ORIGINAL ARTICLE

Protein-Polysaccharide Hydrogel Particles Formed by Biopolymer Phase Separation

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Abstract Biopolymer-based hydrogel particles were fabricated using a segregation-aggregation phase separation mechanism. Pectin-coated whey protein isolate (WPI) hydrogel particles (d~2 μm) were formed when solutions of heat-denatured (90 °C/10 min) WPI (1.5 %) and pectin (1.5 %) were mixed at pH 7, and then adjusted to pH 5 with constant stirring. Hydrogel particle properties and suspension appearance were strongly influenced by pH (2 to 8) and calcium chloride (0–5 mM) due to the importance of electrostatic interactions. At pH 2 to 3, extensive biopolymer complexation occurred leading to highly viscous solutions. At pH 6 to 8, biopolymer complexes disintegrated due to weakening of electrostatic attraction leading to clear solutions. Calcium addition promoted biopolymer aggregation and an increase in viscosity. All systems containing intact hydrogel particles had a whitish appearance similar to that of milk. These biopolymer-based hydrogel particles may be suitable as texture modifiers, fat replacers, or delivery systems for utilization in foods.

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Introduction

The increased incidences of chronic diet-related disorders, such as diabetes and obesity, has been linked to consumption of high-calorie diets [1]. The food industry is responding to this problem by developing a range of healthier food products with reduced levels of total calories, fats and digestible carbohydrates (starches) [2]. However, it is a challenge to develop low calorie products that possess similar physical and sensory qualities as the corresponding full calorie products. This is because fats and starch granules play important roles in determining the desirable appearance, flavor, and textural properties of many food products, e.g., sauces, dressings, desserts, beverages, and dips. For example, decreasing the level of fat droplets within a food product can reduce the desirable appearance, texture, and flavor profile of food products [3–5]. There is therefore a need to develop novel ingredients or structuring approaches that can reduce the calories of foods, but that still impart desirable physicochemical and sensory attributes.

There has also been increasing interest in the development of delivery systems to encapsulate bioactive food components so as to increase their performance in food products, such as flavors, colors, antimicrobials, vitamins, and nutraceuticals [6–8]. These delivery systems should be fabricated from food-grade ingredients and processing operations that are economically viable, they should be compatible with the food matrix, and they should have the desired functional attributes (such as retention, protection, and release characteristics) [9]. There is interest in creating delivery systems that can trigger the release of encapsulated active components under specific conditions, e.g., a change in pH, ionic strength, temperature, or enzyme activity.

In this study, we examined the possibility of forming biopolymer-based hydrogel particles from proteins and

dietary fibers that could be used as texture modifiers, fat replacers, starch replacers, or delivery systems. Proteins and dietary fibers are particularly useful building blocks for assembling hydrogel particles because they are natural ingredients with potential health benefits. In this study, whey protein isolate (WPI) was used as a protein-based building block and pectin was used as a dietary fiber-based building block. These biopolymers are already widely used in the food industry as emulsifiers, stabilizers, or texture modifiers [10–14]. Whey protein consists of a mixture of different globular proteins isolated from the whey fraction of milk [15]. Pectin is an anionic polysaccharide isolated from various types of plant-materials, including apples, citrus fruit, and sugar beet [16–18].

A two-stage biopolymer phase separation method was used to fabricate the hydrogel particles in this study [9, 19]. First, two biopolymer solutions were mixed together under conditions that promoted phase separation due to thermodynamic incompatibility, i.e., at a pH where the two biopolymers have similar charges and therefore electrostatically repel each other. This system was then sheared to produce a water-in-water (W/W) "emulsion" consisting of one biopolymer solution dispersed as small particles in the other biopolymer solution. The biopolymer solution that occupies the smaller volume fraction in the system typically becomes the dispersed phase, while the other biopolymer solution becomes the continuous phase [19–22]. Second, the solution conditions are adjusted to promote adsorption of biopolymers in the continuous phase to the surfaces of the biopolymers in the disperse phase, i.e., by adjusting the pH so that the two biopolymers have opposite charges. The resulting system then consists of spheroid hydrogel particles dispersed in an aqueous medium. Previous studies have shown that this approach can be used to assemble hydrogel particles from various combinations of proteins and polysaccharides: caseinate and pectin [19, 22, 23]; whey protein and pectin [24]; and, whey protein and carrageenan [25]. In the current study, we focused on the influence of environmental conditions that impact electrostatic interactions (pH and ionic strength) on the formation and properties of hydrogel particles assembled from whey protein and pectin.

