurred when the mixture was adjusted to a pH 7 which was close to the pI of LDL and then heated at 70 °C. Hydrophobic interactions among LDL molecules and electrostatic interaction between LDL and pectin molecules were the two driving forces to form nanogels.

As shown in Table 2, the LDL/pectin nanogels were optimized from 8 different formulations. Firstly, the final pH of LDL/pectin mixture before heating was investigated in the range of 5-8. As previously discussed, the pI of LDL was between 6 and 7 so that under the pH of 5 and 6, LDL carried positive surface charge (Table 1). However, when the mixture was heated under these two pH conditions, the electrostatic interactions between LDL and pectin became so intense that large particles were produced with diameter of 914 and 194 nm, respectively. The PDI for these two samples was also very high, indicating a heterogeneous distribution of size. Under pH 6.5 and 7 which were close to its pI, LDL carried overall negligible surface charge, which reduced the magnitude of electrostatic interactions between LDL and pectin when the mixture was heated, and pectin could still electrostatically react with positive patches on the lipoproteins. It is worth noting that under pH 7 LDL aggregated and formed microparticles even without heating (Table 1), while in the presence of pectin the mixture remained clear and transparent before and after heating. This suggested that pectin successfully coated onto the surface of LDL nanoparticles and controlled partial denaturation, facilitating the formation of nanogels. Generally, the average size of nanogels was between 55 to 77 nm, depending on the formulation. The size of nanogels prepared under final pH 7 was significantly smaller than that prepared under pH 6.5, but they had similar PDI and zeta potential values. This phenomenon might be due to the fact that under pH 6.5 the magnitude of partial denaturation of LDL

284 molecules was greater than under pH 7, resulting in a greater extent of aggregation of LDL via
285 stronger hydrophobic interactions and thus larger particle size. In other words, the larger particle
286 size was caused by the aggregation of LDL instead of thicker pectin coating, which was further
287 confirmed in the Section 3.4. By further increasing the pH of LDL/pectin mixture to pH 8, the
288 characteristics of nanogels did not significantly change. But the count rate, which is the
289 scattering signal by the sample and correlates to the particle numbers (Teng, Li, Luo, Zhang, &
290 Wang, 2013; Weber, Coester, Kreuter, & Langer, 2000), was significantly smaller compared to
291 the nanogels prepared under pH 7 (data not shown). This suggested that less nanogels were
292 formed under pH 8 due to less extent of partial denaturation of LDL as well as weaker
293 interactions between LDL and pectin. Besides the final pH of LDL/pectin mixture, the initial pH
294 of pectin solution also affected the formation of nanogels. If LDL was mixed with pectin at its
295 original acidic condition (pH 4.4), the nanogels had a significantly higher PDI and lower
296 magnitude of zeta potential. This may be explained by that pectin was partially protonated and
297 thus carried less negative charge than at pH 7.0, resulting in the insufficient electrostatic
298 interactions to form compact and homogeneous nanogels. The mass ratio between LDL and
299 pectin also played a role in determining the particle size and yield of the nanogels. The size
300 increased as the increase of pectin mass ratio in the mixture, explaining a thicker pectin layer on
301 the surface of nanogels. Meanwhile, a PDI value of 0.382 in the sample with 1:1 of LDL/pectin
302 mass ratio revealed that nanogels became heterogeneous if excess pectin was present. Similar
303 phenomenon was also observed in our recent work that excess coating material negatively
304 affected the formation of nanocomplex (Luo, et al., 2015). In summary, the optimal fabrication
305 condition was as follows: LDL (pH 4) was mixed with pectin (pre-adjusted to pH 7) at the mass
306 ratio of 4:1, and it was essential to adjust the mixture to pH 7 prior to the heating treatment. The

optimal nanogels had a size of 58 nm with high zeta potential (-41 mV) and small PDI value (0.249). It is worth mentioning that the LDL/pectin in our study had similar zeta potential and PDI but much smaller particle size than that of LDL/carboxymethyl cellulose nanogels (about 90 nm) reported previously (He, et al., 2015). The morphology of nanogels prepared at optimal condition was observed under both TEM and SEM, as shown in Fig. 2. The size of nanogels under electron microscope was in good agreement with our DLS measurement. The nanogels had a spherical shape with smooth surface, which was commonly observed for protein/polysaccharide complex nanoparticles or nanogels (Jones, Lesmes, Dubin, & McClements, 2010; Liang, et al., 2015; Luo, Teng, & Wang, 2012; Luo, et al., 2013).

