Encapsulation of protein nanoparticles within alginate microparticles: Impact of pH and ionic strength on functional performance

Liqiang Zou^{a1}, Zipei Zhang^{b1}, Ruojie Zhang^b, Wei Liu^{a*}, Chengmei Liu^a, Hang Xiao^b, David Julian McClements^{b,c*}

^a State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang, No. 235 Nanjing East Road, Nanchang 330047, Jiangxi, China

^b Department of Food Science, University of Massachusetts, Amherst, MA 01003

^c Department of Biochemistry, Faculty of Science, King Abdulaziz University, P. O. Box 80203 Jeddah 21589 Saudi Arabia

¹ These authors contributed equally to this manuscript.

* corresponding author:

Wei Liu, State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang, 330047, Jiangxi, China Tel: + 86 791 88305872x8106. Fax: +86 791 88334509. E-mail: liuwei@ncu.edu.cn.

David Julian McClements, Department of Food Science, University of Massachusetts, Amherst, MA 01003, USA Tel: (413) 545-1019. Fax: (413) 545-1262. E-mail: mcclements@foodsci.umass.edu.

Journal: Journal of Food Engineering

Submitted: December 2015

Abstract

Incorporation of bioactive proteins into functional foods is often challenging due to their instability to aggregation, sedimentation, or hydrolysis. In this study, core-shell protein nanoparticles, consisting of a zein core and a whey protein shell, were fabricated by antisolvent precipitation. The protein nanoparticles were then incorporated into biopolymer microgels fabricated by electrostatic complexation of casein and alginate. Protein nanoparticles were retained in microgels at low pH (3 to 5.5), but released at higher pH (6 to 7) due to microgel dissociation promoted by electrostatic repulsion between anionic casein and alginate. These microgels may be useful for retaining and protecting protein nanoparticles within acidic environments (*e.g.*, stomach), but releasing them under neutral environments (*e.g.*, small intestine). Protein nanoparticles were retained within microgels over a wide range of ionic strengths (0 to 2 M NaCl, pH 5). Protein nanoparticle encapsulation within microgels may improve their pH and salt stability in functional foods.

Keywords: zein nanoparticle; hydrogel particle; stability; delivery system.

1. Introduction

There is growing interest in the utilization of proteins and peptides (referred to collectively as “polypeptides” for convenience) as functional ingredients in foods because of their beneficial health effects, such as antioxidant, antimicrobial, and anti-hypertension activities (Hernandez-Ledesma et al., 2011; Samaranayaka and Li-Chan, 2011; Sarmadi and Ismail, 2010; Udenigwe and Aluko, 2012). Typically, the activity of polypeptides depends on their three-dimensional structures and specific amino acid sequences (Udenigwe and Aluko, 2012). Polypeptide activity may therefore be altered in food products during manufacture, storage, or transportation, due to changes in solution or environmental conditions that alter protein structure, such as pH, ionic strength, ingredient interactions, or temperature (Hettiarachchy et al., 2012). In addition, their activity may be altered after they are ingested and pass through the gastrointestinal tract (GIT) because they are exposed to digestive enzymes (proteases and peptidases) and environmental conditions (pH, ionic strength, and ingredient interactions) that may alter their structure (Mohan et al., 2015). Within the GIT, digestive enzymes and highly acidic gastric conditions may hydrolyze polypeptide chains at particular bond locations, thereby generating new peptides (Moreno, 2007). This process may be undesirable if it leads to loss of the bioactivity of an ingested polypeptide, or it may be desirable if it leads to the generation of new peptides with improved bioactivity. Consequently, it is often important to design food matrices that can control the gastrointestinal fate of polypeptides within foods and within the GIT so as to improve their bioactivity profiles (Mohan et al., 2015; Zhang et al., 2015b).