These hydrogel particles are often unstable to coalescence and have a tendency to separate back into two biopolymer phases. To reduce this instability, the continuous and/or dispersed aqueous phases can be gelled. Calcium chloride has previously been used to induce gelation of whey protein isolates and/or pectin molecules through electrostatic screening and bridging effects [26–30]. Whey protein and/or pectin hydrogels can be formed by either *cold-set* or *heat-set* gelation [26–28, 31]. In *cold-set* gelation, the globular protein solution is heated above the thermal denaturation temperature under conditions that induce a strong repulsion between molecules, i.e., low ionic strength and pH away from isoelectric point [32]. At this step, protein molecules are partially unfolded

and form linear aggregates. To keep the molecules separated, the solution must have a pH far from the isoelectric point of the protein and a low ionic strength. Gelation is then promoted by inducing attractive interactions between the denatured protein molecules at pH close to the isoelectric point or by adjusting the ionic strength of the systems to promote electrostatic screening [26, 27, 33]. Heat-set gelation is usually a one-step process whereby the globular protein solution is heated above the thermal denaturation temperature under conditions where there is a significant non-specific attraction between the unfolded proteins, i.e., elevated ionic strength and/or pH close to isoelectric point [31]. The rheological, optical, and water binding properties of the gels formed can be controlled by altering the pH and/or ionic strength during preparation. In this study, we examined the influence of calcium addition and pH adjustment on the formation and stability of the gels.

The hydrogel particles formed by this process may have potential application as fat or starch mimetics for production of low calorie foods and beverages, as texture modifiers, or as delivery systems for bioactive food components. In commercial applications, hydrogel particles would be incorporated into complex food matrices that contain various other types of ingredients. In particular, there are considerable differences in the pH and ionic strength of the aqueous phases of different food and beverage products, which will alter the electrostatic forces acting between proteins and polysaccharides. As a result, the stability of the hydrogel particles formed to dissolution and aggregation will depend on solution conditions. A major focus of this study was therefore to examine the influence of pH and ionic strength on the microstructure and properties of the hydrogel particles, as this may impact their functional performance as texture modifiers or delivery systems in different food products.

Materials and Methods

Materials

Commercial whey protein isolate (WPI) powder was kindly provided by Davisco Foods International (BiPro, Le Sueur, MN, USA). The WPI was reported to contain 97.9 wt.% protein, 0.2 wt.% fat, and 1.9 wt.% ash. Low methoxyl pectin (LMP) (Pretested LM 35, Lot# 504045, DE 32 %, <1 % Ash) was donated by TIC Gums (Belcamp, MD). Calcium chloride dihydrate ($CaCl_2.2H_2O$), hydrochloric acid (HCl), sodium azide, sodium hydroxide pellet, and Fluorescein isothiocynate isomer I, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Double distilled water was used to prepare all solutions.

Protein Hydrogel Particles Fabrication

Stock solution of 3 wt.% whey protein isolate (WPI) was prepared by dispersing a weighed amount of WPI powder into double distilled water at room temperature (~25 °C) with continuous stirring (200 rpm) until fully hydrated. The pH of the solution was adjusted to pH 7.0 with 0.1 M sodium hydroxide. Heat-denatured WPI was produced by heating the native WPI solution to 90 °C for 10 min with continuous stirring (200 rpm) and then cooling in an ice bath for 1 h. A stock solution of 3 wt.% low methoxyl pectin (LMP) (pH 7.0) was prepared by dispersing a weighed amount of LMP powder in double distilled water at 25 °C and stirring (200 rpm) overnight to ensure full hydration. Equal amounts of WPI and LMP solutions were then mixed together to obtain a final mixture of 1.5 wt.% WPI and 1.5 wt.% LMP (pH 7.0) with continuous stirring for 3 min at 200 rpm.