The FT-IR spectroscopy (Fig. 3) was conducted to analyze the structural characteristics of pectin and LDL as well as their intermolecular interactions during the formation of nanogels. As shown in Fig. 3A, pectin showed its characteristic absorption peaks at 3356, 2934, 1734, and 1605 cm^{-1} , which were assigned to stretching of O-H, C-H, carboxyl groups, and C=O bond of ester carbonyl groups, respectively (Chen, Fu, & Luo, 2015; Kyomugasho, Christiaens, Shpigelman, Van Loey, & Hendrickx, 2015). In the spectrum of LDL (Fig. 3B), a relative sharp absorption peak of O-H was observed at 3284 cm^{-1} , which was due to the water molecules present in the protein-lipid interface. Two intense bands shown at 2921 and 2852 cm^{-1} were respectively attributed to asymmetric and symmetric methylene stretching vibrations from lipid chains, which were also observed in human plasma LDL (Krillov, Balarin, Kosovic, Gamulin, & Brnjac-Kraljevic, 2009). The band at 1741 cm^{-1} was attributed to the stretching vibrations from phospholipid, triglycerides and cholesterol ester, and the bands in the region of 1700 – 1500 cm^{-1} were due to the stretching of protein peptide bonds (Krillov, et al., 2009). The intensity of stretching from lipids was greater than that from protein peptide bonds in egg yolk LDL, while

the human plasma LDL had an opposite situation (Krillov, et al., 2009). This explained that egg yolk LDL contained higher content of lipids and lower content of proteins compared with human plasma LDL. The vibration bands at 970 and 721 cm^{-1} were assigned to the choline in phospholipid and $-\text{CH}_2$ stretching from lipid chains, respectively (Fookson & Wallach, 1978). After formation of nanogels, the intermolecular interaction between LDL and pectin were explained by several major changes in FT-IR spectra (Fig. 3C). First, the O–H stretching became flatter and shifted to 3282 cm^{-1} , indicating the hydrogen bonds was involved; Second, the intensity of methylene stretching vibrations from lipid chains was significantly reduced, which revealed that the hydrophobic interactions was the driving force. Third, the shift of amide I (1653 cm^{-1} , representing α -helix composition) to 1616 cm^{-1} (representing mixture of β -strand and random coil) and the disappearance of amide II indicated the partial protein denaturation (Krillov, et al., 2009) and confirmed the thermal-induced strong hydrophobic interactions (Graff, Pastrana-Rios, Venyaminov, & Prendergast, 1997). Forth, the disappearance of choline and $-\text{CH}_2$ stretching from lipid chains depicted the rearrangement of lipid fractions in the LDL molecules and the shielding effect of pectin coating. Fifth, the band of carboxyl groups of pectin at 1734 cm^{-1} disappeared or merged with the band of lipid ester of LDL showing only one peak at 1741 cm^{-1} in the nanogels, together with the new appearance of the band at 1616 cm^{-1} in the region of amide bonds, which suggested that the electrostatic interactions between carboxyl groups of pectin and amino groups of LDL also significantly contributed to the formation of nanogels.

