Continuous Production of Core-shell Protein Nanoparticles by Antisolvent Precipitation using Dual-channel Microfluidization: Caseinate-coated Zein Nanoparticles

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Abstract

Antisolvent precipitation is commonly used to fabricate protein nanoparticles using a simple batch method that involves injecting a protein-solvent mixture into an antisolvent. In this study, the potential of producing core-shell protein nanoparticles by antisolvent precipitation using a continuous dual-channel microfluidization method was investigated. The solvent phase (zein in ethanol) and antisolvent phase (casein in water) were made to impinge on each other at high velocity, which generates intense shear, turbulent, and cavitation forces that ensure thorough mixing and breakup of the phases. Relatively small core-shell protein nanoparticles ($d < 125$ nm) could be produced using this method when the conditions were optimized. The mean particle diameter decreased with increasing antisolvent-to-solvent ratio, increasing homogenization pressure, increasing ethanol content in the solvent phase, and decreasing zein content in the solvent phase. Depending on the processing conditions employed, zein particles in the range of about 120 nm to over 1000 nm could be produced. The operating conditions were further optimized to increase the final zein concentration and decrease the organic solvent content while still obtaining small particles. The surface potential of the core-shell protein nanoparticles went from positive at low pH to negative at high pH, with a point of zero charge around pH 5. Electron microscopy indicated that the protein particles formed had a roughly spherical shape. The results suggest that the dual-channel microfluidizer could be used to continuously form protein nanoparticles by antisolvent precipitation. Nevertheless, when the microfluidization method was compared with the simple batch method the size of the particles produced under similar conditions were fairly similar.

Keywords: microfluidizer; antisolvent precipitation; zein; caseinate; nanoparticles; core-shell; delivery systems

1. Introduction

Protein nanoparticles and microparticles may be utilized as natural functional ingredients in numerous applications in the food industry, including as colloidal delivery systems, stabilizers, lightening agents, and texture modifiers [1-4]. The ability of protein particles to provide these desirable functional attributes depends on particle properties, such as composition, size, morphology, charge, and polarity [5]. Consequently, there is considerable interest in developing processing methods to fabricate protein particles with well-defined physicochemical and structural properties [6]. A particularly promising method for producing protein particles is antisolvent precipitation [7-10]. Traditionally, this method is carried out using a simple batch process where a protein/solvent mixture is injected into an antisolvent, which leads to the spontaneous formation of small protein particles. These particles form due to self-association of the protein molecules when the solvent quality changes. For hydrophobic proteins, an organic liquid (such as ethanol) is used as the solvent phase, and water is used as the antisolvent phase [11]. Conversely, for hydrophilic proteins, water is used as the solvent phase, and an organic liquid is used as the antisolvent phase [12, 13].

In the current study, a hydrophobic protein (zein) was used to form the core of the protein-nanoparticles, whereas as an amphiphilic protein (caseinate) was used to form the shell. This combination was employed because it has previously been shown that relatively small stable core-shell protein nanoparticles can be formed with the antisolvent precipitation method [10, 14, 15]. In this case, a mixture of zein and ethanol are injected into an aqueous buffer solution consisting of caseinate molecules dissolved in water (at a pH away from the isoelectric point). After injection, the zein molecules associate with each other when the ethanol diffuses into the surrounding aqueous phase, and the local ethanol concentration surrounding the zein molecules drops below the solubilization limit thereby causing protein precipitation [16]. The caseinate is needed in the aqueous solutions to adsorb to the surfaces of the zein particles and cover any exposed non-polar patches. In the absence of this emulsifier, the bare zein nanoparticles have a strong tendency to aggregate due to a strong hydrophobic attraction between the exposed non-polar patches. The adsorption of the caseinate molecules reduces the surface

hydrophobicity, and can increase the steric and electrostatic repulsion between the particles, thereby improving their aggregation stability.

As mentioned earlier, protein nanoparticles are normally formed by the antisolvent precipitation using a simple batch approach that involves injecting a protein-solvent mixture into an antisolvent. Recently, it was shown that protein nanoparticles can also be produced by antisolvent precipitation using an impinging jet approach [17]. The authors constructed a mechanical device that consisted of two syringes, one containing the protein-solvent and the other containing the antisolvent. The syringe outlets were connected to a specially designed series of channels that made the two fluid streams impinge upon each other at high velocity. This procedure led to the formation of small protein particles, which was mainly attributed to the intense mixing forces generated when the two liquids collided with each other. In the present study, we investigated the possibility of using a commercially available dual-channel microfluidizer (PureNano[®], Microfluidics, Newton, MA) to continuously form protein nanoparticles using the antisolvent precipitation method. The principle behind the microfluidization method is similar to the impinging jet approach, but the former is a continuous method that could be used at an industrial scale. As part of this study, we examined the impact of various processing parameters on particle properties so that we could form small protein particles with low amounts of organic solvent. We also compared the properties of the protein particles produced using the microfluidization method with those produced using the simple batch injection method to compare their potential advantages and disadvantages.