Influence of Ionic Strength (Cold-set Gelation)

A calculated volume of calcium chloride (0.1 M $CaCl_2$) was added to the mixtures at pH 7.0 to obtain final concentrations of 0, 1.25, 2.5 and 5 mM $CaCl_2$ in the solutions. These mixtures were stirred continuously for 3 min at 200 rpm. The solution was then adjusted to pH 5.0 with 10 mM sodium hydroxide, and the resulting systems were stored for 24 h at room temperature.

Influence of pH Stability

Systems without $CaCl_2$ were divided into a number of aliquots and their pH was adjusted to different values (i.e., pH 2 to 8). All systems were stored for 24 h at room temperature before analysis. The pH of the systems were re-adjusted prior to analysis.

Sample Characterization

Particle Size Measurement

The particle size distribution was measured using a laser diffraction particle size analyzer (Mastersizer 2000, Malvern Instruments, Ltd., Worcestershire, U.K.). Samples were diluted by adding small aliquots into a measurement chamber containing double distilled water (at the same pH as the sample) until the instrument gave an optimum obscuration rate between 10 and 20 %. The particle size distribution was calculated from the light scattering pattern using Mie theory. A refractive index of 1.33 was used for the aqueous phase, and 1.472 for the hydrogel phase. Particle size measurements are reported as volume-weighted mean diameters (d_{43}).

Microstructure Analysis

The microstructure of the systems was examined using confocal laser scanning microscopy with a 60× objective lens and 10× eyepiece (Nikon DEclipse C1 80i, Nikon, Melville, NY, U.S.). The protein phase was dyed with Fluorescein isothiocynate (FITC) isomer I (1 mg FITC/1 mL dimethyl sulfoxide) (~0.1 mL of dye solution per 2 mL of sample). A small aliquot of each dyed sample was placed on a microscope slide and covered with a glass cover slip prior to analysis. The excitation and emission wavelengths for FITC were 488 and 515 nm respectively. The microstructure images were taken and analyzed using instrument software (Nikon, Melville, NY): EZ-CS1 (version 3.8).

Optical Properties

Lightness ($L*$) of the hydrogel particles was measured using a colorimeter (ColorFlez EZ, HunterLab, Reston, VA) with a tristimulus absorption filter. The lightness value ranges from 0 (i.e., black) to 100 (i.e., white).

Visual Appearance

Digital photographs of the hydrogel particle systems were taken after 24 h storage. Fresh samples were poured into glass tubes, sealed with a cap, and then stored at room temperature (25 °C).

Zeta Potential

The electrical charge (ζ-potential) was measured using a particle electrophoresis instrument (Zetasizer Nano ZA series, Malvern Instruments Ltd. Worcestershire, UK). Samples were diluted 2.5 fold using 0.01 M double distilled water (same pH as sample) prior to analysis to prevent particle interaction effects. The instrument software was used to calculate the ζ-potentials from the measured electrophoretic mobility data.

Statistical Analysis

Replicate analyses were performed for all measurements. Means, standard deviations and statistical differences between means ($p < 0.05$) were calculated using one-way analysis of variance (ANOVA) using Microsoft Excel 2011.

Results and Discussion

Hydrogel Particle Formation

Initially, we characterized the properties of hydrogel particles fabricated by phase separation of WPI and pectin solutions (pH 5, 0 mM $CaCl_2$).