The encapsulation of polypeptides within colloidal delivery systems offers a potentially effective means of controlling their stability both in food products and within the GIT after ingestion (Mohan et al., 2015; Sagalowicz and Leser, 2010; Zhang et al., 2015b). For food applications, polypeptides are typically trapped within a colloidal particle that is fabricated from food-grade ingredients, such as lipids, carbohydrates, proteins, surfactants, or minerals (McClements, 2014). Polypeptides

may be hydrophilic, hydrophobic, or amphiphilic depending on their amino acid composition and structural organization (Udenigwe and Aluko, 2012). Consequently, an appropriate encapsulation material and structure must be selected for the particular polypeptide involved. Numerous kinds of colloidal delivery systems have been explored for their potential to encapsulate polypeptides and other bioactive components, including microemulsions, nanoemulsions, emulsions, solid lipid nanoparticles, multiple emulsions, biopolymer particles, and microgels (Du and Stenzel, 2014; du Plessis et al., 2014; McClements, 2014; Mohan et al., 2015). Each of these delivery systems has advantages and disadvantages in terms of its ease of preparation, storage, and handling, cost, stability characteristics, encapsulation efficiency, loading capacity, and food matrix compatibility.

Previously, complex coacervation has been used by our group to develop hydrogel particles containing encapsulated lipid droplets (Li and McClements, 2011; Zhang et al., 2015a; Zhang et al., 2015b). These filled hydrogel particles have a number of potential applications in the food industry, including improving physical or chemical stability, targeted delivery, and regulation of lipid digestion and satiety (Zhang et al., 2015c). In the current study, we investigated the possibility of encapsulating protein nanoparticles within polysaccharide-based hydrogel particles (“microgels”), and studied the influence of solution and environmental conditions on their properties. The main focus of this study was to examine the influence of solution conditions (pH and ionic strength) on the stability and properties of the protein nanoparticle-loaded microgels. This information is useful for establishing their potential application in food products, and their potential behavior within the gastrointestinal tract.

2. Materials and methods

2.1. Materials

Zein (Lot# SLBD5665V) and sodium alginate (Lot 50K0180) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sodium caseinate powder was obtained from the American Casein Company (MP Biomedicals LLC). Whey protein isolate

(WPI) powder was obtained from Davisco Foods International Inc. (Le Sueur, MN, USA). **Alginic acid (sodium salt)** (Lot# 180947) was purchased from the Sigma Chemical Company (St. Louis, MO). All other chemicals were of analytical grade. Double distilled water was used to prepare all solutions and colloidal suspensions.

2.2. Solution preparation

A weighed amount (2.64 g) of zein powder was added to 100 mL ethanol solution (80% v/v), and stirred at 500 rpm (IKA R05, Werke, GmbH) for 1 h, and then filtered with filter paper (Fisher Science, P5). WPI (0.25% w/v) was solubilized in phosphate buffer solution (10 mM, pH 6.5). 2% (w/w) sodium caseinate and 2% (w/w) alginate were prepared separately in 10 mM phosphate buffer at pH 7 and stirred until fully dissolved.

2.3. Fabrication of Colloidal Particles

2.3.1. Protein nanoparticles preparation

Protein nanoparticles were fabricated from zein using an antisolvent precipitation method. Initially, zein (26.4 mg/mL) was dissolved in ethanol solution (80% v/v). Then, 25 mL of aqueous ethanol solution was rapidly injected into 75 mL of whey protein solution (0.25% WPI, PBS, pH 6.5) that was continuously stirred at 1200 rpm using a magnetic stirrer (IKA R05, Werke, GmbH). The resulting colloidal dispersion was then stirred for another 30 min at the same speed. **The ethanol remaining in the final colloidal dispersions (around 16% v/v) was evaporated at 40 °C using a rotary evaporator (Rotavapor R110, Büchi Corp., Switzerland), and the same volume of pH 6.5 PBS was added to compensate for the lost ethanol.**

2.3.2. Unfilled hydrogel particle preparation

2 M sodium hydroxide was used to adjust caseinate (2%) and alginate (2%) solutions to pH 7. Then these two stock solutions and phosphate buffer were mixed together at different volume ratios under continuous stirring to form final compositions of 0.33% sodium caseinate/1.33% alginate (mass ratio 1:4). The mixtures were then acidified to pH 5 using 1 M citric acid at a rate of 1 drop/10 s with continuous stirring at

129 500 rpm to promote complex formation.