2. Materials and methods

2.1. Materials

Powdered zein ($\geq 95\%$) was purchased from Sigma Aldrich Corp. (St. Louis MO, USA). Sodium caseinate and ethanol (100 % proof) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Other chemicals, reagents, and solvents were purchased from Sigma-Aldrich or Thermo Fisher Scientific (Waltham, MA, USA) and were of analytical grade, unless otherwise stated.

2.1. Solution preparation

For the solvent phase, hydrophobic zein from maize was dissolved in a mixture of ethanol and 10 mM citrate phosphate buffer (pH 7). The solution was stirred for 2 h at room temperature to ensure complete hydration of the protein. Solutions were subsequently vacuum filtered with a Whatman Filter (Nr. 42). 1 % sodium caseinate was dissolved in 10 mM citrate phosphate buffer (pH 7.0) to prepare the antisolvent phase.

2.2. Nanoparticle Preparation

Continuous Method: The fabrication of nanoparticles was carried out using a high pressure dual-channel microfluidizer (Microfluidics PureNano, Newton, MA, USA) with Y- and Z-type interaction chambers. The impact of operating pressure (5 to 19 kpsi), solvent-to-antisolvent ratio (1:10 to 1:1), zein concentration (1 to 10 % w/v), and ethanol concentration (60-85 % v/v) was varied. Based on preliminary experiments, the aqueous phase composition was kept constant, consisting of 1% (w/v) sodium caseinate dissolved in citrate phosphate buffer solution (10 mM, pH 7.0).

The solvent and antisolvent were poured into the two input hoppers of the microfluidizer, and then forced into the mixing chamber under pressure at variable relative flow rates (to alter the solvent-to-antisolvent ratio). Antisolvent precipitation was achieved using a single pass of the samples through the device. After particle formation, ethanol was removed using a vacuum rotary evaporator at 40 °C (Buchi RE 111, Flawil, Switzerland).

Batch Method: A simple batch injection method was also used to prepare protein particles by antisolvent precipitation under similar conditions as the dual-channel microfluidizer (with the exception of pressure variation). In this case, the solvent was poured into the antisolvent at a stirring speed of 600 rpm and then the sample was stirred for a further 5 min. Ethanol was removed using the rotary evaporation method mentioned earlier.

Sodium azide (0.02 % w/v) was added to all samples after particle formation to prevent any microbial growth during storage.

2.3. Nanoparticle Characterization

Particle size and charge: The size and charge of the protein particles was measured using a combined dynamic light scattering / particle electrophoresis instrument (Nano ZS, Malvern Instruments, Malvern, UK). Samples were diluted in 10 mM citrate phosphate buffer solutions at the appropriate pH prior to characterization to prevent multiple scattering effects. The instrument reports the mean particle diameter (Z-average size), polydispersity index (PDI), and ζ -potential of the protein particles. Samples were equilibrated at 25 °C prior to analysis.

pH stability: The ζ -potential *versus* pH profiles of zein nanoparticles, caseinate/zein-coated nanoparticles, and caseinate solutions (1 % w/v) were measured. All samples were prepared at pH 7 and adjusted to pH values ranging from 2 to 9 with phosphoric acid and sodium hydroxide, respectively.

Particle morphology: Aqueous protein particle suspensions were freeze-dried and then the resulting powder was visualized using SEM to provide information about particle morphology. A thin layer of the powder was immobilized on double-sided carbon tape. The samples were subsequently sputter coated with 3–6 nm of platinum. The samples were then visualized on a Magellan 400 FESEM (FEI, Hillsboro, OR, USA) at 5 kV and 13 pA.

2.4. Statistical analysis

All measurements were performed at least twice on duplicate freshly prepared samples. Means and standard deviations were calculated from these four measurements using Excel (Microsoft, Redmond, VA, USA). One-way analysis of variance was carried out with IBM SPSS Statistics (IBM Corp, Armonk, NY, USA). Differences among pairs of means were compared using a Tukey-test and a significance level of 0.05.

