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STUDY OF THE PROPERTIES AND COLLOIDAL STABILITY FOR THE TECHNOLOGICAL APPLICATION OF ZEIN-BASED NANOSPHERES

ESTUDIO DE LAS PROPIEDADES Y ESTABILIDAD COLOIDAL PARA LA APLICACIÓN TECNOLÓGICA DE NANOESFERAS DE ZEÍNA

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Abstract

Nanospheres of α-zein fabricated by antisolvent precipitation are promising carriers for hydrophobic bioactives in foods. The objective of this research was to study the changes in the properties of zein nanoparticles (size, ζ-potential, PDI, loading efficiency and pH colloidal stability) by using different copolymer coatings (sodium caseinate and pectin), ethanol concentrations (75 to 95% v/v) and solvent/antisolven or zein/krill oil ratios. Smallest particles (< 200 nm) can be obtained with the use of sodium caseinate as coating. This is the first time that zein nanospheres are used as encapsulating agents for a krill oil extract. These nanoparticles with a ζ-potential value around -25 mV are stable at neutral to basic pH values based on their ζ-potential and visual analysis. The use of pectin as coating produced the largest particles (< 400 nm) indicating that an increase in hydrophobic interactions allows the formation of bigger assemblies. Pectin-coated nanoparticles increased the LE of krill oil in about 9% compared with non-coated particles. Moreover, the krill oil/zein ratio was found to be a suitable way to increase the LE of the nanoparticles. The encapsulation of krill oil within the zein nanoparicles improved the antioxidant activity of the particles (non-coated and casein-coated particles). All types of nanoparticles were capable to nano-entrap appreciable amounts of a hydrophobic krill oil extract.

Keywords: α-zein, krill oil, nanospheres, colloidal stability.

Resumen

Las nanoesferas de α-zeína fabricadas por precipitación antisolvente son potenciales acarreadores de bioactivos poco solubles en la industria de alimentos. El objetivo de esta investigación fue estudiar los cambios en las propiedades de las nanopartículas de zeína (tamaño, potencial, PDI, eficiencia de encapsulación y estabilidad coloidal frente a pH) mediante el uso de diferentes copolímeros estabilizantes (caseinato de sodio y pectina), concentraciones de etanol (75 a 95 % v / v) y variadas procporciones solvente/antisisolven o aceite de zeína/krill. Las nanopartículas más pequeñas (<200 nm) se pueden obtener con el uso de caseinato de sodio como recubrimiento. Esta es la primera vez que se utilizan nanoesferas de zeína como agentes de encapsulación para un extracto de aceite de krill. Estas nanopartículas con un valor de potencial zeta de alrededor de -25 mV son estables a valores de pH neutro a básico. El uso de pectina como recubrimiento produjo las partículas más grandes (> 400 nm) lo que indica que un aumento en las interacciones hidrófobas permite la formación de partículas más grandes. Las nanopartículas recubiertas con pectina aumentaron la eficiencia de encapsulación del aceite de kril en aproximadamente el 9% en comparación con las partículas no recubiertas. Además, se encontró que la proporción de aceite de kril/zeína era una forma adecuada de aumentar la eficiencia de encapsulación de las nanopartículas. La encapsulación de aceite de krill aumentó la actividad antioxidante de las nanopartículas (no cubiertas y recubiertas con caseinato de sodio. Todos los tipos de nanopartículas fueron capaces de nano-atrapar cantidades apreciables de un extracto de aceite de krill.

Palabras clave: α-zeína, aceite de krill, nanoesferas, estabilidad coloidal.

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1 Introduction

Delivery systems can be fabricated from various food-grade materials. Among the many types of biopolymer molecules that exist in nature, core-shell nanoparticles can be assembled from proteins and/or polysaccharides using various bottom-up and top-down methods (Matalanis, Jones, & McClements, 2011). These methods include solvent extraction-evaporation (Freitas, Merkle, & Gander, 2005), coacervation/phase separation (Reza, 1990), and liquid antisolvent-precipitation (Joye, Davidov-Pardo, & McClements, 2015). Liquid antisolvent-precipitation was used to fabricate protein-based nanoparticles to encapsulate and protect a variety of bioactive compounds (Parris, Cooke, & Hicks, 2005).

Proteins used as nanoparticle-based materials include gelatin, whey proteins, soy proteins, and corn proteins (Lakkis, 2007). Zein, the prolamin of corn, is a mixture of alcohol-soluble proteins. α-zein is the most abundant fraction, amounting to 70-85% of the total zein. All zein fractions are amphiphilic polymers due to their particular amino acid sequence (Shukla & Cheryan, 2001). The overall amino acid composition of zein reveals a high content of hydrophobic amino acids including leucine, alanine, and proline, which are also related to antioxidant activity. Owing to its amphiphilic character, zein molecules are able to self-assemble into various microstructures, including films, rods, microparticles, and spheres or nanoparticles (Y. Wang & Padua, 2010).

