Utilization of Anionic Polysaccharides to Improve the Stability of Rice Glutelin Emulsions: Impact of Polysaccharide Type, pH, Salt, and Temperature

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Abstract

The objective of this study was to improve the physical stability of emulsions containing oil droplets stabilized by hydrolyzed rice glutelin (HRG) by coating them with anionic polysaccharides. Polysaccharide type and concentration had a strong influence on the properties of the coated droplets. Smaller particles with better pH-stability could be formed from xanthan gum or pectin than from alginate or gum arabic. The impact of environmental stresses on the stability of oil droplets coated with HRG-xanthan gum or HRG-pectin complexes was tested by measuring the impact of salt addition (0-500 mM NaCl, pH 3.5) and thermal processing (30-90 °C 30 min, 0 mM NaCl, pH 3.5). The emulsions containing xanthan gum were stable at all NaCl concentrations, whereas those containing pectin were only stable at low salt levels. Emulsions containing xanthan gum or pectin were stable to thermal processing. The improved stability of the emulsions containing the polysaccharides can be attributed to an increase in electrostatic and steric repulsion between the droplets. These results are useful for expanding the application of rice glutelin as a functional ingredient in the food industry.

Keywords: rice glutelin; emulsion; stability; polysaccharide; environmental stress

1. Introduction

There is considerable interest in replacing synthetic surfactants with natural emulsifiers in the food industry due to consumer demands for more label-friendly products (Huck-Iriart, Rincon-Cardona, & Herrera, 2014; McClements & Gumus, 2016; Ozturk, Argin, Ozilgen, & McClements, 2015). Rice glutelin (RG) is hypoallergenic, has a high nutritive value, and contains many essential amino acids (Du, et al., 2013; Zhao, et al., 2012). However, the application of RG as an emulsifier is limited because of its relatively low water-solubility. In our previous studies, we showed that the solubility and emulsifying properties of RG can be enhanced by limited enzymatic hydrolysis to alter its surface hydrophobicity, charge characteristics, and molecular flexibility (Xu, et al., 2016a). In particular, it was demonstrated that stable emulsions could be formed using a hydrolyzed rice glutelin (HRG) with 2% degree of hydrolysis. These emulsions had good thermal stability, but they were highly sensitive to droplet aggregation when the pH or ionic strength was altered, which would limit the utilization of HRG as an emulsifier in many food products. The poor pH and salt stability of protein-coated oil droplets can be attributed to the fact that they are primarily stabilized by electrostatic repulsion (McClements, 2004).

The aggregation stability of protein-coated oil droplets can be enhanced by covering their surfaces with one or more layers of polysaccharide (Dickinson, 2011, 2013; McClements, 2010). Polysaccharides are usually used as functional food ingredients in emulsions due to their thickening and gelling properties (Niu, et al., 2015). However, they can also be used to improve the stability of protein-coated droplets by forming interfacial protein-polysaccharide complexes that reduce the van der Waals attraction and increase the steric and electrostatic repulsion between droplets (Dickinson, 2011; Guzey & McClements, 2006b; Harnsilawat, Pongsawatmanit, & McClements, 2006). Consequently, protein-polysaccharide-coated droplets are often more resistant to environmental stresses (*e.g.*, pH, ionic strength, heating, and freezing) than those coated by protein only (Dickinson, 2008; Guzey & McClements, 2006a). The ability of polysaccharides to improve the stability of protein-coated oil droplets

71 depends on the nature of the proteins and polysaccharides used, as well as the
72 environmental conditions during and after interfacial complexation (Cheng, Ma, Li,
73 Yan, & Cui, 2015; Niu, et al., 2016; Qiu, Zhao, & McClements, 2015). In particular,
74 the formation of stable multilayer emulsions is highly dependent on the level of
75 polysaccharide used (Guzey, et al., 2006a; Salminen & Weiss, 2014). At too low
76 polysaccharide concentrations, the emulsions become unstable due to bridging
77 flocculation, *i.e.*, the adsorption of a single polysaccharide molecule to the surfaces of
78 two or more droplets. At intermediate polysaccharide concentrations, the droplet
79 surfaces are completely covered by polysaccharide molecules and the droplets become
80 stable to aggregation due to strong electrostatic and steric repulsion. At too high
81 polysaccharide concentrations, the emulsions may become unstable due to depletion
82 flocculation, *i.e.*, the presence of non-adsorbed polysaccharide molecules in the
83 aqueous phase generates a strong osmotic attraction between the droplets.

84 Previous studies have shown the stability of rice glutelin-coated oil droplets can be
85 improved by covalently cross-linking the protein to an anionic polysaccharide
86 (carrageenan) using the Maillard reaction (Du, et al., 2013). However, the utilization of
87 chemically modified biopolymers is often unfavorable because they can no longer be
88 considered as natural ingredients, which may lead to regulatory and labeling concerns.
89 For this reason, there is interest in improving the stability of rice glutelin-coated
90 droplets using non-covalent interactions with polysaccharides. **However, there is**
91 **currently little information about the nature of the interactions between rice glutelin and**
92 **polysaccharides at oil-in-water interfaces, and their impact on emulsion properties.**

93 In the present research, we therefore examined the ability of four anionic
94 polysaccharides to improve the stability of rice glutelin emulsions based on an
95 electrostatic deposition approach: Alginate, pectin, xanthan gum (XG), and gum arabic
96 (GA). Alginate is a linear polysaccharide whose negative charge is due to the presence
97 of carboxylic acid groups on the mannuronic and guluronic acid subunits (Paliandre,
98 Decker, & McClements, 2007). Xanthan gum is a rigid linear polysaccharide with
99 trisaccharide side-chains whose negative charge is due to carboxylic acid groups on the
100 guluronic acids in the side-chains (Dário, Hortêncio, Sierakowski, Neto, & Petri, 2011).