Particle Size and Microstructure

Light scattering measurements of hydrogel particles in the absence of CaCl$_2$ showed that they had a multimodal particle size distribution (Fig. 1). The majority of particles had diameters between 1 and 5 μm with a mode diameter around 2 μm. However, there was also a population of larger particles with diameters ranging from 13 to 300 μm. Confocal microscopy images confirmed that the majority of hydrogel particles had diameters around 2 to 3 μm (Fig. 2). However, no large particles (13 to 300 μm) were observed using confocal microscopy, which is different from the light scattering measurements (Fig. 1). The large particles observed by light scattering may have consisted of pectin, which is not stained by the fluorescent used and would therefore not be visible in the microscopy analysis. The confocal images also indicated that the hydrogel particles were protein-rich so only the WPI was stained with a fluorescent dye in this study. Overall, the results indicate that protein-rich hydrogel particles were formed using the biopolymer phase separation method. A similar particle preparation method has been used to form hydrogel particles using other combinations of proteins and polysaccharides, such as casein and pectin [19, 21, 22].

Optical Properties

The optical properties of the hydrogel suspensions were quantified by measuring their lightness (L*) using a colorimeter as biopolymer particles will influence the appearance of food products. The hydrogel suspensions had an L* value of about 76 (Table 1), which can be attributed to light scattering by the
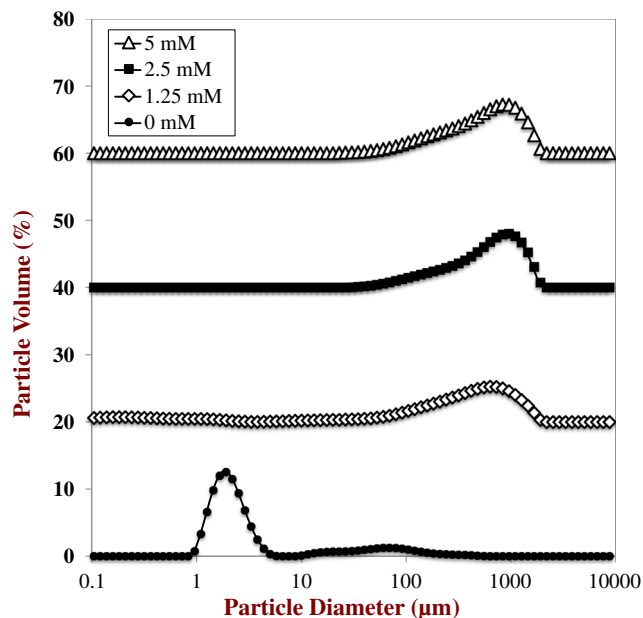

Fig. 1 Particle size distribution of hydrogel particles at pH 5 (1.5 % WPI and 1.5 % LMP) containing different CaCl2 concentrations

protein-rich hydrogel particles. This L* value is similar to that reported for suspensions of raw starch granules (L*=77 for 3.5 % starch), but appreciably higher than that for swollen starch granules (L*=38 for 3.5 % starch) [34]. The L* value of the hydrogel suspensions was similar to that of oil-in-water emulsions containing low fat contents (L*=75 for 1 % fat), but considerably lower than that for emulsions containing high fat contents (L*>90 for>5 % fat) [35]. These differences in optical properties can be attributed to differences in the scattering efficiency of particles, which is related to their size, concentration, and refractive index contrast [36]. Raw starch granules and fat droplets are small particles with high refractive index contrasts, and therefore scatter light efficiently, whereas swollen starch granules are large particles with low refractive index contrasts and therefore only scatter light weakly. The hydrogel particles fabricated in this study appeared to have optical characteristics somewhere between those of fat droplets and swollen starch granules. Consequently, it may be necessary to blend different levels of hydrogel particles, fat droplets, starch granules, and/or other lightening agents to get the desired final optical properties in a commercial product.