3. Results and Discussion

Suspensions of protein particles, hereafter referred to as hydrosols, were prepared by mixing solvent and antisolvent, using either the dual-channel microfluidizer or the simple batch approach. A solvent phase containing 3 % zein (w/v) dissolved in 80 % ethanol (v/v) solution, and a solvent-to-antisolvent ratio of 1-to-5 were used as initial parameters for particle formation based on previous studies (Davidov-Pardo, Joye, and McClements 2015; Zhong and Jin 2009). Preliminary experiments showed that no protein purification of the solvent phase, in form of centrifugation, was needed prior to particle preparation by antisolvent precipitation, as there was no significant difference ($p > 0.05$) among these samples concerning particle size and PSD. The ζ -potential was approximately -30 mV for all samples, except for samples prepared by varying the zein concentration in the solvent.

3.1. Influence of operating pressure

Increasing the operating pressure of the microfluidizer should generate more intense disruptive forces when the protein-solvent and antisolvent mixtures collide, which would be expected to influence the particle size. The influence of the operating pressure on the particle size was therefore investigated, keeping the other system parameters constant (Figure 2). At all operating pressures, the particle size distribution was monomodal, the polydispersity index was relatively small ($PDI < 0.21$), and the mean particle diameter was < 200 nm, indicating that even low pressures were suitable for producing core-shell protein nanoparticles. Nevertheless, the mean particle diameter did decrease with increasing operating pressure from 5 to 15 kpsi, and then remained fairly constant (Fig. 2a). Statistical analysis indicated that only the mean diameters of the protein particles produced at 5 and 7 kpsi were significantly different ($p > 0.05$) from those produced at 9 to 19 kpsi. Thus, it may be beneficial to use higher pressures in applications where smaller particles are required. Presumably higher pressures lead to better mixing of the solvent and antisolvent phases, and may help break up any aggregated protein particles that may be formed. Protein particle aggregation may occur if the bare zein nanoparticles collide with each other before they have time to be coated by caseinate molecules. Nevertheless, overall these experiments suggest that the operating pressure of

the microfluidizer does not have a major impact on the size of the protein particles produced. This is consistent with a previous study that reported that there was a decrease in protein particle size when the velocity that the solvent and antisolvent were made to impinge on each other increased [17].

3.2. Influence of solvent to antisolvent ratio

An increase in the concentration of protein nanoparticles using the microfluidization methods would be beneficial for the industrial application of this technology. One means of increasing the nanoparticle level in the final systems is to increase the solvent-to-antisolvent ratio utilized. The impact of varying the solvent-to-antisolvent mixing ratio was therefore examined while all the other parameters were kept constant: 3% zein (w/v) in 80 % ethanol solution (v/v) for the solvent phase, 1% sodium caseinate (w/v) in the water phase; and an operating pressure of 13 kpsi.

There was little impact of the mixing ratio on the particle size distribution or mean particle diameter from 1:10 to 1:4 solvent-to-antisolvent, with the mean particle diameter remaining appreciably below 200 nm (**Figure 3**). However, there was an appreciable increase in the particle size when the solvent-to-antisolvent ratio was increased further, with very large particles (> 2000 nm) being formed at a 1:1 ratio. Interestingly, all of the systems had a monomodal particle size distribution, regardless of the mixing ratio used. The reason that small particles were produced at low mixing ratios may be attributed to the impact of solvent composition on saturation, nucleation, and growth processes [7]. When there is a large amount of antisolvent (water) present, the ethanol rapidly diffuses out of the protein-solvent particles and the critical saturation concentration where protein precipitation occurs is reached rapidly. Consequently, relatively small particles are formed under these conditions. Conversely, when there is a much lower amount of antisolvent present, the critical saturation concentration is reached more slowly. Consequently, the particles formed may have a more diffuse and irregular structure that is more prone to aggregation. Moreover, there would have been less caseinate present at the higher solvent-to-antisolvent ratios, which means that the protein particles could have had a greater tendency to aggregate due to hydrophobic attraction.

These results indicate that there is a range of solvent-to-antisolvent ratios that can be utilized to produce small protein nanoparticles using the microfluidization method.

3.3. Influence of ethanol content

From an industrial viewpoint, it is advantageous to minimize the amount of organic solvent required to fabricate the protein nanoparticles from an economical and environmental perspective [18]. To investigate the minimal needed amount of organic solvent required, the ethanol content in the solvent phase was varied from 60-85 % (v/v), with the remainder being citrate phosphate buffer. This range was determined to be the solubility limit of the hydrophobic biopolymer zein in the ethanol-buffer mixture. The protein was found to precipitate from the solvent phase when the ethanol level was below 60 % or above 85 %, which is in accordance to previous studies on similar systems [11].