Pectin consists of a linear anionic backbone with regions of neutral side branches attached at certain regions, whose charge is due to carboxylic acid groups on galacturonic acids in the linear backbone (Courts, 2013). Gum arabic is a complex mixture of polysaccharides and glycoproteins whose negative charge is mainly due to carboxylic acid groups on guluronic acids in the polysaccharide fraction (Gulao, de Souza, Andrade, & Garcia-Rojas, 2016).

In this study, we characterized the major factors affecting the formation of stable oil-in-water emulsion coated by HRG-polysaccharide layers. The stability of these emulsions to environmental stresses (pH, ionic strength, and thermal treatment) was analyzed to ascertain the type of foods they may be utilized within. This study provides valuable information for optimizing the application of biopolymers as natural stabilizers in the food industry.

2. Materials and Methods

2.1. Materials

Rice glutelin (93.42 wt%, dry basis) was kindly provided by Golden Agriculture Biotech Company Limited (Jiangxi, China). Corn oil was obtained from a commercial food supplier (Mazola, ACH Food Companies, Memphis, TN). Xanthan gum with a reported molecular weight of 6000 kDa (Qiu, et al., 2015)) and gum arabic with a reported molecular weight of 383 kDa (Chang, Hu, & McClements, 2016)) were purchased from TIC Gums (Maryland, USA). Pectin with a reported molecular weight of 73 kDa (Cuevas-Bernardino, Lobato-Calleros, Román-Guerrero, Alvarez-Ramirez, & Vernon-Carter, 2016)) and sodium alginate with a reported molecular weight of 350 kDa (Pamies, Schmidt, Martínez, & de la Torre, 2010) were obtained from the Sigma Chemical Co. (St. Louis, MO). Sodium chloride, sodium azide and all other chemicals were of analytical grade and were also obtained from the Sigma Chemical Co. (St. Louis, MO). Double-distilled water was used throughout to prepare all solutions and emulsions.

128 **2.2 HRG preparation**

129 HRG was prepared according to a method described previously (Xu, et al., 2016a).
130 Briefly, 2 g of RG was added to double-distilled water (30 mL) with constant stirring
131 for 1 h at 25 °C, and then adjusted to pH 8 and 50 °C. The pH of the suspension was
132 kept constant throughout the entire period of hydrolysis by addition of 2.5 M NaOH.
133 After 4.2 min of hydrolysis by trypsin, the dispersion was heated to 95 °C for 10 min to
134 inactivate the enzyme, followed by adjustment of the solution to pH 7.0 using HCl or
135 NaOH, and immediate cooling in water to room temperature. The mixture was
136 centrifuged (TGL-20B, Anting Scientific Instrument Factory, Shanghai, China) at 4800
137 g for 10 min and the supernatant was freeze-dried. The degree of hydrolysis (DH) was
138 calculated from the amount of base required to maintain a constant pH using the pH-stat
139 method (Adler-Nissen, 1986).

140 **2.3 Solution preparation**

141 Protein solution was prepared by dispersing HRG into 5 mM phosphate buffer (pH
142 7), stirring for at least 3 h and then storing overnight at 4 °C to ensure complete
143 hydration. Polysaccharide solutions (1 wt%) were prepared by dispersing
144 corresponding weight amounts of powdered materials into buffer solution (pH 7.0), and
145 stirring overnight at 4 °C to ensure complete hydration. The pH of all the solutions was
146 readjusted to 7.0 if required.

147 **2.4 Emulsion preparation**

148 A primary oil-in-water emulsion was prepared by homogenizing 10 wt% corn oil
149 with 90 wt% aqueous protein solution (5 wt%, pH 7.0) using a high-shear mixer at
150 10,000 rpm for 2 min (M133/1281-0, Biospec Products, Inc., ESGC, Switzerland),
151 followed by passing through a microfluidizer (M110Y, Microfluidics, Newton, MA) at
152 12,000 psi for 3 passes. A secondary emulsion (1 wt% corn oil, 0.3 wt% HRG, 0-0.4 wt%
153 polysaccharide, pH 7) was formed by diluting the primary emulsion with different

ratios of polysaccharide solution and buffer solution, then stirring for 30 min. Finally, 0.005% (w/w) of sodium azide was added as a microbial preservative.

2.5 Particle size measurements

The particle size distribution of the emulsions was analyzed by laser light scattering (Mastersizer 2000, Malvern Instruments, Worcestershire, UK). To avoid multiple scattering, samples were diluted with a buffer solution with the same pH and ionic composition as the sample being measured. Particle size data is represented as the surface-weighted mean diameter (d_{32}) calculated from the full particle size distribution.