130 2.3.3. *Filled hydrogel particle preparation*

131 2 M sodium hydroxide was used to adjust caseinate solutions, alginate solutions,
132 and protein nanoparticle dispersions to pH 7. After pH adjustment, 6.6 mg/mL protein
133 nanoparticle dispersion and 2% sodium caseinate solution were mixed together at a 1:1
134 volume ratio. Then, this system was mixed (500 rpm) with 2% alginate solution at a
135 volume ratio of 1:2. Finally, the resulting mixture was acidified to pH 5 using 1 M
136 citric acid with continuous stirring at 500 rpm to promote complex formation. The final
137 composition of the resulting system was 1.1 mg/mL protein nanoparticles, 0.33%
138 sodium caseinate, and 1.33% alginate.

139 2.4. ζ -potential measurements

140 The electrical charge (ζ -potential) of biopolymers and colloidal particles was
141 measured at different pH values (3.0–7.0) using a particle electrophoresis instrument
142 (Zetasizer Nano ZA series, Malvern Instruments Ltd. Worcestershire, UK). Samples
143 were diluted using 10 mM phosphate buffer (at the same pH as the sample) prior to
144 analysis to avoid multiple scattering effects. All measurements were made on at least
145 two freshly prepared samples and each sample was measured in duplicate.

146 2.5. Particle size analysis

147 The particle size distribution was measured using a static light scattering
148 instrument (Mastersizer 2000, Malvern Instruments, Worcestershire, United Kingdom).
149 This instrument infers the size of the particles from measurements of their angular
150 scattering pattern. All measurements were made on at least two freshly prepared
151 samples. Samples were diluted in 10 mM phosphate buffer (pH 3.0–7.0) by adding
152 small aliquots into a measurement chamber. Particle size measurements were reported
153 as surface-weighted mean diameters (d_{32}).

2.6. Microstructural analysis

The microstructure of the colloidal dispersions was characterized using confocal scanning fluorescence microscopy (Nikon D-Eclipse C1 80i, Nikon, Melville, NY, U.S.). Prior to analysis, the protein phase of the samples was dyed with fluorescein thiocyanate isomer I (FITC) solution (1 mg/mL dimethyl sulfoxide) by adding 0.1 mL of FITC dye solution to 2 mL of sample. All images were captured with a 10× eyepiece and a 60× objective lens (oil immersion). The microstructure images for confocal microscopy were digitally acquired and then analyzed using image analysis software (NIS-Elements, Nikon, Melville, NY).

2.7. Stability of environmental conditions

The particle size and microstructure of the colloidal dispersions were determined after they were exposed to various environmental conditions.

Effect of pH: Freshly prepared colloidal dispersions were mixed with equal volumes of 10 mM phosphate buffer with pH values ranging from 3.0 to 7.0. The samples were then adjusted to the desired pH values with 1 mol/L NaOH or HCl.

Effect of salt: Protein nanoparticles were mixed with equal volumes of phosphate buffer at pH 6.5 containing sodium chloride (50–400 mM NaCl). While, nanoparticle-loaded microgels were mixed with equal volumes of phosphate buffer at pH 5.0 containing sodium chloride (0.5–2 M NaCl). If necessary, the samples were then adjusted to pH 6.5 or pH 5.0 with 1 mol/L NaOH or HCl.

2.8. Statistical analysis

All experiments were carried out on two or three freshly prepared samples. The results are expressed as means $\pm$ standard deviations (SD). Data were subjected to statistical analysis using SPSS software (version 18.0). Means were subject to Duncan's test and a *P*-value of <0.05 was considered statistically significant.

3. Results and discussion

3.1. Properties of initial particles

3.1.1. Protein nanoparticles

Initially, we characterized the properties of the core-shell protein nanoparticles produced using the antisolvent precipitation method (pH 6.5). Based on the fabrication method used these protein nanoparticles should consist of a core of hydrophobic zein molecules and a shell of amphiphilic whey protein molecules (Chen and Zhong, 2015). The protein nanoparticles formed had relatively small mean diameters ($d_{32} = 260$ nm) (**Table 1**), and a monomodal particle size distribution with the majority of particles being between 100 and 1000 nm (**Figure 1a**). Confocal microscopy images indicated that the particles were uniformly distributed throughout the sample, indicating that they were stable to extensive aggregation (**Figure 2b**). Visual images of the protein nanoparticle suspensions indicated that they had a uniform milky white appearance, supporting the observation that they were stable to aggregation and sedimentation (**Figure 2a**). The optical opacity of the colloidal suspensions can be attributed to the fact that the protein particles had dimensions (260 nm) that were fairly similar to the wavelength of visible light (380 to 780 nm) and therefore they scattered light strongly (McClements, 2002). The protein nanoparticles had a relatively high negative charge ($\zeta = -45$ mV) at pH 6.5 (**Table 1**) due to the fact that this pH is above the isoelectric point of the protein nanoparticles. Interestingly, the electrical characteristics (ζ -potential versus pH profile) of the protein nanoparticles (which consisted of a zein core and a whey protein shell) were very similar to those of a solution of whey protein molecules (**Figure 3**), which suggests that the proteins at the surfaces of the nanoparticles dominated their overall electrical characteristics. In addition, the isoelectric point of pure zein nanoparticles has been reported to be around pH 6.0 (Patel et al., 2010), which is considerably higher than the value of pH 4.5 measured for the whey protein-coated zein nanoparticles used in our study (**Figure 3**), again suggesting that it is the adsorbed whey protein layer that dominates the overall electrical characteristics.