For all ethanol levels used, the mean diameter of the protein particles produced was relatively small (< 180 nm) and the particle size distribution was monomodal (**Figures 4 and 4b**). Nevertheless, there was a moderate decrease in mean particle diameter with increasing ethanol concentration, from around 175 to 158 nm. Interestingly, the opposite effect was reported in a previous study where antisolvent precipitation was carried out using a jet impingement method [17]. However, again the mean particle diameter did not depend strongly on ethanol concentration in that study either, varying from around 120 to 160 nm. Overall, these results suggest that ethanol level did not have a major influence, and that it would be possible to form relatively small particles over a range of ethanol levels.

3.4. Influence of zein concentration

An alternative approach to increase the total concentration of protein nanoparticles in the final hydrosol formed after antisolvent precipitation is to increase the zein level in the initial protein-solvent solution [11, 15]. The impact of varying the zein concentration in the solvent phase from 1 to 10 % (w/v), while keeping all other parameters fixed was therefore investigated.

There was an increase in mean particle diameter from around 141 to 556 nm with increasing zein concentration in the solvent phase (Figure 5a). From about 1 to 4% protein the mean particle diameter was relatively small (< 200 nm) and the particle size distribution was monomodal (Figure 5b), but at higher values that was a steep increase in

particle diameter, and the polydispersity increased (Figure 5b). This effect may be attributed to a number of factors. First, there may have been insufficient caseinate present in the antisolvent (water) phase to completely cover all of the zein particles formed, thereby leading to bridging flocculation [19]. Second, the high initial protein level in the solvent phase will have led to a more concentrated suspension of protein particles being formed, thereby increasing the chance of particle aggregation due to the increase in the particle collision frequency in more concentrated systems [7]. Third, the increase in the viscosity of the protein-solvent solution may have hindered ethanol diffusion out of the droplets and/or inhibited the intense mixing of the solvent and antisolvent.

Measurements of the surface potential of the protein particles was carried out to provide additional insights into the origin of this effect. The ζ -potential of the protein particles became more negative with increasing zein concentration, changing from around -27 to -34 mV as the protein level was increased from 1 to 10% (data not shown). At neutral pH, the ζ -potential of the caseinate molecules was less negative than that of the zein nanoparticles (see later). This suggests that the zein particles may not have been fully coated by sodium caseinate at high zein levels, which could have led to some particle aggregation. The particles may have aggregated due to hydrophobic attraction between exposed non-polar groups on the zein particle surfaces, or due to bridging flocculation caused by a single caseinate molecule adsorbing to the surface of multiple zein particles. In future studies, it would therefore be interesting to examine the influence of caseinate concentration on the formation of stable core-shell nanoparticles.

3.5. Optimized parameters for antisolvent precipitation

Based on the results of the previous experiments, the following optimized parameters were selected for creating core-shell protein nanoparticles with small mean particle diameters and monomodal particle size distributions: *solvent phase*: 4 % zein (w/v) and 80 % ethanol (v/v) in buffer solution; *antisolvent phase*: 1% sodium caseinate in buffer solution; solvent-to-antisolvent mixing ratio = 1 to 5; and, operating pressure = 13 kpsi.

3.6. Electrical characteristics of core-shell protein particles

A number of important functional attributes of protein particles depend on their electrical characteristics, such as their aggregation stability, tendency to adsorb to charged surfaces, and interaction with other charged species. The electrical properties of proteins are pH-dependent, which effects their ability to exhibit their desired functional attributes in different types of food products or within the gastrointestinal tract where the pH can change considerable. For this reason, the ζ -potential *versus* pH profile of the caseinate-coated zein protein nanoparticles was compared to bare zein nanoparticles and caseinate molecules (**Figure 6**).

The surface potential of all the samples ranged from highly positive to highly negative as the pH was increased from highly acidic (pH 2) to highly basic (pH 9) (Fig. 6a). This behaviour is typical of proteins and can be attributed to the presence of both amine groups and carboxylic groups on the polypeptide chains. In general, a protein is negatively charged above its isoelectric point (pI), but positively charged below it. The caseinate had a lower pI (4.5) than the bare zein nanoparticles (5.9). Extensive protein aggregation and sedimentation was observed in these samples because the relatively weak electrostatic repulsion between them was insufficient to overcome the attractive interactions (such as van der Waals and hydrophobic attraction). As expected, the core-shell protein nanoparticles had a pI (5) between that of the bare zein nanoparticles and casein molecules. In addition, the ζ -potential of the core-shell nanoparticles was appreciably different from that of the bare nanoparticles at most pH values, which suggests that the caseinate molecules were absorbed. The interaction of zein and sodium caseinate may be attributed to hydrophobic interactions, or interactions between cationic groups of one protein and anionic groups of the other. At very high (pH 9) and very low (pH 2) pH-values the core-shell nanoparticles had surface potentials about the same magnitude as bare zein nanoparticles, which suggests that the caseinate may not have adsorbed under these conditions. Presumably, there was a large electrostatic repulsion between the two proteins under these conditions, which was large enough to overcome the hydrophobic attraction.