2.6 ζ -potential measurements

The ζ -potential of the particles in the samples was determined by particle electrophoresis (Zetasizer Nano ZS-90, Malvern Instruments, Worcestershire, UK). To avoid multiple scattering effects, emulsions were diluted to 100-fold with buffer solutions of the same pH and ionic composition prior to measurement. The attenuation value was kept between 5 and 10. It should be noted that the ζ -potential calculated by the particle electrophoresis instrument is based on the measured electrophoretic mobility and an appropriate theory (Smoluchowski). In highly aggregated systems, the assumptions underlying the theory may no longer be applicable, and therefore the results should be treated with caution.

2.7 Confocal laser scanning microscopy (CLSM)

Confocal imaging of emulsions was performed using an optical microscope (Leica TCS SP5, Leica Microsystems, Wetzlar, Germany) using a HCX PLAPO 60 $\times$ objective at room temperature. 2 mL of emulsion was stained with 0.1 mL Nile Red solution (1 mg/mL in ethanol) and 0.1 mL FITC (10 mg/mL in dimethyl sulfoxide) to dye the oil and protein, respectively. An aliquot of sample was placed on a slide for visualization. Fluorescent dyes were excited using an Argon 488 nm laser and then the emitted light was collected at 515 nm for protein and 621 nm for oil, respectively.

2.8 Emulsion physical stability

The stability of the emulsions was analyzed after they experienced a variety of different environmental stresses representative of those that might be experienced during manufacture, transport, or utilization.

2.8.1 pH stability

The initial primary and secondary emulsions (pH 7) were adjusted to pH values ranging from 7 to 2 by adding HCl or NaOH solutions while continuously stirring. After 1 h stirring, the pH was readjusted back to the required value if it had changed.

2.8.2 Ionic strength stability

The pH of the initial primary and secondary emulsions (pH 7) was adjusted to pH 3.5, and then different ratios of salt and buffer solution (pH 3.5) were added to obtain a range of salt concentrations (0 to 500 mM NaCl). The pH was measured and then adjusted back if necessary.

2.8.3 Temperature stability

The pH of the initial primary and secondary emulsions (pH 7) was adjusted to pH 3.5, and then the emulsions were placed in individual glass test tubes. The test tubes were then incubated in a water bath for 30 min at a fixed temperature (30 to 90 °C), and then they were cooled to room temperature by placing them in an ice water.

After exposure to these environmental stresses, samples were maintained at room temperature for 24 h before their particle size, ζ -potential, and microstructure were measured. The visual appearance of samples stored for 48 h was also recorded.

2.9 Statistical analyses

All analysis was carried out in triplicate and repeated twice per sample. The mean and standard deviations were calculated from these values using statistical analysis software (SPASS 19.0, SPSS Inc., Chicago, IL).

3 Results and discussion

3.1 Influence of polysaccharide addition on emulsion characteristics

The purpose of these experiments was to examine the influence of polysaccharide addition on the stability of oil-in-water emulsions containing HRG-coated lipid droplets. Secondary emulsions (1 wt% corn oil, 0.3 wt% HRG, 0-0.4 wt% polysaccharide, pH 7) were formed by diluting primary emulsions with different ratios of polysaccharide solution and buffer solution, and then adjusting the emulsions to pH 3.5. The mean particle diameter (d_{32}), electrical characteristics (ζ -potential) and appearance of the emulsions at pH 3.5 and 7 were compared.

3.1.1 Stability characteristics

pH 3.5: At this pH, the mean particle diameter of the primary emulsion was relatively large ($d_{32}= 13.9 \mu\text{m}$) in the absence of polysaccharides and the emulsions were unstable to creaming (Fig. 1), which can be attributed to aggregation of the HRG-coated droplets near their isoelectric point ($\text{pH}\approx 4$). In this pH range, the electrostatic repulsion between the droplets is not large enough to overcome attractive interactions such as van der Waals and hydrophobic attraction. An increase in mean particle diameter was observed when relatively low concentrations (0.01 wt%) of polysaccharide were added (Fig. 1a) and the emulsions were still highly unstable to creaming (Fig. 1c). These results suggest that addition of this low level of polysaccharide actually promoted further droplet aggregation, which can be attributed to charge neutralization and bridging flocculation (Guzey, et al., 2006a). The positive charge on the droplets was reduced by the adsorption of the anionic polysaccharide molecules (Section 3.2), thereby reducing the electrostatic repulsion between the droplets. Furthermore, when the amount of polysaccharide present is insufficient to fully coat the droplet surfaces, then one polysaccharide molecule adsorbs to the surfaces of several droplets resulting in bridging flocculation (Joye, Nelis, & McClements, 2015). At higher polysaccharide concentrations, the aggregation

232 stability of the emulsions depended on the type of polysaccharide used (Fig. 1).