Electrical Characteristics

The electrical charge (ζ-potential) of the hydrogel particles was around −40 mV (Table 1). This relatively high negative charge can be attributed to the adsorption of a layer of anionic pectin molecules around the WPI-rich hydrogel particles at pH 5. At this pH, the protein molecules have little net charge because they are close to their isoelectric point (pI), but they do have appreciable cationic patches on their surfaces to which anionic pectin molecules can attach.

Influence of Ionic Strength

In the second part of the study, we determined the influence of calcium chloride (CaCl$_2$) addition on the physical properties of the hydrogel suspensions.

Particle Size and Microstructure

The light scattering measurements indicated that all hydrogel suspensions containing CaCl$_2$ had a broad monomodal particle size distribution (Fig. 1). The mean particle diameter (d_{43}) of the hydrogel particles increased with increasing calcium content. This result indicates that CaCl$_2$ addition promoted aggregation of the hydrogel particles, presumably by screening the electrostatic repulsive forces between them and forming salt bridges between negatively charged groups [27–30].

There were noticeable changes in the microstructure of the hydrogel particles with increasing salt concentration observed

Fig. 2 Confocal micrographs (60×magnification) of hydrogel particles fabricated with WPI (1.5 %) and LMP (1.5 %) with 0 to 5 mM CaCl2 at pH 5. Protein phase was stained in green using FITC. Scale bar at bottom right represents 5 μm
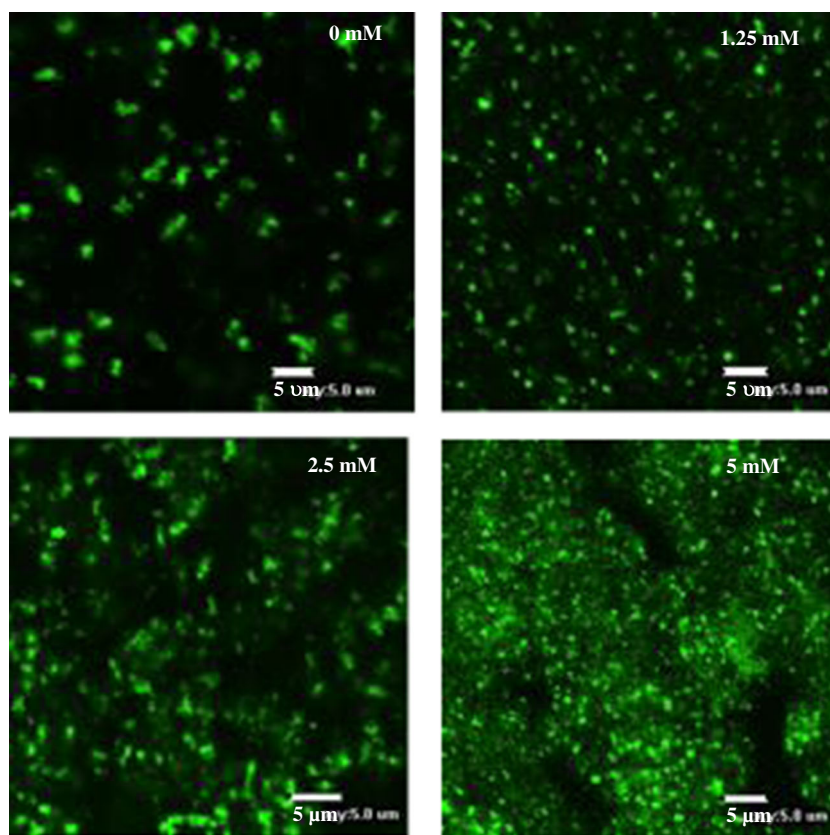
using confocal microscopy (Fig. 2). At 0 and 1.25 mM CaCl$_2$, the hydrogel particles were approximately spherical and evenly dispersed throughout the continuous phase. At higher salt concentrations (2.0 and 5 mM), a dense network of aggregated hydrogel particles were formed, which could be due to electrostatic screening or salt bridge formation of the protein hydrogel particles [27].