The particle size measurements and visual observations indicated that there was extensive aggregation in all three systems at pH 5 (Fig. 6b), which is close to the

isoelectric point of the proteins (**Fig. 6a**). The caseinate solutions appeared optically clear at pH 2, 3, 7 and 9, which suggests that the proteins were dissolved under these conditions. The extensive aggregation observed at pH 5 in all the samples can be attributed to the reduction in the electrostatic repulsion between the protein molecules and particles.

3.7. Comparison of continuous and batch antisolvent precipitation methods

In this section, the sizes of the particles produced using the continuous dual-channel microfluidization method were compared with those produced using the traditional batch antisolvent precipitation method. Initially, we hypothesized that much smaller particle sizes would be produced using the microfluidization method due to the intense mixing conditions generated when the solvent and antisolvent phases were made to collide together. However, in practice it was found that in most cases the size of the protein particles produced using the batch method were very similar to those produced using the microfluidization method using similar system conditions (*e.g.*, similar zein concentration, ethanol concentration, and solvent-to-antisolvent mixing ratio). This effect is illustrated in Figure 7, where the mean diameters of the particles produced using the batch method are plotted against those produced using the microfluidizer method under similar conditions. Under conditions where relatively small protein particles were formed ($d < 500$ nm) there was a strong linear correlation between the two different antisolvent precipitation methods ($r^2 = 0.89$, slope = 0.90). Conversely, under conditions where very large particles tend to be produced (such as high zein levels) the microfluidizer generates considerably smaller particles than the batch method, presumably because the intense disruptive forces in the mechanical device can partially breakup large aggregates. These results suggest that the mechanism of nanoparticle formation does not depend strongly on the mixing conditions used. Overall, it seems that the microfluidizer is not able to produce smaller particles, but that its main advantage is that it is a rapid continuous process that could produce protein particles on a larger scale than the simple batch process. In addition, it was observed that the reproducibility of the particle size produced using a given set of conditions was better (smaller standard deviation from batch-to-batch) for the microfluidizer (Mean Error = 4.8%) than for the

batch method (Mean Error = 7.3%), which may also be important for some commercial applications.

3.8. Microstructure of nanoparticles

Finally, the microstructure of the protein particles produced using the batch and continuous (microfluidizer) approach was characterized using scanning electron microscopy (SEM). In general, both samples contained a large number of roughly spherical particles (Figure 8). The roughness of the protein particle surfaces may have been an artifact introduced by the freeze-drying and electron microscopy preparation procedure. Interestingly, an occasional SEM image (about 1 in 10) of the samples prepared using the batch method indicated that they contained some large particles, whereas those of the samples produced using the microfluidizer method always contained small particles (data not shown). The presence of these large particles was not detected by the dynamic light scattering method used, since the mean particle diameters were fairly similar for both the batch and microfluidizer methods (Figure 7). This may have been because these large particles rapidly sediment to the bottom of the measurement cells in the dynamic light scattering instrument and are therefore not detected. These results suggest that there may be an advantage in using the microfluidization method if a highly uniform particle suspension is required. It was not possible to verify the expected core-shell structure of the protein nanoparticles using the SEM method because it only gives information about the surface topology. In future studies, it would be useful to utilize TEM for this purpose.

4. Conclusions

This study has shown that protein nanoparticles can be successfully produced by antisolvent precipitation using a dual-channel microfluidizer. The main advantage of the microfluidization method over conventional batch methods for this purpose is that it can rapidly and continuously produce particles, which may make it more suitable for large-scale production purposes. A number of process variables were examined to determine the magnitude of their impact on the size of the protein particles produced by the

microfluidizer. The particle size could be reduced by increasing homogenization pressure, reducing the solvent-to-antisolvent mixing ratio, increasing the ethanol content in the solvent phase, or increasing the zein level in the solvent phase. Under optimized conditions a suspension of relatively small ($d < 130$ nm) caseinate-coated zein particles with a monomodal particle size distribution could be produced. Interestingly, the size of the particles produced by the microfluidizer method were similar to those produced using the conventional batch antisolvent precipitation method under conditions where small particles were generated. This observation suggests that the highly intense mixing conditions produced within the microfluidizer did not play a critical role in the formation of small protein nanoparticles by antisolvent precipitation. Nevertheless, they were capable of partially breaking up aggregates under conditions where large particles are formed.