233 For alginate and gum arabic, the mean particle diameter decreased when the
234 polysaccharide concentration exceeded 0.05%, but the final values were fairly similar
235 to those of the initial polysaccharide-free emulsions (Fig. 1a). In addition, the
236 emulsions were still highly unstable to creaming (Fig. 1c), which can be attributed to
237 their relatively large particle size. These results suggest that the polysaccharide
238 molecules wrapped around flocs containing numerous protein-coated oil droplets,
239 rather than around individual protein-coated droplets. Interestingly, it has previously
240 been reported that the addition of alginate to WPI-stabilized oil-in-water emulsions
241 actually improved their aggregation stability at pH 5 (Fioramonti, Martinez, Pilosof,
242 Rubiolo, & Santiago, 2015a). These differences in the addition of polysaccharides on
243 emulsion stability may be attributed to a number of phenomena. First, whey protein
244 and rice glutelin have different molecular characteristics, such as molecular weight,
245 conformation, flexibility, and electrical charge distribution (Huck-Iriart, et al., 2014;
246 Xu, et al., 2016b), which would be expected to affect the interfacial properties of the
247 protein-coated droplets in the emulsions. Second, the molecular characteristics of
248 alginates used in different studies are likely to be different, which may have influenced
249 the nature of the polysaccharide coating formed.

250 For xanthan gum and pectin, the mean particle diameter was much lower at 0.1 and
251 0.2 wt% polysaccharide than at lower levels (Fig. 1a) and the emulsions became stable
252 to creaming (Fig. 1c), which suggested that the polysaccharides wrapped around
253 individual protein-coated droplets, rather than promoting bridging flocculation. At
254 higher polysaccharide concentrations (0.4%), the particles in the emulsions containing
255 xanthan gum remained relatively small, but those in the emulsions containing pectin
256 increased appreciably (Fig. 1a). This suggests that the pectin molecules promoted
257 depletion flocculation when they were present in the aqueous phase above a critical
258 level, whereas the xanthan molecules did not (Aoki, Decker, & McClements, 2005;
259 Guzey, et al., 2006a).

260 The observed differences in the impact of the different polysaccharides on
261 emulsion stability are likely due to differences in their molecular characteristics, such

as molecular weight, conformation, branching, and charge density (Guzey, et al., 2006a). Differences in these molecular characteristics impact the affinity of the polysaccharide molecules for the protein-coated oil droplet surfaces, as well as the rate of polysaccharide adsorption (Chang, et al., 2016). In addition, they alter the strength and range of the colloidal interactions between the droplets, such as electrostatic, steric, and van der Waals.

pH 7: At this pH, the mean particle diameter of the protein-coated oil droplets in the primary emulsions was relatively small (0.18 μm), which can be attributed to the relatively strong electrostatic repulsion between the highly charged droplets. There was a slight increase in the mean particle diameter of the secondary emulsions with increasing polysaccharide concentration for all systems (Fig. 1b). In addition, creaming instability was observed in the emulsions containing alginate and pectin at higher polysaccharide levels, but not in the emulsions containing gum arabic and xanthan gum (Fig. 1c). These results suggest that droplet aggregation had occurred in the emulsions. The addition of non-adsorbed polysaccharides to emulsions is known to increase the attractive osmotic forces between the oil droplets due to a depletion mechanism (McClements, 2015). As a result, the droplets may become flocculated above a critical polysaccharide concentration, which can then promote coalescence because the droplets are forced together. The critical polysaccharide concentration (wt%) required to promote depletion flocculation in an emulsion depends on the number and size of the polymer molecules (Dickinson, 2010). Typically, this critical concentration decreases as the number density and effective size of the polysaccharide molecules in solution increases. The gum arabic molecules may not have promoted creaming because they have relatively small molecular dimensions (low radius of hydration) due to the fact that they are highly branched, and therefore they were not present at a sufficiently high concentration to cause depletion flocculation. Conversely, the xanthan gum molecules may not have promoted creaming for a number of reasons. First, xanthan gum molecules have a relatively high charge density, rigidity, and molecular weight and so they may have adsorbed to positive patches on the surfaces of the protein-coated droplets, which would increase the electrostatic and

steric repulsion between them (Protonotariou, Evageliou, Yanniotis, & Mandala, 2013; Qiu, et al., 2015). Second, xanthan gum is highly effective at increasing the viscosity of aqueous solutions, which may have prevented the oil droplets from creaming, even if they were flocculated (Chen, et al., 2016).

3.1.2 Electrical characteristics

pH 3.5: At this pH, the ζ -potential of the HRG-coated droplets was +18 mV in the absence of polysaccharides (Fig. 2a). This positive charge can be attributed to the fact that the adsorbed proteins were below their isoelectric point ($pI \approx 4$) at pH 3.5. The ζ -potential went from positive to negative when the polysaccharide level was increased, which suggested that anionic polysaccharide molecules adsorbed to the surfaces of cationic HRG-coated droplets. The negative charge reached a relatively constant value when the polysaccharide level exceeded about 0.1 wt%, suggesting that the droplet surfaces had become saturated with polysaccharide, which is in agreement with previous studies (Li & McClements, 2013). The final values of the droplet charge depended on polysaccharide type: -40 mV for alginate; -37 mV for xanthan gum; -22 mV for gum arabic; and, -18 mV for pectin. These values will depend on the electrical characteristics of the polysaccharides used, as well as their packing and orientation at the droplet surfaces. Interestingly, there appeared to be little correlation between the electrical properties of the polysaccharide-coated droplets (Fig. 2a), and the aggregation stability of the emulsions (Fig. 1a). Alginate and xanthan gum led to the highest negative charge on the droplets, but alginate led to unstable emulsions, whereas xanthan gum led to stable ones. Moreover, pectin and gum arabic led to the lowest negative ζ -potentials on the droplets, but pectin led to stable emulsions at intermediate concentrations, whereas gum arabic led to unstable ones at all concentrations. As reported previously, the stability of emulsions containing protein-polysaccharide coated droplets is not only influenced by the electrical characteristics of the adsorbed layer, but also by the thickness and porosity of the polysaccharide layer (Chang, et al., 2016; Gu, Decker, & McClements, 2005). A possible reason for the poor stability of the emulsions containing alginate and gum arabic is that they formed relatively thin