Optical Properties

There was no appreciable difference in the lightness of hydrogel suspensions containing different CaCl$_2$ concentrations, with L^* values ranging from around 75 to 78 (Table 1). This suggests that the observed changes in the structural organization of the particles within the hydrogel suspensions due to calcium addition did not have a major impact on their optical properties. Studies using oil-in-water emulsions have also reported that there was little change in the overall optical properties when the particles aggregated [37]. Thus light scattering appears to be dominated by the individual hydrogel particles, rather than by the large aggregates of hydrogel particles.

Electrical Characteristics

The electrical charge (ζ-potential) of the particles in the hydrogel suspensions did not change appreciable upon addition of different levels of CaCl$_2$ (Table 1). This suggests that the calcium ions were mainly bound to whey protein molecules trapped within the hydrogel particles, rather than on the pectin components that were in the continuous phase.

Influence of pH

The impact of pH on the properties of the hydrogel suspensions was also evaluated since different food products have different pH values, and pH is known to modulate the electrostatic interactions between proteins and polysaccharides.

Table 1 Optical properties and electrical characteristics of hydrogel suspensions and controls (WPI and Pectin solutions) at pH 5

System	CaCl$_2$ (mM)	Lightness (L^*)	Zeta Potential (mV)**
Hydrogel particles	0.00	76.32±0.01	−39.98±1.31[a]
	1.25	76.55±0.06	−44.30±2.46[ab]
	2.50	78.02±0.02	−46.28±4.15[b]
	5.00	74.95±0.09	−41.44±4.51[a]
WPI	0.00	–	−4.37±0.87
Pectin	0.00	–	−41.38±1.34

**Data sharing the same alphabetical superscript are not statistically different ($p < 0.05$)

Table 2 Influence of pH on mean particle diameter and lightness of hydrogel particle suspensions

pH	Diameter, d_{43} (μm)	Lightness (L^*)
2	485.42±47.40	61.84±0.11
3	291.11±36.49	80.15±0.02
4	97.20±12.77	81.46±0.01
5	39.53±11.73	76.32±0.01
6	0.49±0.08	34.77±0.18
7	0.50±0.02	36.51±0.10
8	0.55±0.04	32.33±0.25

Particle Size and Microstructure

The particle size measurements revealed that the average diameter (d_{43}) of the hydrogel particles decreased with increasing pH (Table 2). Systems with pH 2 to 5 had monomodal particle size distributions, whereas systems with pH 6 to 8 had multimodal distributions (data not shown). The small hydrogel particles formed at pH 6 to 8 indicate that the protein hydrogel particles disintegrated. This could be due to reduction of the electrostatic attraction between the WPI and pectin molecules above the isoelectric point of the protein, since then both of the biopolymers are then negatively charged (see later). Conversely, at pH values below the *pI* the two biopolymers have opposite charges and are therefore attracted to each other and form hydrogel particles. However, if this pH is reduced too much, then the net charge on the hydrogel particles tends towards zero, thereby reducing the electrostatic repulsion between them and leading to aggregation (see later).

The confocal micrographs also showed that the structure of the hydrogel suspensions depended strongly on pH (Fig. 3): at *pH 2 to 3*, large clumps of hydrogel particles were formed; at *pH 4 and 5*, individual hydrogel particles distributed evenly throughout the aqueous phase were observed; at *pH 6 to 8*, the images appeared to have a homogeneous green color, which suggested that the hydrogel particles had dissociated and released the protein molecules. This latter phenomenon can be attributed to the strong electrostatic repulsion between anionic protein and anionic pectin molecules above the isoelectric point (*pI*).