In summary, the dual channel microfluidization method is capable of rapidly and continuously producing core-shell protein nanoparticles using the antisolvent precipitation approach. These features may make it suitable for the large-scale commercial production of protein nanoparticles that could be used as delivery systems, stabilizers, lightening agents, or texture modifiers. The same approach may also be useful for producing other types of food-grade nanoparticles. For example, nanoemulsions can be produced by passing oil through one channel and water through the other, whereas nutraceutical nanocrystals can be formed by passing a hydrophobic nutraceutical dissolved in an organic solvent through one channel and water through the other.

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References

- Augustin, M. A. 2003. "The Role of Microencapsulation in the Development of Functional Dairy Foods." *ResearchGate* 58 (2): 156–60.
- Bai, Long, Siqi Huan, Jiyu Gu, and David Julian McClements. 2016. "Fabrication of Oil-in-Water Nanoemulsions by Dual-Channel Microfluidization Using Natural Emulsifiers: Saponins, Phospholipids, Proteins, and Polysaccharides." *Food Hydrocolloids* 61 (December): 703–11. doi:10.1016/j.foodhyd.2016.06.035.
- Bai, Long, and David Julian McClements. 2016. "Development of Microfluidization Methods for Efficient Production of Concentrated Nanoemulsions: Comparison of Single- and Dual-Channel Microfluidizers." *Journal of Colloid and Interface Science* 466 (March): 206–12. doi:10.1016/j.jcis.2015.12.039.
- Davidov-Pardo, Gabriel, Iris J. Joye, and David Julian McClements. 2015. "Encapsulation of Resveratrol in Biopolymer Particles Produced Using Liquid Antisolvent Precipitation. Part 1: Preparation and Characterization." *Food Hydrocolloids* 45 (March): 309–16. doi:10.1016/j.foodhyd.2014.11.023.
- Grodowska, Katarzyna, and Andrzej Parczewski. 2010. "Organic Solvents in the Pharmaceutical Industry." *Acta Poloniae Pharmaceutica* 67 (1): 3–12.
- Guzey, Demet, and D. Julian McClements. 2006. "Formation, Stability and Properties of Multilayer Emulsions for Application in the Food Industry." *Advances in Colloid and Interface Science*, In Honor of Professor Nissim Garti's 60th Birthday, 128–130 (December): 227–48. doi:10.1016/j.cis.2006.11.021.
- Hinrichs, J. 2001. "Incorporation of Whey Proteins in Cheese." *International Dairy Journal*, Cheese Ripening and Technology, 11 (4–7): 495–503. doi:10.1016/S0958-6946(01)00071-1.
- Joye, Iris Julie, and David Julian McClements. 2013. "Production of Nanoparticles by Anti-Solvent Precipitation for Use in Food Systems." *Trends in Food Science & Technology* 34 (2): 109–23. doi:10.1016/j.tifs.2013.10.002.
- Kryder, David, Stanley Kowalski, and Anatole Krattiger. 2000. "The Intellectual and Technical Property Components of pro-Vitamin A Rice (GoldenRice™): A Preliminary Freedom-To-Operate Review." *Legal Scholarship*, January. http://scholars.unh.edu/law_facpub/167.

- Luo, Yangchao, Zi Teng, Thomas T. Y. Wang, and Qin Wang. 2013. "Cellular Uptake and Transport of Zein Nanoparticles: Effects of Sodium Caseinate." *Journal of Agricultural and Food Chemistry* 61 (31): 7621–29. doi:10.1021/jf402198r.
- Panagiotou, Thomai, Steven V. Mesite, and Robert J. Fisher. 2009. "Production of Norfloxacin Nanosuspensions Using Microfluidics Reaction Technology through Solvent/Antisolvent Crystallization." *Industrial & Engineering Chemistry Research* 48 (4): 1761–71. doi:10.1021/ie800955t.
- Patel, Ashok, Yingchun Hu, Jyoti Kumar Tiwari, and Krassimir P. Velikov. 2010. "Synthesis and Characterisation of Zein–curcumin Colloidal Particles." *Soft Matter* 6 (24): 6192–99. doi:10.1039/C0SM00800A.
- Patel, Ashok R., Elisabeth C. M. Bouwens, and Krassimir P. Velikov. 2010. "Sodium Caseinate Stabilized Zein Colloidal Particles." *Journal of Agricultural and Food Chemistry* 58 (23): 12497–503. doi:10.1021/jf102959b.
- Siró, István, Emese Kápolna, Beáta Kápolna, and Andrea Lugasi. 2008. "Functional Food. Product Development, Marketing and Consumer acceptance—A Review." *Appetite* 51 (3): 456–67. doi:10.1016/j.appet.2008.05.060.
- Zhao, Hong, Jie-Xin Wang, Qi-An Wang, Jian-Feng Chen, and Jimmy Yun. 2007. "Controlled Liquid Antisolvent Precipitation of Hydrophobic Pharmaceutical Nanoparticles in a Microchannel Reactor." *Industrial & Engineering Chemistry Research* 46 (24): 8229–35. doi:10.1021/ie070498e.
- Zhong, Qixin, and Minfeng Jin. 2009. "Zein Nanoparticles Produced by Liquid–liquid Dispersion." *Food Hydrocolloids* 23 (8): 2380–87. doi:10.1016/j.foodhyd.2009.06.015.
1. Jones, O.G. and D.J. McClements, *Functional biopolymer particles: design, fabrication, and applications*. Comprehensive Reviews in Food Science and Food Safety, 2010. 9(4): p. 374-397.