layers, and so the steric repulsion between the droplets was less than that for the other polysaccharides (Razzak, Kim, & Chung, 2016).

pH 7: In the absence of polysaccharides, the ζ -potential of the HRG-coated droplets was strongly negative (around -40 mV), which can be attributed to the fact that pH 7 was well above the isoelectric point of the proteins. The ζ -potential remained highly negative for all polysaccharide types and concentrations added to the emulsions, although there were some differences (Fig. 2b). At this pH, both the polysaccharides and protein-coated droplets are negatively charged, and therefore one might expect a limited interaction between them because of electrostatic repulsion (Fioramonti, Martinez, Pilosof, Rubiolo, & Santiago, 2015b). The observed changes in the measured ζ -potential may have arisen from two main effects: (i) the adsorption of polysaccharides to the droplet surfaces: (ii) the contribution of non-adsorbed polysaccharides to the electrophoretic signal used to calculate the droplet potential (Fioramonti, et al., 2015b).

3.2 Influence pH on emulsion properties

3.2.1 Electrical characteristics

Emulsions used in food products are exposed to a range of different pH values, and so it is practically important to investigate the influence of pH on emulsion stability. The ζ -potential of the protein-coated droplets in the primary emulsions went from negative (-39 mV) to positive (+26 mV) when the pH was reduced from 7 to 2, with a value of zero charge around pH 4 (Fig. 3a). This effect can be attributed to changes in the electrical properties of the adsorbed proteins when solution pH is altered from above to below their isoelectric point. In general, the ζ -potential of the secondary emulsions was more negative (or less positive) than that of the primary emulsions at relatively low pH values, which suggested that anionic polysaccharides adsorbed to the protein-coated droplet surfaces in this pH range (Qiu, et al., 2015). The nature of the protein-polysaccharide interaction depends on both the number and distribution of the charges on the two biopolymer molecules at a specific pH (Gu, et al., 2005). Anionic polysaccharide molecules adsorb onto protein-coated oil droplets around and below the

isoelectric point of the protein molecules due to the presence of cationic regions on the protein surfaces (Dickinson, 2011). Conversely, little or no adsorption occurs at neutral pH because the strong negative charge on the protein-coated oil droplet surfaces repels the anionic polysaccharide molecules (Fioramonti, et al., 2015b; Qiu, et al., 2015). In our research, the alginate-/XG-HRG complexes were formed when pH was lower than 6, while the pectin-/GA-HRG complexes could be found when pH was lower than 5. These results are consistent with the fact that the alginate and XG have a higher charge density than the pectin and GA, as demonstrated by the higher surface charge of the droplets at saturation (Fig. 2a) and by the charge characteristics of the molecules in solution (Fig. 3b).

The pH dependence of the ζ -potential of the polysaccharide molecules in aqueous solutions was measured to obtain additional information about their electrical characteristics (Fig. 3b). In general, the negative charge on the polysaccharide molecules increased with increasing pH, but there were distinct differences in the magnitude of the ζ -potentials. For example, at neutral pH, the magnitude of the negative charge decreased in the following order: XG > alginate > pectin > GA (Fig.3b), which is indicative of differences in the charge densities of the polysaccharide molecules. The relatively low magnitude of the ζ -potential of the polysaccharides observed under the most acidic conditions can be attributed to the fact that this pH is well below the pK_a value of their carboxyl groups (Bokkhim, Bansal, Grondahl, & Bhandari, 2015; Joye, et al., 2015; Lim & Roos, 2015; Moreau, Kim, Decker, & McClements, 2003; Xiang, Liu, Fan, & Gao, 2015). The dependence of the ζ -potential on pH was governed by the nature of the polysaccharides used (Fig. 3a). Interestingly, the ζ -potential values of the secondary emulsions containing pectin were fairly similar to those containing GA, even though the charge densities of these two types of polysaccharide molecules in solution were appreciably different (Fig. 3b). Similarly, the ζ -potentials of the secondary emulsions containing alginate and XG were fairly similar, even though the charge densities of the two polysaccharides in solution were quite different (Figs. 3a and 3b). These results may be due to differences in the location and orientation of the charged groups on the polysaccharide molecules after

they adsorb to the droplet surfaces (Fig.3b). Furthermore, for gum arabic, the ζ -potential of the primary and secondary emulsions only deviated below pH 5, suggesting that the polysaccharide molecules did not adsorb to the droplet surfaces until relatively low pH values were reached. For pectin, the ζ -potentials deviated below pH 6, suggesting that this polysaccharide adsorbed to the droplet surfaces at a slightly higher pH than gum arabic. For xanthan gum and alginate, the difference in ζ -potentials of the primary and secondary emulsions was already pronounced at pH 6, which suggested that these polysaccharides adsorbed at a considerably higher pH than the other two types. This effect can be attributed to the higher charge density of xanthan gum and alginate compared to pectin and gum arabic. Our results are consistent with those of an earlier study (Qiu, et al., 2015) that reported that xanthan gum interacted with cationic patches on protein-coated droplets at relatively high pH values.