3.3 Stability under simulated gastric and intestinal conditions

As a potential oral delivery system, LDL/pectin nanogels need to be stable under gastric and intestinal conditions without significant change in particle size and PDI. For the gastric

environment, the pH 2 was used to mimic the fast-status gastric condition while pH 4 was tested as fed-status gastric condition, and both conditions had pepsin added. As shown in Table 3, the particle size of nanogels increased to 134 nm at pH 2, which was due to the lower magnitude of zeta potential, resulting from the protonation of pectin and reduction of electrostatic interactions, thus causing slight aggregation of nanogels particles. When the nanogels were incubated at pH 4 with the presence of pepsin, the particle size slightly increased to 77 nm and the magnitude of zeta potential slightly decreased, but PDI remained the same as its original condition. After incubated under intestinal environment (pH 7.5) with pancreatin for 4 h at 37 °C, the nanogels were able to maintain the particle size and PDI, although a reduction of zeta potential was observed which was partly due to the screening effects of salts in the intestinal fluid. Under all incubation conditions, the dissociation of nanogels into smaller fragments was not detected based on the DLS measurement that only one single peak of size distribution was observed (data not shown), suggesting that the nanogels did not dissociate or were not digested into smaller particles (Teng, Li, Luo, Zhang, & Wang, 2013). These results demonstrated that LDL/pectin nanogels were stable in simulated gastric and intestinal conditions even with the presence of digestive enzymes. This excellent stability evidenced the protective effects of pectin coating against the enzymatic digestion of LDL molecules.

3.4 Nano Spray drying of nanogels

In order to avoid extensive gelation during drying process, the innovative Nano Spray Drying technology was adopted in the present study to produce ultrafine powders of nanogels. The representative SEM images of powders dried under various conditions are presented in Fig. 4. Drying parameters, such as inlet temperature, concentration of nanogels dispersions, and pH of nanogels, were found to be critically important in obtaining ultrafine powders from dispersion

(Murugesan & Orsat, 2012). At low inlet temperature at 70 °C, large particles with a lot of agglomerates of small particles were obtained (Fig. 4A), owing to the inefficient removal of water under low temperature. By raising temperature to 120 °C, although the size of powders slightly reduced, many particles had irregular shape and rough surface (Fig. 4B) which may be attributed to the additional gelation process induced by excess heating. When the temperature was set at 100 °C, the powders with the most spherical shape and smooth surface were obtained and the size was smaller than 500 nm (Fig. 4C). To shorten the spray drying time and increase the yield, the concentration of nanogels at 2 mg/mL was tested for spray drying. However, the particles were dramatically larger ($> 2.0 \mu\text{m}$, Fig. 4D) than those spray dried at a diluted concentration (0.5 mg/mL). As previously discussed in Table 1, because the pI of LDL was within pH 6 – 7 and the nanogels prepared at final pH 6.5 showed similar characteristics to the nanogels prepared at pH 7, the nanogels prepared at pH 6.5 were also investigated for spray drying under optimal condition. However, these nanogels exhibited many holes throughout the surface of dried powders (Fig. 4E). Based on DLS result, at pH 6.5 the as-prepared nanogels had a larger particle size than the one prepared at pH 7 (Table 2). This phenomenon might be explained by the fact that the magnitude of heat-induced partial denaturation of LDL molecules may be greater under pH 6.5 than pH 7, resulting in a greater extent of aggregation of LDL due to the stronger hydrophobic interactions. The damage of nanogels morphology after spray drying also indicated the insufficient protection from pectin coating (Fig. 4E). As a control, the pure LDL solution was also spray dried under the same condition, however, irregular shape with severe aggregation was observed (Fig. 4F). This suggested that appropriate pectin coating was critical to obtain ultrafine powders without aggregation or agglomeration.

The spray-dried nanogels were also subjected to FT-IR measurement, as shown in Fig. 3D. The O–H stretching shifted from 3282 cm^{-1} in nanogels dispersion to 3291 cm^{-1} after spray drying, indicating that hydrogen bond was re-enforced during spray drying process. It is interesting to note that the lipid stretching at 2920 and 2851 cm^{-1} were also re-intensified and the re-appearance of amide II band at 1540 cm^{-1} , although intensity was lower than in original LDL (Fig. 3B), explaining that the water removal process and high temperature treatment during spray drying further induced the change of secondary structure of LDL molecules. The spray-dried nanogels were able to re-disperse into water with similar particle size observed under SEM. The nanogels powder as observed in Fig. 4C exhibited the best re-dispersibility in water, showing the particle size of 231.1 nm and zeta potential of -36.7 mV.