3.1.2. Unfilled and filled microgels

The properties of the microgels formed by the electrostatic complexation method were characterized in the absence and presence of the protein nanoparticles (pH 5.0). The unfilled and filled microgels had mean particle diameters (d_{32}) around 4.4 and 6.0 μm respectively (**Table 1**), and had monomodal particle size distributions (**Figure 1a**). Confocal fluorescence microscopy images indicated that the microgels in both systems had a spheroid shape, and were non-aggregated (**Figure 1b**). Electrophoresis measurements indicated that the unfilled and filled microgels had a relatively high negative charge ($\zeta = -73$ and -69 mV respectively) (**Table 1**), which can be attributed to the fact that they were constructed from an anionic polysaccharide (alginate). Visual observation of the filled microgel suspensions indicated that they had a uniform turbid appearance (**Figure 2b**). In summary, the presence of the protein nanoparticles led to an increase in the dimensions of the microgels formed by electrostatic complexation, but had little impact on their electrical characteristics.

3.2. Influence of pH on stability

Colloidal delivery systems utilized within foods and beverages may experience appreciably different pH environments depending on the nature of the product. In addition, they may be exposed to considerable variations in pH as they pass through the human gastrointestinal tract after ingestion. Consequently, it is useful to understand the influence of pH on the properties of any colloidal delivery system intended for use in commercial food and beverage products.

3.2.1 Protein-nanoparticles

Initially, we examined the influence of pH on the physicochemical properties of protein nanoparticle suspensions. Visual observation of the suspensions indicated that their appearance was highly dependent on pH (**Figure 2a**). At relatively high pH values (6.5 to 7) the suspensions had a uniform white appearance. At intermediate pH values (5.5. to 4.0), a layer of white sediment was observed at the bottom of the test tubes with a clear or slightly turbid layer above. At relatively low pH values (3 to 3.5),

a slightly turbid suspension that appeared uniform throughout was observed. This kind of behavior can be attributed to the influence of pH on the electrostatic interactions between the protein nanoparticles. The electrical potential (ζ) on the protein nanoparticles went from highly negative at high pH values (e.g., -32 mV at pH 7) to highly positive at low pH values (e.g., +24 mV at pH 3), with a point of zero charge around pH 4.5 (**Figure 3**). Consequently, there would be a strong electrostatic repulsion between the protein nanoparticles at low and high pH values, but only a weak one at intermediate pH values. It is therefore likely that the attractive forces between the protein nanoparticles (such as van der Waals) were strongly enough to overcome the repulsive forces (such as electrostatic and steric) at intermediate pH values, leading to aggregation and sedimentation (McClements, 2014). The tendency for aggregation to occur is highlighted in measurements of the influence of pH on the particle size and microstructure of the protein nanoparticle suspensions (**Figures 4, 5 and S1**). At high pH values (6.5 to 7), the particles were relatively small ($d_{32} < 1 \mu\text{m}$), had a monomodal distribution, and were evenly distributed throughout the sample. At intermediate pH values (pH 5.5. to 4), the particle size was relatively big ($d_{32} > 10 \mu\text{m}$) and large irregular shaped aggregates were observed in the microscopy images. At low pH values (3 to 3.5), there was an appreciable increase in particle size detected by light scattering, and also evidence of some large aggregates in the microscopy images (**Figures 4, 5 and S1**). It is possible that these aggregates formed as the suspensions were moved from high pH to low pH, and were not fully broken down under acidic conditions. The reason for the difference in appearance of the protein nanoparticle suspensions at low and high pH values also indicated that the nature of the particles present had changed due to an alteration in the strength of the electrostatic interactions in the system.