The ζ -potential values were around zero for all the secondary emulsions at pH 2, which would be expected to lead to a low electrostatic repulsion between the droplets. However, the secondary emulsions containing pectin were fairly stable to droplet aggregation and creaming at this low pH value (Fig. 3c and 3d). Previous researchers have reported that this may be due to steric repulsion by polysaccharides that have long neutral side chains, such as pectin (Azarikia & Abbasi, 2016; Ray & Rousseau, 2013; Wang, Chen, & Lü, 2014). In general, the stability of biopolymer-coated droplets to aggregation depends on numerous factors. For the rice-glutelin emulsions, electrostatic repulsion was the only long-range repulsive force operating between the droplets (in addition to short-range steric repulsion due to the thin protein layer). Thus, the larger the magnitude of the ζ -potential in the primary emulsions, the stronger the repulsive forces, and the less droplet aggregation would be expected. For the rice glutelin-polysaccharide emulsions, the range of the steric repulsion would be much longer due to the thick polysaccharide layer present, thereby improving their aggregation stability by generating both electrostatic and steric repulsion. Thus, the overall emulsion stability is not only influenced by the electrical characteristics of the interfacial layer, but also by other factors that affect the thickness and porosity of the polysaccharide layer.

3.2.2 Stability characteristics

The pH dependence of the mean particle diameter and creaming stability of the primary and secondary emulsions was also measured (Figs. 3c and 3d). In the absence of polysaccharide, the primary emulsions were stable at pH 6 and 7, but became highly unstable to aggregation and creaming when the pH was at 5 or below, which can be attributed to the reduction in droplet charge around the isoelectric point (Fig. 3a). In the presence of the polysaccharides, the stability of the emulsions was highly dependent on polysaccharide type.

Interestingly, the secondary emulsions containing pectin remained relatively stable to droplet aggregation (Fig. 3c) and creaming (Fig. 3d) across the entire pH range. These results suggest that pectin is a highly effective polysaccharide for improving the stability of protein-coated oil droplets, which has also been reported in other recent studies (Qiu, et al., 2015; Zhao, Wei, Wei, Yuan, & Gao, 2015). Presumably, the pectin molecules adsorbed to the protein-coated droplet surfaces before the droplets could aggregate with each other, and then formed a thick charged interfacial layer that increased the electrostatic and steric repulsion between the droplets.

The secondary emulsions containing xanthan gum were relatively stable to droplet aggregation and creaming from pH 7 to 4, but unstable at lower pH values (Figs. 3c and 3d). The reason that the xanthan gum-covered droplets aggregated at low pH, whereas the pectin-covered ones did not, can be attributed to differences in the steric repulsion generated by the interfacial layers. Xanthan gum has relatively short anionic side chains, whereas pectin has relatively long neutral side chains, which may protrude into the surrounding aqueous phase and generate a strong long-range steric repulsion (Wang, et al., 2014). Interestingly, the emulsions containing alginate were unstable to aggregation and creaming over a wider pH range (pH 2 to 5) than the ones containing xanthan gum, even though the ζ -potential *versus* pH profile of the two systems was very similar (Fig. 3a). One possible reason for this phenomenon is that alginate is a linear polysaccharide without side chains (Razzak, et al., 2016). As a result, it may lay flat against the droplet surfaces and therefore not be as effective as

xanthan or pectin at generating a steric repulsion (Gulao, et al., 2016).

The secondary emulsions containing gum arabic were stable to droplet aggregation and creaming at pH 7 and 6, but highly unstable from pH 5 to 2 (Fig. 3c). Consequently, their pH stability was very similar to that of the primary emulsions. The most likely reason for this similarity is that the gum arabic molecules only had a relatively low charge density, and therefore they did not adsorb to the surfaces of the protein-coated droplets before they aggregated with each other. Indeed, this hypothesis is supported by the ζ -potential measurements, which indicate that the gum arabic only adsorbed strongly to the droplet surfaces at pH 4 and below (Fig. 3a), which is less than the pH value where the droplets in the primary emulsion had strongly aggregated (Fig. 3c).

These results show that the emulsions containing pectin or xanthan gum were the most stable, and so only these systems were examined in the subsequent experiments.