2. Joye, I.J. and D.J. McClements, *Biopolymer-based nanoparticles and microparticles: Fabrication, characterization, and application*. Current Opinion in Colloid & Interface Science, 2014.
3. Xiao, J., Y.Q. Li, and Q.R. Huang, *Recent advances on food-grade particles stabilized Pickering emulsions: Fabrication, characterization and research trends*. Trends in Food Science & Technology, 2016. **55**: p. 48-60.
4. Chen, L.Y., G.E. Remondetto, and M. Subirade, *Food protein-based materials as nutraceutical delivery systems*. Trends in Food Science & Technology, 2006. **17**(5): p. 272-283.
5. McClements, D.J., *Nanoparticle- and Microparticle-based Delivery Systems*. First ed. 2015, Boca Raton, FL: CRC Press.
6. Matalanis, A., O.G. Jones, and D.J. McClements, *Structured biopolymer-based delivery systems for encapsulation, protection, and release of lipophilic compounds*. Food Hydrocolloids, 2011. **25**(8): p. 1865-1880.
7. Joye, I.J. and D.J. McClements, *Production of nanoparticles by anti-solvent precipitation for use in food systems*. Trends in Food Science & Technology, 2013. **34**(2): p. 109-123.
8. Thorat, A.A. and S.V. Dalvi, *Liquid antisolvent precipitation and stabilization of nanoparticles of poorly water soluble drugs in aqueous suspensions: Recent developments and future perspective*. Chemical Engineering Journal, 2012. **181**: p. 1-34.
9. Patel, A., et al., *Synthesis and characterisation of zein-curcumin colloidal particles*. Soft Matter, 2010. **6**(24): p. 6192-6199.
10. Patel, A.R., E.C.M. Bouwens, and K.P. Velikov, *Sodium Caseinate Stabilized Zein Colloidal Particles*. Journal of Agricultural and Food Chemistry, 2010. **58**(23): p. 12497-12503.
11. Zhong, Q.X. and M.F. Jin, *Zein nanoparticles produced by liquid-liquid dispersion*. Food Hydrocolloids, 2009. **23**(8): p. 2380-2387.
12. Gulseren, I., Y. Fang, and M. Corredig, *Whey protein nanoparticles prepared with desolvation with ethanol: Characterization, thermal stability and interfacial behavior*. Food Hydrocolloids, 2012. **29**(2): p. 258-264.
13. Gulseren, I., Y. Fang, and M. Corredig, *Zinc incorporation capacity of whey protein nanoparticles prepared with desolvation with ethanol*. Food Chemistry, 2012. **135**(2): p. 770-774.
14. Li, K.K., et al., *Preparation of water-soluble antimicrobial zein nanoparticles by a modified antisolvent approach and their characterization*. Journal of Food Engineering, 2013. **119**(2): p. 343-352.
15. Davidov-Pardo, G., I.J. Joye, and D.J. McClements, *Encapsulation of resveratrol in biopolymer particles produced using liquid antisolvent precipitation. Part 1: Preparation and characterization*. Food Hydrocolloids, 2015. **45**: p. 309-316.
16. Zhong, Q.X., et al., *Application of supercritical anti-solvent technologies for the synthesis of delivery systems of bioactive food components*. Food Biophysics, 2008. **3**(2): p. 186-190.
17. Li, K.K., et al., *Continuous preparation of zein colloidal particles by Flash NanoPrecipitation (FNP)*. Journal of Food Engineering, 2014. **127**: p. 103-110.