Optical Properties

The influence of pH on the lightness of the hydrogel suspensions was also measured as it may have a strong influence on the appearance of a commercial food product. The lightness increased from pH 2 to 4, attained a maximum value at pH 4, and then decreased from pH 5 to pH 8 (Table 2). The pH dependence of the lightness can be attributed to changes in the structural organization of the protein and polysaccharide molecules in the system. At intermediate pH values, the strongest light scattering occurs because dense particles that are formed have dimensions similar to the wavelength of light. At high pH values, the light scattering decreases because the hydrogel particles dissociate. At low pH values, the light scat-
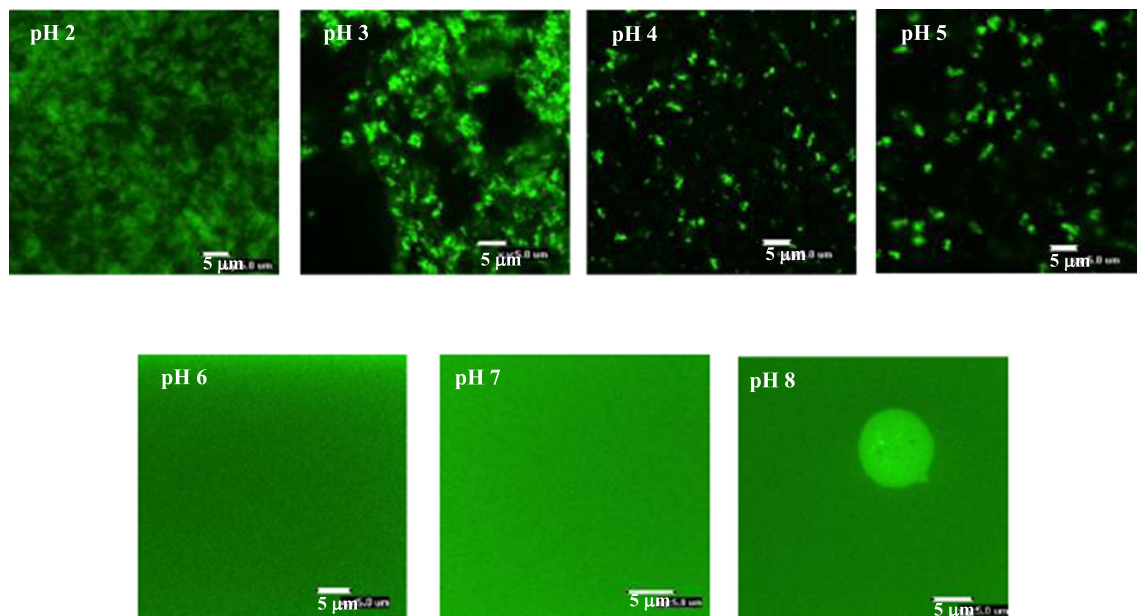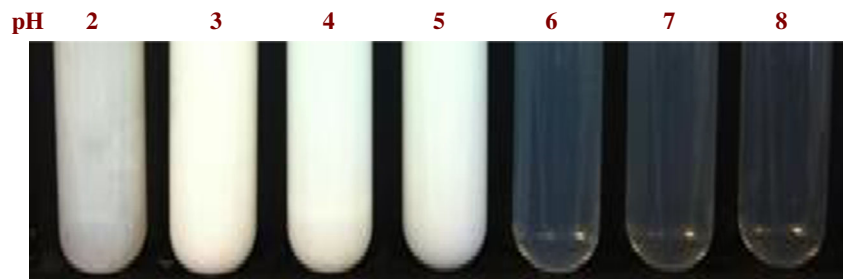

Fig. 3 Confocal micrographs (60×magnification) of hydrogel particles fabricated with WPI (1.5 %) and LMP (1.5 %) (0 mM CaCl2) at different pH conditions. Protein phase was stained in green using FITC. Scale bar at bottom right represents 5 mm

Fig. 4 Visual appearance of hydrogel particle systems (0 mM CaCl2) in different pH conditions

tering decreases because the hydrogel particles fuse together, and so the refractive index contrast is reduced (Fig. 4).