3.2.3 Microstructure

Confocal fluorescence microscopy was used to obtain information about the impact of pH on the microstructure of selected emulsions (Fig. 3e). At pH 7, the microscopy images indicated that the primary emulsions contained small uniformly distributed oil droplets, indicating that these systems were stable to aggregation due to the strong electrostatic repulsion between the droplets. Secondary emulsions containing xanthan gum or pectin were also stable at pH 7, which can again be attributed to the strong electrostatic repulsion between the droplets, and the fact that the level of polysaccharide used was not high enough to induce depletion flocculation. In contrast, at pH 3.5, the droplets in the primary emulsions were highly aggregated due to the reduced electrostatic repulsion between them. However, extensive droplet aggregation was inhibited in the secondary emulsions containing xanthan gum or pectin at pH 3.5. This behavior can be attributed to the stronger electrostatic and steric repulsion between polysaccharide coated droplets (Qiu, et al., 2015; Zhao, et al., 2015). The microscopy results therefore strongly supported the light scattering measurements and visible observations discussed earlier.

3.3 Influence of ionic strength on emulsion stability

Emulsions may be used in food and beverage products with different salt levels, and therefore it is important to establish the impact of ionic strength on droplet stability. The influence of NaCl level on the mean particle diameter and ζ -potential of emulsions containing 0.1% xanthan gum or pectin at pH 3.5 was therefore measured (Figs. 4a and 4b). In the absence of the polysaccharide covering the droplets were highly unstable to aggregation (data not shown). The secondary emulsion containing pectin was stable to droplet aggregation and creaming from 0 to 100 mM NaCl (Fig. 4a). This result suggests that the combination of the repulsive interactions (electrostatic and steric repulsion) acting between the pectin-covered droplets was strong enough to overcome the combination of attractive interactions (van der Waals, depletion, and hydrophobic) (Shi, et al., 2015). However, as higher NaCl levels, the droplets became unstable to aggregation and creaming. This effect was attributed to the decrease in electrostatic repulsion between the oil droplets upon the addition of NaCl, *i.e.*, electrostatic screening (Niu, et al., 2015). In addition, the presence of high levels of salt may have weakened the attractive electrostatic interactions between the protein and polysaccharide molecules at the droplet surfaces, which could have caused some of the polysaccharide molecules to become attached to more than one droplet and promote bridging flocculation.

Compared with pectin, the oil droplets covered with xanthan gum had relatively high stability to salt addition across the whole range studied, with little evidence of droplet aggregation or creaming (Fig. 4a). Interestingly, there was actually an initial decrease in mean particle diameter in the emulsions containing xanthan gum with increasing salt content: from around 1.01 μm to 0.81 μm when the NaCl level was increased from 0 to 100 mM. There are a number of possible reasons for this decrease associated with a reduction in the strength of electrostatic interactions in the presence of salt. First, salt addition will have weakened intra-molecular electrostatic repulsion between negatively charged groups on the xanthan molecules, thereby causing the effective size of the molecules to decrease because the chains could pack closer

together (Protonotariou, et al., 2013; Rochefort & Middleman, 1987). This effect may have reduced the thickness of the adsorbed xanthan gum coating. Second, salt addition will have weakened electrostatic attraction between the negative groups on the xanthan molecule and positive groups on the protein-coated droplets (Protonotariou, et al., 2013). This may have enabled some rearrangement of the xanthan gum molecules at the droplet surfaces leading to a more compact and thinner polysaccharide coating (Protonotariou, et al., 2013). Alternatively, it may have caused some of the xanthan gum molecules to be desorbed from the droplet surfaces, again leading to a thinner polysaccharide coating.

The HRG-xanthan gum and HRG-pectin coated droplets were negatively charged at all NaCl levels used, but the magnitude of the ζ -potential decreased as the NaCl level increased: from -17 to -2 mV for pectin and from -33 to -15 mV for xanthan gum (Fig. 4b). This effect may be the result of a number of phenomena, including electrostatic screening, ion binding, polysaccharide rearrangement, and polysaccharide desorption (Guzey, et al., 2006a; Li, Li, Shen, Niu, & Fu, 2016).

Additional information about the impact of ionic strength on the stability of the secondary emulsions was obtained using confocal fluorescence microscopy (Fig. 4c). At low NaCl levels, there was no significant change in the microstructure of the emulsions containing pectin or XG compared to that containing no salt, indicating that the repulsive interactions between the droplets were still strong enough to overcome the attractive interactions (Shi, et al., 2015). However, large aggregates were observed in the secondary emulsions containing pectin at higher salt levels (500 mM NaCl), which suggested that some droplet flocculation or coalescence had occurred. As discussed earlier, this may have been due to electrostatic screening, ion binding, interfacial rearrangements, and/or polysaccharide desorption (Guzey, et al., 2006a; Li, et al., 2016). In contrast, the secondary emulsions containing xanthan gum were fairly stable to aggregation even at high salt concentration. The microscopy results are therefore in good agreement with the light scattering measurements and visual observations of the samples discussed earlier.