3.2.2 Filled microgels

We then examined the influence of pH on the properties of the protein-nanoparticle filled microgels. Visual observation of the microgel suspensions indicated that they remained stable to creaming or sedimentation across the entire pH range (**Figure 2b**). Thus, the rapid sedimentation of the free protein nanoparticles that was observed at

intermediate pH values was not observed when they were encapsulated within filled microgels. The most likely reason for this is that there was a strong electrostatic repulsion between the microgels at all pH values where they remained intact. Indeed, the electrophoresis measurements indicated that the microgel samples had a relatively high negative charge from pH 3 to 7 (**Figure 3**). This high negative charge can mainly be attributed to the presence of relatively high levels of anionic alginate molecules within the microgels. The light scattering measurements indicated that the dimensions of the microgels remained relatively constant from pH 3 to 6, with a monomodal particle size distribution (**Figures 4 and S1b**). However, the particle size distribution became bimodal at pH 6.5 and 7, with a population of particles around 180 nm and another population around 4 μm . We hypothesize that the microgels dissociated at these higher pH values because the alginate and caseinate molecules both had strong negative charges and so there was a strong electrostatic repulsion between them. Consequently, the microgels may have released the protein nanoparticles, which would account for the population of small particles observed in the particle size distributions at these pH values (**Figure S1b**). This observation was supported by the confocal microscopy images of the influence of pH on the microstructure of the system (**Figure 5b**). At pH 3 to 5, the microgels remained as discrete intact particles, *i.e.*, the images showed discrete regions of high fluorescence intensity (protein-rich particles) surrounded by a black (protein-free) background. At pH 5.5, there was evidence that some of the protein had leaked out of the microgels, *i.e.*, the images showed discrete regions of high fluorescence intensity (protein-rich particles) surrounded by a green (protein-rich) background. At pH 7, it appeared that the microgels had almost completely disintegrated and the protein was evenly distributed throughout the system. In these systems, we hypothesize that the protein nanoparticles had been released from the microgels and formed a stable colloidal dispersion. This hypothesis is based on the fact that a population of small particles was observed in the light scattering data (**Figure S1b**), the overall systems appeared uniformly turbid (**Figure 2b**), and the protein was evenly spread throughout the samples (**Figure 6**). **It should be noted that the microgels also contained protein (caseinate) and this may also have been released**

with the protein nanoparticles when they dissociated.

3.3. Influence of ionic strength on stability

Colloidal delivery systems may be used in foods and beverages that contain different types and amounts of salts in the aqueous phase, or they may be exposed to different ionic environments as they pass through the gastrointestinal tract. Consequently, it is important to understand the influence of ionic strength on the properties of colloidal delivery systems intended for utilization within commercial food and beverage products.

3.3.1 Protein-nanoparticles

Initially, the influence of ionic strength on the stability of the protein nanoparticles was examined by adding different amounts of salt to them (pH 6.5). This pH was selected because in the absence of salt, the protein nanoparticles appeared stable to aggregation and sedimentation as demonstrated by the relatively small particle diameter and visual absence of phase separation (Figures 6a and 7). As discussed earlier, this effect can be attributed to a strong electrostatic repulsion between the protein nanoparticles due to their relatively high ζ -potential at this pH (Figure 3). The suspensions remained stable to particle aggregation and sedimentation after addition of 50 mM NaCl, which suggests that the electrostatic repulsion was still strong enough to overcome any attractive colloidal interactions. However, after addition of 100 mM NaCl, there was evidence of an increase in particle size (Figures 7 and S2a) indicating some aggregation occurred, which would account for the increase in opacity of the suspensions (Figure 6a). After addition of 200 mM NaCl or higher, there was a further increase in particle size and evidence of rapid sedimentation. In this case, the positively charged counter ions (Na^+) from the salt screened the negative charge on the protein nanoparticle surfaces, thereby reducing the magnitude and range of the electrostatic repulsion. The confocal fluorescence microscopy (Figure 8) and particle size distribution measurements (Figure S2a) clearly showed evidence of a population of large aggregates within the protein-nanoparticle suspensions at high salt

concentrations. In summary, the protein nanoparticles were not very stable to salt addition due to the fact that electrostatic repulsion played a major role in preventing their aggregation.