3.5 Characterization of curcumin-encapsulated nanogels

The pH-driven process has been recently reported to encapsulate hydrophilic molecules (folic acid) into a soy protein/soy polysaccharide by adjusting the pH from neutral to acidic condition to induce complexation and encapsulation process (Ding & Yao, 2013). In our previous work, an innovative pH-driven encapsulation process of hydrophobic molecules (curcumin and rutin) into casein nanoparticles has been developed (Luo, et al., 2015; Pan, et al., 2014). This process is based on the deprotonation and solubilization of curcumin in aqueous solution at pH 12 and then during the following neutralization process curcumin migrates into the core of micelles formed by amphiphilic molecules via hydrophobic interactions. The alkaline degradation of curcumin has been previously reported (Tønnesen, Másson, & Loftsson, 2002). In our study, the degradation rate of curcumin at pH 12 within the first 60 min (Fig. 5A) was 15% which concurred with the observation from our previous work (Pan, et al., 2014). When curcumin was co-incubated with LDL at the same condition, the degradation rate was significantly reduced to

only 5.8% (Fig. 5B), suggesting that LDL micelles protected curcumin from degradation at alkaline pH.

Four different mass ratios of curcumin/LDL were investigated by the pH-driven process. The characteristics of curcumin loaded nanogels were depicted in Table 4. Compare to control nanogels (Table 1), the particle size (>135 nm) was significantly increased and zeta potential (absolute value < 30 mV) was dramatically decreased. The increase in particle size might be attributed to the hydrophobicity of curcumin in the core causing the expansion of LDL micelles, which may further change the surface properties of the nanogels and therefore the zeta potential. An opposite change in particle size and zeta potential was observed in casein/pectin nanocomplex particles (Pan, et al., 2014) which may be owing to the different molecular structure between LDL and casein. When the mass ratio of curcumin/LDL was 1 and 2.5%, the particle size and zeta potential of nanogels were not significantly different, while the size dramatically increased to about 300 nm when the mass ratio increased to 5 and 7.5%. By increasing the mass ratio from 5% to 7.5%, the EE dramatically decreased to 66% but the LC remained the same at 3.5%, explaining that curcumin in nanogels reached its plateau and saturated at 5% mass ratio. Along with the above observation, 2.5% was selected as optimal mass ratio for further experiment. In the FT-IR spectrum of curcumin loaded nanogels (Fig. 3E), a significant shift of O–H band from 3291 to 3329 cm^{-1} and a dramatic reduction of stretching intensity in the bands of lipid chains at 2923 and 2852 cm^{-1} demonstrated that hydrogen bonding and hydrophobic interactions were the two major interactions between curcumin and LDL. Besides, many characteristics of curcumin peaks (data not shown) were not observed in the curcumin loaded nanogels, confirming the successful encapsulation of curcumin in the LDL/pectin matrix.

3.6 Controlled release

The controlled release of curcumin from nanogels was investigated at three conditions, pH 2 and 4, which simulated the gastric fast and fed condition, and pH 7.5 mimicking the intestinal condition. As shown in Fig. 6 A and B, the release of curcumin was faster at pH 2 than pH 4, because the protonation of pectin at lower pH reduced the electrostatic interactions. This result agreed with the stability data (Table 3) in the fact that nanogels had loosen structure and expanded size at pH 2. The diffusion of free curcumin was linear at pH 2 and 4 until it reached the plateau at cumulative percentage of 70% in both conditions within less than 2 h, while the cumulative release of curcumin from nanogels was 50% and 25%, respectively. At simulated intestinal condition, the kinetic profile of free curcumin was found to be first-order with accumulative percentage reaching 65% within 3 h, while the release of curcumin from nanogels was dramatically slower with only 20% released in 3 h before reach its plateau. It is worth mentioning that the release of curcumin from nanogels had a lag phase, with no curcumin detected for the first 30 min. This probably because under intestinal condition the nanogels maintained its compact structure due to strong electrostatic interactions, and the curcumin started to release when the nanogels began to expand as the water molecules gradually diffused into the nanogels. However, in the case of oral treatment, the nanogels will have enough time to expand in gastric site and this lag phase may not exist. Burst release was only observed from nanogels at pH 2 due to the pH was lower than the pKa of pectin (pH 3.5) and its protonation, causing dissociation of pectin and LDL complex structure. The absence of burst release at pH 4 and 7.5 revealed that curcumin had been completely entrapped into the lipid core of LDL molecules and there was no curcumin being weakly adsorbed or bound on the surface (Kumari, Yadav, & Yadav, 2010; Soppimath, Aminabhavi, Kulkarni, & Rudzinski, 2001).