Zein-based structures obtained by antisolvent precipitation have already been used for the encapsulation and controlled delivery of flavors, micronutrients, and bioactive compounds with improvements in their stability, water-dispersibility and release in gastrointestinal conditions (Parris *et al*., 2005). Additionally, when zein is assembled into particles such as microspheres, the protein may also serve as an enterocoating agent and protect bioactive components from stomach acid (Suzuki *et al*., 1989).

To study the ability of zein nanoparticles to entrap and protect bioactive compounds, especially hydrophobic molecules, essential oils have been incorporated into zein particles. The particles were subjected to analysis to elucidate the effect of nanoparticles on the antioxidant properties of essential oils (Wu, Luo, & Wang, 2012). The authors concluded that entrapping essential oils in zein nanoparticles could increase their solubility without affecting their antioxidant ability, thus furthering the use of nanodelivery systems in foods.

Recently, krill oil has gained importance as dietary supplement due to its biological functions as a nutraceutical for the prevention and treatment of various diseases (Massrieh, 2008). Krill oil is considered a rich source of eicosapentaenoic acid (EPA, C20:ω-3) and docosahexaenoic acid (DHA, C22:6ω-3) (Castro-Gómez, Holgado, Rodríguez-Alcalá, Montero, & Fontecha, 2015). A distinctive property of krill oil is that EPA and DHA fatty acids are almost exclusively bound to phospholipids such as phosphatidylcholine. Esterification of EPA/DHA to phospholipids seems to improve their bioefficacy and bioavailability (Wijendran *et al*., 2002). Another important distinction between fish and krill oil is that the last one contains astaxanthin, which is a carotenoid pigment considered as a nutraceutical, as well as a general enhancer of immune responses (Guerin, Huntley, & Olaizola, 2003). It has been reported that astaxanthin is more effective than β-carotene and lutein at preventing UV light photooxidation of lipids (Santocono, Zurria, Berrettini, Fedeli, & Falcioni, 2006) and has stronger antioxidant activity than vitamin E (Miki, 1991). Therefore, EPA/DHA may be protected against oxidation, but the overall benefits of krill may be reduced as a result of the loss of antioxidant capacity (Choubert, Dentella, Atgié, & Baccaunaud, 2005). In addition, the low water solubility of krill oil has limited its technological applications. In past years, several studies have been performed to improve the solubility or stability of krill oil and astaxanthin such as microencapsulation into chitosan matrix (Higuera-Ciapara, Felix-Valenzuela, Goycoolea, & Argüelles-Monal, 2004), encapsulation into polymeric nanospheres (Tachaprutinun, Udomsup, Luadthong, & Wanichwecharungruang, 2009), incorporation into emulsions, and complexation with SIMBOLO-cyclodextrin (Shi, Beamer, Yang, & Jaczynski, 2018). Moreover, zein has been used for the encapsulation and controlled delivery of flavors, micronutrients, and bioactive compounds (Patel, Bouwens, & Velikov, 2010; Zhong & Jin, 2009), and can be used as an encapsulation alternative to other encapsulation methods or for novel compounds (Avendaño-Morales, 2017; Enciso-Sáenz, 2018; Fuentes-Ortega, 2017; Pascual-Pineda, 2018). Encapsulated products have been prepared by several methods based on zein's ability to self-assemble when the solution polarity changes toward a more hydrophilic environment. Upon solvent evaporation zein capsules

or nanostructures can be useful in the snacks, confectionery, baking, and prepared food industries. For the beverage industry, their dispersion in water can be facilitated by the use of stabilizers such as sodium caseinate and chitosan.

The aim of the present research was to study the best conditions for the formation of stable protein-copolymer particles for the nanoencapsulation of hydrophobic nutraceuticals (krill oil). The nanoparticles are based on a fraction of hydrophobic zein proteins (α-zein), capable of binding hydrophobic molecules, and two different hydrophilic copolymers: sodium caseinate and pectin. The approach for the fabrication of these nanoparticles involves liquid antisolven precipitation. Biopolymeric nanoparticles can be fabricated using antisolvent precipitation by decreasing the quality of the solvent in which the biopolymers are dissolved. This can be achieved by the addition of a non-solvent to induce supersaturation of the biopolymer, thus providing the driving force for nucleation and subsequent particle growth (Joye *et al*., 2015). Additional components, such as copolymers or surfactants, can be dissolved in the antisolvent phase to control particle formation and stability. Furthermore, functional ingredients, such as bioactive compounds, that co-precipitate with the biopolymer can be encapsulated by incorporating them in the original solvent.