3.3.2 Filled microgels

The salt-stability of the filled microgels was very different from that of the free protein nanoparticles. In this case, increasing amounts of salt (0 to 2 M NaCl) were added to microgel suspensions at pH 5. This pH value was selected because the microgels remained intact under these conditions, *i.e.*, they did not dissociate or aggregate. At all salt levels used, the microgel suspensions appeared to be homogeneous (**Figure 6b**), which suggested that they were stable to particle aggregation and gravitational separation. In addition, the light scattering measurements (**Figure S2b**) and the microscopy images (**Figure 8b**) indicated that the microgels remained as discrete particles. The fact that the fluorescence intensity (protein) was only relatively high within the microgels suggested that the protein-nanoparticles were retained within them at all salt levels. Thus, salt addition did not promote dissociation or aggregation of the filled microgels, which may be an important attribute for application of these microgels in certain types of food and beverage products. For example, they may be useful for delivering proteins in products such as dressings, sauces, and meat products that have relatively high salt contents.

4. Conclusions

This study has shown that protein nanoparticles can be successfully incorporated into biopolymer microgels formed from electrostatic complexation of casein and alginate. The protein nanoparticles appeared to be retained within the microgels at pH values from 3 to 5.5, but were released at higher pH values due to dissociation of the casein-alginate complexes. At higher pH values both the casein and alginate are negatively charged, which causes them to dissociate and release the encapsulated protein nanoparticles. This phenomenon may be useful for retaining protein nanoparticles within acidic environments (such as the stomach), but release them under

neutral environments (such as the small intestine). The protein nanoparticles were retained within the microgels at all salt levels studied (0 to 2 M, pH 5), which suggests that the attraction between the biopolymers that made up the microgels was strong enough to resist high ionic strengths. Overall, our results suggest that encapsulation of protein nanoparticles within microgels may be a viable method of improving their stability to pH and ionic strength within foods, and possibly of controlling their fate within the gastrointestinal tract. **Nevertheless, further studies are required to monitor the potential gastrointestinal fate of the filled microgels under simulated or real gastrointestinal conditions.**

5. Acknowledgements

This material was partly based upon work supported by the USDA, NRI Grants (2013-03795 and 2014-67021). We also thank the National Aero and Space Administration (NASA) for partial funding of this research (NNX14AP32G). This project was also partly funded by the Deanship of Scientific Research (DSR), King Abdulaziz University, Jeddah, under grant numbers 87-130-35-HiCi. The authors, therefore, acknowledge with thanks DSR technical and financial support.