Electrical Characteristics

The electrical charge (ζ-potential) of the different hydrogel suspensions and individual biopolymer solutions (whey protein isolate and pectin solutions) were measured. The ζ-potential of the mixed systems went from highly negative at pH 8 to slightly positive at pH 2 (Fig. 5). Interestingly, the ζ-potential versus pH profile of the mixed systems was fairly similar to that of pure pectin solutions, which suggests that the electrical characteristics of the hydrogel particles were dominated by the outer pectin layer. The decrease in negative charge on the pectin at low pH values is due to protonation of the carboxyl groups ($-COO^-$ to $COOH$) on the backbone of the pectin molecules below their pK_a values (around pH 3.5). WPI and pectin both had high negative charges at pH 6 to 8, which would account for the disintegration of the hydrogel particles observed by confocal microscopy under these conditions (Fig. 5).
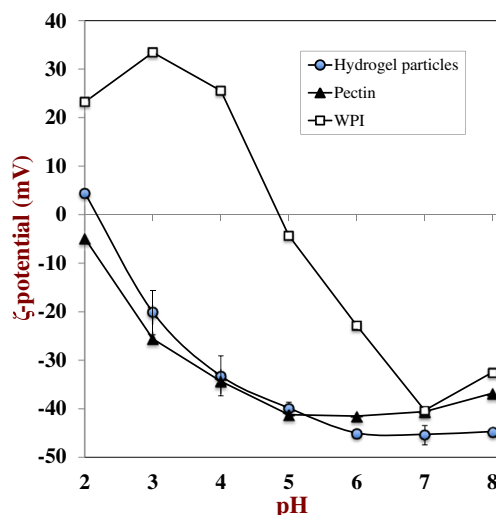

Fig. 5 Influence of pH on the electrical characteristics of hydrogel particles, whey protein isolate, and pectin systems

Conclusions

Hydrogel particles consisting of a protein-rich core and a pectin-rich shell were formed using a two-step biopolymer phase separation method that relies on both segregative (pH 7) and aggregative (pH 5) phenomena. Suspensions fabricated at pH 5 contained hydrogel particles with mean diameters around 2 µm. These particles scattered light strongly to give the hydrogel suspensions a milky white appearance, and they also led to an appreciable increase in viscosity or gel-like characteristics. These characteristics may be important for the development of fat mimetics that can contribute to the creamy appearance, texture, and mouthfeel of food products. When the pH was increased above the isoelectric point of the whey proteins, the hydrogel particles disintegrated due to electrostatic repulsion between the anionic polysaccharide and anionic protein molecules. This phenomenon may be useful for developing products that "melt" in the mouth to improve the sensory perception of low fat products, or to develop pH-triggered release mechanisms. When the pH was decreased, the hydrogel particles fused together, which was attributed to a reduction in electrostatic repulsion associated with a loss of electrical charge on the outer layer of pectin molecules that occurred around their pK_a values.

Addition of calcium addition led to aggregation of the hydrogel particles, with a notable increase in the viscosity of the suspensions with increasing calcium concentration. Overall, the hydrogel particles formed in this study have potential for use as fat mimetics in low calorie food products due to their ability to increase opacity and viscosity, or as delivery systems that may trigger the release of an active component due to pH changes. The properties of these hydrogel suspensions were modulated by adding salt or altering pH, which can be attributed to the influence of these parameters on the electrostatic interactions operating within and between hydrogel particles. This type of information is important for ensuring that hydrogel particles can be successfully incorporated into commercial food or beverage products that have different pH or ionic strength conditions.

In future studies, it would be beneficial to compare the ability of different kinds of hydrogel particles to act as fat mimetics and delivery systems, so as to establish their relative

advantages and disadvantages for commercial applications. For example, it would be useful to compare their ease and cost of preparation, their effectiveness at scattering light and increasing viscosity, and their sensitivities to changes in environmental conditions, such as pH, ionic strength, and temperature. In addition, it would be useful to carry out sensory analysis to establish their impact on the perceived mouthfeel of food products. The food industry could then select the most appropriate hydrogel particles for a specific application.

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