2 Methods and materials

2.1 Materials

Alpha-Zein, pectin, and low molecular weight chitosan were purchased from Sigma-Aldrich (St. Louis, MO). Sodium caseinate (P 86%) was obtained from AMCO Proteins (Burlington, NJ). Krill oil was obtained from Nano-nutrition (Naucalpan Edo. de Mexico, Mexico). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO); they were analytical grade and used as received.

2.2 Preparation of non-loaded zein nanoparticles

Nanoparticles were fabricated following the procedure described by Joye *et al*., (2015) with some modifications. Briefly, 6.0 g of α-zein were dissolved in 95% (v/v) ethanol (solvent solution). The protein solution was subsequently added to the antisolvent solution (phosphate buffer 20 mM, pH 8.0) at either 1:5 or 1:3 (solvent/antisolvent) ratios and a flow rate of 1 mL/min under continuous stirring (1000 rpm) at room temperature. After addition of the solvent into the antisolvent solution, the particle suspension was stirred for additional 10 min. Ethanol was removed from the mixture using a rotary evaporator at 40 ºC (Buchi RE 111, Flawil, Switzerland). For the production of coated particles, the titration of the solvent solution was carried out in the antisolvent phase containing sodium caseinate (1% w/v) or pectin (0.1% w/v). The pH of the antisolvent phase containing the copolymers was adjusted to 4.8 for pectin, while the pH of the sodium caseinate was fixed to 6.5.

2.3 Preparation of krill oil-loaded zein nanospheres

For the production of loaded nanoparticles, different concentrations of krill oil (1.0, 1.5, and 3.0% w/v final concentration) were added to the solvent phase (i.e., the initial zein solution) and the solution was stirred for 20 min. Our initial studies indicated that the krill oil sample had a limited solubility in aqueous ethanol. In 95% v/v aqueous ethanol, the krill oil could be dissolved up to approximately 10% w/v, measured by visual inspection of transparent solutions. For the optimized nanoparticle systems, the solvent solution was titrated into the antisolvent solution containing the hydrophilic copolymers (coatings) as previously described. The entire process took a few minutes and the suspension of the nanoparticles was kept at 4 ºC to further analysis.

2.4 Characterization of krill oil-loaded nanospheres

2.4.1 Particle size

The particle size distribution of the α-zein nanoparticles was measured using dynamic light scattering (DLS) instrument (Zetasizer Nano-ZS, Malvern Instruments Ltd., Malvern, UK). Biopolymer particle suspensions were diluted 1:100 with deionized water immediately before analysis. The deionized water had been filtered using a 0.45μm membrane prior measurements to remove any particulate contaminates. Samples were paced in the measurement cell and equilibrated to 25 ºC. A refractive index of 1.335 and viscosity of 0.8872 mPas was used for the aqueous phase properties in the

calculation of the particle size, which were calculated automatically by the instrument software assuming the aqueous phase was pure water.

2.4.2 *ζ-potential*

Particle electrical characteristics (ζ-potential) were carried out using a particle electrophoresis instrument (Zetasizer Nano-ZS, Malvern Instruments Ltd., Malvern, UK). Samples were diluted 1:100 with deionized water adjusted to the pH of the suspension being analyzed.

2.4.3 *SEM studies*

The morphology of the zein particles was analyzed using scanning electron microscopy (DSM-940, Zeiss, Oberkochen, Germany) at a voltage of 20 kV. An aqueous dispersion of the biopolymer nanoparticles was diluted 10 times with pH-adjusted distilled water, and then a small aliquot (10 μL) was drop-casted onto a carbon-coated surface. The sample was then air-dried at room temperature before loading into microscope.

2.4.4 *Determination of the total krill oil content by UV analyses*

The total krill oil content (in equivalents of astaxanthin) in the zein nanoparticles was determined using a UV-visible spectrophotometric method (UV-1800 Shimadzu uv spectrophotometer, Shimadzu Scientific Instruments, Kyoto, Japan). The oil was quantitatively extracted from the zein nanoparticles using acetone without interference from zein aggregates, which were not soluble in acetone. Briefly, an aliquot of the biopolymer particles suspension was diluted 1:20 with acetone and the amount of krill oil released from the particles was determined by measuring the absorbance at 477 nm (wavelength of the maximum absorbance of astaxanthin in acetone) with the value of 2198 for the specific optical extinction coefficient ($E^{1\%}_{1cm}$) (Chen & Meyers, 1984) using eq (1) (Davies, 1976):

$$x = \frac{A_y}{E^{1\%}_{1cm}} \times 100 \tag{1}$$

where x is the amount of carotenoid astaxanthin (g), y is the amount of solvent (mL) and A is the absorbance.