6. References

- Chen, H., Zhong, Q., (2015). A novel method of preparing stable zein nanoparticle dispersions for encapsulation of peppermint oil. *Food Hydrocolloids* 43, 593-602.
- Du, A.W., Stenzel, M.H., (2014). Drug Carriers for the Delivery of Therapeutic Peptides. *Biomacromolecules* 15(4), 1097-1114.
- du Plessis, L.H., Marais, E.B., Mohammed, F., Kotze, A.F., (2014). Applications of Lipid based Formulation Technologies in the Delivery of Biotechnology-based Therapeutics. *Current Pharmaceutical Biotechnology* 15(7), 659-672.
- Hernandez-Ledesma, B., Contreras, M.D., Recio, I., (2011). Antihypertensive peptides: Production, bioavailability and incorporation into foods. *Advances in Colloid and Interface Science* 165(1), 23-35.
- Hettiarachchy, N., Sato, K., Marshall, M., Kannan, A., (2012). *Food Proteins and Peptides: Chemistry, Functionality, Interactions, and Commercialization*. CRC Press, Boca Raton.
- Li, Y., McClements, D.J., (2011). Controlling lipid digestion by encapsulation of protein-stabilized lipid droplets within alginate-chitosan complex coacervates. *Food Hydrocolloids* 25(5), 1025-1033.
- McClements, D.J., (2002). Theoretical prediction of emulsion color. *Advances in Colloid and Interface Science* 97(1-3), 63-89.
- McClements, D.J., (2014). *Nanoparticle-and microparticle-based delivery systems: Encapsulation, protection and release of active compounds*. CRC Press, Boca Raton.
- Mohan, A., Rajendran, S., He, Q.S., Bazinet, L., Udenigwe, C.C., (2015). Encapsulation of food protein hydrolysates and peptides: a review. *Rsc Advances* 5(97), 79270-79278.
- Moreno, F.J., (2007). Gastrointestinal digestion of food allergens: Effect on their allergenicity. *Biomedicine & Pharmacotherapy* 61(1), 50-60.
- Patel, A.R., Bouwens, E.C.M., Velikov, K.P., (2010). Sodium Caseinate Stabilized Zein Colloidal Particles. *Journal of Agricultural and Food Chemistry* 58(23), 12497-12503.
- Sagalowicz, L., Leser, M.E., (2010). Delivery systems for liquid food products. *Current Opinion in Colloid & Interface Science* 15(1-2), 61-72.
- Samaranayaka, A.G.P., Li-Chan, E.C.Y., (2011). Food-derived peptidic antioxidants: A review of their production, assessment, and potential applications. *Journal of Functional Foods* 3(4), 229-254.
- Sarmadi, B.H., Ismail, A., (2010). Antioxidative peptides from food proteins: A review. *Peptides* 31(10), 1949-1956.
- Udenigwe, C.C., Aluko, R.E., (2012). Food Protein-Derived Bioactive Peptides: Production, Processing, and Potential Health Benefits. *Journal of Food Science* 77(1), R11-R24.
- Zhang, Z., Zhang, R., Decker, E.A., McClements, D.J., (2015a). Development of food-grade filled hydrogels for oral delivery of lipophilic active ingredients: pH-triggered release. *Food Hydrocolloids* 44, 345-352.
- Zhang, Z., Zhang, R., Tong, Q., Decker, E.A., McClements, D.J., (2015b). Food-grade filled hydrogels for oral delivery of lipophilic active ingredients: Temperature-triggered release microgels. *Food Research International* 69, 274-280.
- Zhang, Z.P., Zhang, R.J., Chen, L., Tong, Q.Y., McClements, D.J., (2015c). Designing hydrogel particles for controlled or targeted release of lipophilic bioactive agents in the gastrointestinal tract. *European*

Polymer Journal 72, 698-716.

Fig. 1 (a). Particle size distributions of protein (zein-WPI) nanoparticles, unfilled hydrogels, and protein nanoparticle filled hydrogels.

Fig. 1 (b). Confocal micrographs of protein nanoparticles, unfilled hydrogel particles and protein nanoparticle filled hydrogel particles.

Fig. 2 (a). Appearance of protein (zein-WPI) nanoparticles dispersed in different pH solutions. Extensive particle aggregation and sedimentation occurred from pH 5.5 to 4.0.

Fig. 2 (b). Appearance of protein nanoparticle-filled hydrogels dispersed in different pH solutions.

Fig. 3. The pH-dependence of the ζ -potential of whey protein molecules (WPI) in solution, protein (zein-WPI) nanoparticles, unfilled hydrogels, and protein nanoparticle filled hydrogels.

Fig 4. Effect of pH on mean particle diameters (d_{32}) of protein nanoparticles and filled hydrogel particles. Different letters mean significant differences ($p < 0.05$) of the particle diameter at different pH values (for each type of particle).

Fig. 5 (a). Confocal fluorescence micrographs of protein nanoparticles after incubation at different pH values.

Fig. 5 (b). Confocal fluorescence micrographs of protein nanoparticle-filled hydrogel particles after incubation at different pH values.

Fig. 6 (a) Appearance of protein nanoparticle suspensions at pH 6.5 after incubation in different ionic conditions (from 50 to 400 mM NaCl);

Fig. 6 (b) Appearance of protein nanoparticle-filled hydrogel particle suspensions at pH 5 after incubation in different ionic conditions (from 0.0 to 2 M NaCl).

Fig 7. Effect of ionic strength on mean particle diameter (d_{32}) of suspensions of protein nanoparticles and filled hydrogel particles. Different letters mean significant differences ($p < 0.05$) of the particle diameter at different ionic concentration.