- 1
2
3
4 481 18. Grodowska, K. and A. Parczewski, *Organic solvents in the pharmaceutical industry*.
5 482 Acta Poloniae Pharmaceutica, 2010. **67**(1): p. 3-12.
6
7 483 19. Guzey, D. and D.J. McClements, *Formation, stability and properties of multilayer*
8 484 *emulsions for application in the food industry*. Advances in Colloid and Interface
9 485 Science, 2006. **128**: p. 227-248.
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Figure 1: (a) Schematic illustration of the production of zein nanoparticles using one-step dual-channel microfluidization. A stream of zein in ethanol is made to impinge on a stream of caseinate in water, which results in the formation of zein nanoparticles due to antisolvent precipitation.

Figure 2a: Influence of homogenization pressure of dual-channel microfluidizer on mean particle diameter of zein nanoparticles produced by antisolvent precipitation (3 % zein, 80 % EtOH, solvent-to-antisolvent mixing ratio 1:5, 1 % of sodium caseinate in antisolvent). Different letters above the data points represent statistical difference ($p < 0.05$).

Figure 2b: Influence of homogenization pressure of dual-channel microfluidizer on particle size distribution of zein nanoparticles produced by antisolvent precipitation (3 % zein, 80 % EtOH, solvent-to-antisolvent mixing ratio 1:5, 1 % of sodium caseinate in antisolvent).

Figure 3a. Influence of mixing ratio of solvent-to-antisolvent on the mean particle diameter of zein nanoparticles (3 % zein, 80 % EtOH, pressure 13 kpsi) produced by dual-channel microfluidization. Different letters above the data points represent statistical difference ($p < 0.05$).

Figure 3b: Influence of mixing ratio of solvent-to-antisolvent on the particle size distribution of zein nanoparticles (3 % zein, 80 % EtOH, pressure 13 kpsi) produced by dual-channel microfluidization.

Figure 4a: Influence of ethanol content in the solvent phase on the mean particle diameter of zein nanoparticles produced by dual-channel microfluidization: 3 % zein, mixing ratio 1:5, pressure 13 kpsi, and 1 % sodium caseinate in antisolvent. Different letters above the data points represent statistical difference ($p < 0.05$).

Figure 4b: Influence of ethanol content in the solvent phase on the particle size distribution of zein nanoparticles produced by dual-channel microfluidization: 3 % zein, mixing ratio 1:5, pressure 13 kpsi, and 1 % sodium caseinate in antisolvent.

Figure 5a: Influence of zein concentration in the solvent phase on the mean particle diameter of zein nanoparticles produced using a dual-channel microfluidizer: 80 % ethanol (v/v), mixing ratio 1:5, pressure 13 kpsi, 1 % sodium caseinate in antisolvent. Different letters above the data points represent statistical difference ($p < 0.05$).

Figure 5b: Influence of zein concentration in the solvent phase on the mean particle diameter of zein nanoparticles produced using a dual-channel microfluidizer: 80 % ethanol (v/v), mixing ratio 1:5, pressure 13 kpsi, 1 % sodium caseinate in antisolvent.

Figure 6a: ζ potential versus pH profiles of sodium caseinate solution, bare zein nanoparticles, and composite nanoparticles. The nanoparticles were prepared using optimized conditions (75 % ethanol (v/v), 4 % zein (w/v), 13 kpsi, mixing ratio 1-to-5, 1 % of sodium caseinate in antisolvent).

Figure

6b: Particle size versus pH profiles of bare zein nanoparticles and composite nanoparticles, and impact of pH on appearance of caseinate solutions. The nanoparticles were prepared using optimized conditions (75 % ethanol (v/v), 4 % zein (w/v), 13 kpsi, mixing ratio 1-to-5, 1 % of sodium caseinate in antisolvent).

Figure 7: Comparison of the mean particle diameter of zein nanoparticles prepared by simple injection or by microfluidization. The zein concentration in the solvent was 4 % (w/v), the ethanol content in the solvent was 75 % (v/v), a mixing ratio of solvent-to-antisolvent of was 1-to-5, and a sodium caseinate concentration in the antisolvent of 1 % (w/v) was used to form the nanoparticles.

Batch approach

Continuous approach

Figure 8: SEM images of caseinate-coated zein nanoparticles at 20k times (left) and 80k times (right) magnification. Antisolvent precipitation was carried out using either the *batch* or the *continuous (microfluidizer) approach* and the particle suspensions were freeze-dried prior to SEM analysis. The system consisted of: 4 % (w/v) zein in solvent; 75 % (v/v) ethanol in solvent; 1 % (w/v) sodium caseinate concentration in the antisolvent; and, mixing ratio of solvent-to-antisolvent = 1-to-5.