3.4 Influence of temperature on emulsion stability

In many commercial applications it is important that an emulsion is able to withstand thermal treatments during its production, storage, or utilization. We therefore examined the impact of heat treatments (30-90 °C) on the stability of secondary emulsions containing either xanthan gum or pectin (pH 3.5, 0 mM NaCl). Both types of secondary emulsion appeared visibly stable to creaming after the heat treatments (Fig. 5a), however there were changes in particle size in some of the emulsions (Fig. 5b). The mean particle diameter (d_{32}) of the HRG emulsions coated with pectin and xanthan gum at 90 °C were 2.1 and 1.2 times larger than that of the control (emulsions treated at 30°C), respectively. The mean particle diameter of the emulsions containing pectin remained fairly constant from 30 to 60 °C (d_{32} = 0.33 to 0.37 μ m), but increased somewhat from 70 to 90 °C (d_{32} = 0.43 to 0.70 μ m). On the other hand, the mean particle diameter of the emulsions containing xanthan gum remained relatively stable across the entire temperature range studied (d_{32} = 1.08 to 1.29 μ m), and there was little change in their microstructure (Fig. 5c). The confocal fluorescence microscopy images also showed that there was a slight increase in droplet size in the emulsions containing pectin (Fig. 4c), but that the droplets still remained evenly dispersed, which may account for the fact that no visible creaming occurred. Overall, these results suggest that the emulsions containing xanthan gum were more stable to heating than those containing pectin. This may have been due to a stronger electrostatic and steric repulsion between the droplets coated by xanthan gum.

There was no significant change in the ζ -potential of the secondary emulsions (-35 mV for xanthan gum and -18 mV for pectin, respectively) upon thermal treatment (Fig. 5b), suggesting that heating did not strongly influence the overall composition or structure of the polysaccharide coatings (Güzey & McClements, 2006).

4. Conclusions

This study compared the effectiveness of four different anionic polysaccharides at

improving the physical stability of emulsions containing oil droplets coated by hydrolyzed rice glutelin. Pectin and xanthan gum were much more effective at stabilizing the emulsions than gum arabic or alginate, which was attributed to differences in molecular properties such as charge density, branching, rigidity, and dimensions. The best pH-stability of the emulsions was obtained by covering the HRG-coated droplets with pectin, whereas the best salt- and thermal-stability was obtained by covering them with xanthan gum. In future studies, it would be useful to use more detailed analytical tools to provide information about differences in interfacial properties, such as electron microscopy or interfacial rheology. This study provides valuable information that may increase the utilization of rice proteins as emulsifiers in the food industry.

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709

Figure Captions

Fig. 1. Effect of polysaccharide concentration on the mean particle diameter ($d_{3,2}$) of oil-in-water emulsions (1 wt% corn oil, 0.3 wt% HRG) at (a) pH 3.5 and (b) pH 7. (c) Photographs of emulsions containing different polysaccharide levels after 48 h storage at pH 3.5 and pH 7. Key: XG, Xanthan gum; GA, gum arabic.

Fig. 2. Effect of polysaccharide concentration on ζ -potential of oil-in-water emulsions (1 wt% corn oil, 0.3 wt% HRG) at (a) pH 3.5 and (b) pH 7. Key: XG, Xanthan gum; GA, gum arabic.

Fig. 3. (a) Effects of pH on ζ -potential of emulsions (1 wt% oil droplet) in the absence and presence of different types of polysaccharide (0.1 wt%); (b) Effect of pH on ζ -potential of 0.1 wt% of polysaccharide solution; (c) Effects of pH on mean particle diameter ($d_{3,2}$) of emulsions (1 wt% oil droplet) in the absence and presence of different types of polysaccharide (0.1 wt%); (d) Photographs of emulsions in the absence and presence (0.1 wt%) of polysaccharides after 48 h storage at different pH values. (e) Confocal fluorescence micrographs of the influence of pH on selected emulsions in the absence and presence of polysaccharide (0.1 wt%). Key: XG, Xanthan gum; GA, gum arabic.

Fig. 4. Effect of NaCl concentration on (a) mean particle diameter ($d_{3,2}$) and (b) ζ -potential of emulsions (1 wt% oil droplet) in the absence and presence of polysaccharide (0.1 wt%). (c) Impact of salt addition (0 mM and 500 mM) on the confocal microscopy images of emulsions containing pectin or xanthan gum at pH 3.5. White scale bar indicates 20 μm . Key: XG, Xanthan gum; GA, gum arabic.

Fig. 5. Effect of temperature (0 mM NaCl) on the (a) mean particle diameter ($d_{3,2}$) and (b) ζ -potential of emulsions (1 wt% oil droplet) containing pectin or xanthan gum (0.1 wt%) at pH 3.5. (c) Impact of heat treatment (30 °C and 90 °C, 0 mM NaCl) on the confocal microscopy images of emulsions containing pectin or xanthan gum (0.1 wt%) at pH 3.5. White scale bar indicates 20 μm . Key: XG, Xanthan gum; GA, gum arabic.

Fig.1a

Fig.1b

Fig.1c

Fig.2a

Fig.2b

(b)

Fig.3a

Fig.3b

Fig.3c

Fig.3d

(d)

Fig.3e

Fig.4a

Fig.4b

Fig.4c

(C)

with pectin pH 3.5
0 mM NaCl

with XG pH 3.5
0 mM NaCl

with pectin pH 3.5
500 mM NaCl

with XG pH 3.5
500 mM NaCl

Fig.5a

Fig.5b

Fig.5c

(C)