Fig. 8 (a). The confocal micrographs of protein nanoparticles at pH 6.5 after exposure to different ionic conditions (from 50 to 400mM);

Fig. 8 (b). The confocal micrographs of filled hydrogel particles at pH 5.0 after exposure to different ionic conditions (from 0.5 to 2.0 M NaCl).

Supporting Information

Fig. S1 (a). The pH-dependence of the particle size distribution of protein nanoparticles.

Fig. S1 (b). The pH-dependence of the particle size distribution of protein nanoparticle-filled hydrogel particles.

Fig. S2 (a). The particle size distribution of protein nanoparticles at pH 6.5 after exposure to different ionic conditions (from 50 to 400 mM NaCl);

Fig. S2 (b). The particle size distribution of filled hydrogel particles at pH 5.0 after exposure to different ionic conditions (from 0.5 to 2.0 M NaCl).

Figure Captions

- Fig. 1 (a). Particle size distributions of protein (zein-WPI) nanoparticles, unfilled hydrogels, and protein nanoparticle filled hydrogels.
- Fig. 1 (b). Confocal micrographs of protein nanoparticles, unfilled hydrogel particles and protein nanoparticle filled hydrogel particles.
- Fig. 2 (a). Appearance of protein (zein-WPI) nanoparticles dispersed in different pH solutions. Extensive particle aggregation and sedimentation occurred from pH 5.5 to 4.0.
- Fig. 2 (b). Appearance of protein nanoparticle-filled hydrogels dispersed in different pH solutions.
- Fig. 3. The pH-dependence of the ζ -potential of whey protein molecules (WPI) in solution, protein (zein-WPI) nanoparticles, unfilled hydrogels, and protein nanoparticle filled hydrogels.
- Fig 4. Effect of pH on mean particle diameters (d32) of protein nanoparticles and filled hydrogel particles. Different letters mean significant differences ($p < 0.05$) of the particle diameter at different pH values (for each type of particle).
- Fig. 5 (a). Confocal fluorescence micrographs of protein nanoparticles after incubation at different pH values.
- Fig. 5 (b). Confocal fluorescence micrographs of protein nanoparticle-filled hydrogel particles after incubation at different pH values.
- Fig. 6 (a) Appearance of protein nanoparticle suspensions at pH 6.5 after incubation in different ionic conditions (from 50 to 400 mM NaCl);
- Fig. 6 (b) Appearance of protein nanoparticle-filled hydrogel particle suspensions at pH 5 after incubation in different ionic conditions (from 0.0 to 2 M NaCl).
- Fig 7. Effect of ionic strength on mean particle diameter (d32) of suspensions of protein nanoparticles and filled hydrogel particles. Different letters mean significant differences ($p < 0.05$) of the particle diameter at different ionic concentration.
- Fig. 8 (a). The confocal micrographs of protein nanoparticles at pH 6.5 after exposure to different ionic conditions (from 50 to 400mM).
- Fig. 8 (b). The confocal micrographs of filled hydrogel particles at pH 5.0 after exposure to different ionic conditions (from 0.5 to 2.0 M NaCl).

Supporting Information

Fig. S1 (a). The pH-dependence of the particle size distribution of protein nanoparticles.

Fig. S1 (b). The pH-dependence of the particle size distribution of protein nanoparticle-filled hydrogel particles.

Fig. S2 (a). The particle size distribution of protein nanoparticles at pH 6.5 after exposure to different ionic conditions (from 50 to 400 mM NaCl).

Fig. S2 (b). The particle size distribution of filled hydrogel particles at pH 5.0 after exposure to different ionic conditions (from 0.5 to 2.0 M NaCl).

Table 1. Particle size and ζ -potential of different systems: protein nanoparticles; unfilled hydrogels particles; and protein nanoparticle filled hydrogels particles.

Systems	D_{43} (μm)	D_{32} (μm)	Uniformity	ζ -potential (mV)
Protein nanoparticles	0.31 ± 0.01^a	0.26 ± 0.02^a	0.36 ± 0.03^a	-44.8 ± 0.7^b
Unfilled hydrogel particles	4.41 ± 0.25^b	3.77 ± 0.12^b	0.35 ± 0.03^a	-73.4 ± 2.1^a
Filled hydrogel particles	6.01 ± 0.70^c	5.02 ± 0.64^c	0.38 ± 0.05^a	-69.2 ± 3.5^a