4. CONCLUSIONS

In this work, we successfully characterized nanostructure of egg yolk LDL under different pH conditions and optimized the fabrication condition for LDL/pectin nanogels. The chemical structure of LDL was analyzed by FT-IR to verify the intermolecular interactions between LDL and pectin. The prepared nanogels had smooth surface and spherical shape with a diameter of 58 nm, while the complexation of LDL with pectin significantly increased the size, confirming the pectin coating. The ultrafine powder of nanogels was obtained by controlling the Nano Spray Drying condition and the powder was able to re-disperse into water and maintain nanoscale size. Encapsulation of curcumin into nanogels was effectively achieved by a pH-driven process. The nanogels showed a pH-dependent controlled release profile in gastric and intestinal conditions, demonstrating the promising potential as potential oral delivery vehicles for lipophilic nutrients and drugs.

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Table and Figure Captions:

Table 1. Characterization of LDL in various pH conditions.

Table 2. Characterization of LDL/pectin nanogels

Table 3. Stability of LDL/pectin nanogels under simulated gastrointestinal conditions (with enzymes)

Table 4. Characteristics of curcumin-loaded nanogels.

Scheme 1. The schematic illustration of the formation of LDL/pectin nanogels.

Fig. 1. Size distribution by DLS and TEM image of freshly extracted LDL.

Fig. 2. TEM (A) and SEM (B) images of freshly prepared LDL/pectin nanogels.

Fig. 3. FT-IR spectrum of pectin, LDL, and different nanogels.

Fig. 4. SEM images of nanogels spray-dried under different conditions. (A) 70 °C, 0.5 mg/mL, pH 7.0; (B) 120 °C, 0.5 mg/mL, pH 7.0; (C) 100 °C, 0.5 mg/mL, pH 7.0; (D) 100 °C, 2 mg/mL, pH 7.0; (E) 100 °C, 0.5 mg/mL, pH 6.5; (F) LDL, 100 °C, 2 mg/mL, pH 5.5 (original pH). Scale bars in all figures and inserts are 500 nm.

Fig. 5. Stability of curcumin at pH 12 without (A) or with (B) the presence of LDL. (A) Curcumin in aqueous solution (pH=12); (B) 1% Curcumin/LDL in aqueous solution (pH=12).

Fig. 6. The kinetic release profile of free curcumin and curcumin from nanogels under different simulated gastric and intestinal conditions. (A) pH 2; (B) pH 4; (C) pH 7.5.

4 **Fig. 1.**

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7 **Fig. 2**

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10 **Fig. 3**

12 **Fig. 4.**

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15 **Fig. 5.**
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17 **Fig. 6.**

1 **Table 1**

pH	Size (nm)	PDI	zeta-potential (mV)
3	42.4 ± 2.5 ^a	0.12 ± 0.03 ^a	34.7 ± 1.6 ^g
4	41.8 ± 0.6 ^a	0.13 ± 0.01 ^a	29.1 ± 1.7 ^g
5	44.3 ± 1.3 ^a	0.18 ± 0.01 ^{bc}	17.4 ± 1.7 ^f
6	153.0 ± 19.6 ^d	0.28 ± 0.17 ^b	7.4 ± 2.3 ^e
7	1900.0 ± 180.0 ^c	0.64 ± 0.45 ^d	-1.0 ± 0.9 ^d
8	96.7 ± 6.0 ^b	0.26 ± 0.11 ^b	-10.0 ± 1.0 ^c
9	41.7 ± 1.0 ^a	0.16 ± 0.02 ^{ab}	-14.2 ± 3.3 ^c
10	42.9 ± 3.0 ^a	0.17 ± 0.04 ^{ab}	-45.5 ± 1.0 ^{ab}
11	44.2 ± 2.2 ^a	0.17 ± 0.06 ^{ab}	-53.4 ± 1.2 ^a
12	47.2 ± 0.6 ^a	0.12 ± 0.02 ^a	-43.2 ± 1.2 ^{bc}

2 *Data were expressed as mean ± standard deviation. The values sharing different superscript
3 letters in the same column were statistically different at P<0.05. PDI, polydispersity index.

4 **Table 2.**

No.	Final pH	LDL/pectin (W/W)	Pectin pH	Size (nm)	PDI	zeta-potential (mV)
1	5	4:1	7.0	914.4 ± 93.2 ^d	0.51 ± 0.16 ^c	-45.7 ± 0.6 ^c
2	6	4:1	7.0	193.7 ± 83.8 ^c	0.41 ± 0.05 ^{bc}	-41.1 ± 2.2 ^b
3	6.5	4:1	7.0	76.0 ± 4.2 ^b	0.29 ± 0.01 ^{ab}	-38.3 ± 9.5 ^{abc}
4	7	4:1	7.0	57.8 ± 1.7 ^a	0.25 ± 0.01 ^{ab}	-41.3 ± 1.8 ^b
5	8	4:1	7.0	54.6 ± 0.7 ^a	0.23 ± 0.01 ^a	-37.6 ± 3.5 ^{ab}
6	7	4:1	4.4	74.4 ± 13.2 ^b	0.35 ± 0.02 ^{abc}	-34.0 ± 0.6 ^a
7	7	2:1	7.0	71.8 ± 0.2 ^b	0.28 ± 0.03 ^{ab}	-41.7 ± 5.7 ^b
8	7	1:1	7.0	77.1 ± 1.3 ^b	0.38 ± 0.03 ^{abc}	-43.2 ± 1.5 ^b

5 *Data were expressed as mean ± standard deviation. The values sharing different superscript
6 letters in the same column were statistically different at P<0.05. PDI, polydispersity index.

7 **Table 3.**

pH Condition	Size (nm)	PDI	Zeta potential (mV)
Original	60.8 ± 1.5^b	0.27 ± 0.01^c	-41.9 ± 0.6^d
pH 2	133.7 ± 4.2^d	0.21 ± 0.01^a	2.12 ± 0.2^a
pH 4	77.2 ± 5.4^c	0.25 ± 0.01^{bc}	-31.1 ± 3.1^c
pH 7.5	53.5 ± 2.8^a	0.24 ± 0.05^b	-14.5 ± 0.8^b

8 *Data were expressed as mean $\pm$ standard deviation. The values sharing different superscript
9 letters in the same column were statistically different at $P < 0.05$. PDI, polydispersity index.

10 **Table 4.**

Curcumin/LDL (w/w)	Size (nm)	PDI	Zeta-potential (mV)	EE (%)	LC (%)
1%	152.2 ± 24.1 ^a	0.28 ± 0.08 ^b	-26.7 ± 1.9 ^a	100.0 ± 0.0 ^c	0.8 ± 0.0 ^a
2.5%	134.9 ± 17.6 ^a	0.18 ± 0.02 ^a	-27.7 ± 1.1 ^a	100.0 ± 0.0 ^c	2.0 ± 0.0 ^b
5%	308.7 ± 20.7 ^b	0.47 ± 0.07 ^c	-30.7 ± 1.7 ^b	97.7 ± 0.5 ^b	3.9 ± 0.1 ^c
7.5%	298.8 ± 36.9 ^b	0.48 ± 0.05 ^c	-27.9 ± 1.2 ^a	66.4 ± 3.3 ^a	4.0 ± 0.2 ^c

11 *Data were expressed as mean ± standard deviation. The values sharing different superscript
 12 letters in the same column were statistically different at P<0.05. PDI, polydispersity index; EE,
 13 encapsulation efficiency; LC, loading capacity.

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