2.4.5 *Stability against pH*

The nanoparticle size and electrical characteristics of the colloidal dispersions were determined after they were exposed to various pH values. The effect of pH on the stability of the non-loaded and loaded zein nanoparticles was determined by mixing equal volumes of 20 mM buffer with pH values ranging from 3.0 to 9.0 with freshly prepared nanoparticle suspensions. The samples (when needed) were then adjusted to the desired pH values with 0.1 M HCl or NaOH.

2.4.6 *Chemical stability of astaxanthin-rich extract: Antioxidant activity*

The free radical scavenging capacity of the extracts was determined by the DPPH assay as previously described (Simirgiotis & Schmeda-Hirschmann, 2010). DPPH radical absorbs at 515 nm, but upon reduction by an antioxidant compound its absorption decreases. Briefly, 50 uL of nanoparticles suspension (or astaxanthin-rich extract) was added to 1950 uL of fresh 0.1 mM solution of DPPH in methanol and allowed to react at 25 C in the dark. After 30 minutes the absorbance was measured at 515 nm. The DPPH scavenging ability as percentage was calculated as:

$$DPPH \;\; scanvenging \;\; capacity = \left(1 - \frac{A_{sample}}{A_{control}}\right) \times 100 \tag{2}$$

where, A_{sample} is the absorbance of the sample (inhibited absorbance) and $A_{control}$ is the absorbance of the control (non-inhibited absorbance). Trolox (from 20 to 100 μM, R= 0.987) was used as standard antioxidant compound. The results were expressed as percentage of antioxidant activity.

2.4.7 *Statistical analysis*

All measurements were performed on the three samples at least three times. Means and standard deviations were calculated from these values using Excel Microsoft (Redmond, WA, USA).

3 Results and discussion

The aim of this research was to study the potential use of zein nanospheres formed by antisolvent precipitation method for encapsulation and protection of krill oil (an important hydrophobic nutraceutical).

3.1 Effect of fabrication parameters on the formation of zein nanospheres

The objective of these experiments was to identify the preparation conditions where relatively small (< 200 nm) zein nanoparticles could be formed. Initially, the influence of solvent/antisolvent ratio, type of stabilizing agent (protein or polysaccharide), ethanol (solvent) concentration, and, zein/krill oil ratio on the dimensions, polydispersity index, zeta potential and loading efficiency (in terms of astaxanthin content) of the zein-based nanospheres were evaluated.

i) *Effect of solvent/antisolvent ratio on physical properties (size and z-potential) of zein-based nanospheres*

Zein nanoparticles were produced by dissolving zein (6% w/v) in 95% aqueous ethanol (solvent solution) and then titrating this solution into an aqueous medium (antisolvent solution) to precipitate the colloid. The fabrication of the particles included an evaporation step to eliminate the ethanol used during the particle synthesis. The solvent/antisolvent ratios evaluated were 1:3 and 1:5 and, to prevent particle aggregation, sodium caseinate and pectin were added as electrostatic and steric stabilizers (coatings), respectively. Despite similar fabrication process, the zein particles formed from 1:5 solvent/antisolvent ratio had a mean diameter 10 times smaller than the particles formed from 1:3 solvent/antisolvent proportion (see Table 1). This behavior could be observed for both, the non-coated and the coated particles. These results suggested that the average particle size could be controlled through the solvent system, because the particle fabrication process involves the dilution and/or evaporation of binary or ternary solvents which changes the composition and polarity of the solution, and drives the self-assembly of solutes. During the formation of particles, zein molecules self-assembled into spherical particles upon titration of solvent solution into antisolvent system, mainly due to hydrophobic protein-to-protein interactions induced by ethanol dilution or evaporation, which rendered the remaining solvent increasingly hydrophilic, thus promoting hydrophobic self-assembly. Amphiphilicity is the main driving force for self-assembly (Löwik & van Hest, 2004), the spontaneous formation of organized phases from disordered ones. It is mediated by weak interactions (i.e., van der Waals, capillary, hydrogen bonds) rather than covalent or ionic bonds. The smaller size of zein particles formed from 1:5 solvent/antisolvent ratio can be (partially) explained by the higher hydrophobicity of this protein and, hence, its lower water solubility. A lower water solubility leads to a higher degree of supersaturation, which increases the nucleation rate and, hence, promotes the formation of more and smaller protein nanoparticles (Joye *et al*., 2015). The production of non-coated and coated zein nanoparticles has also been described in earlier reports (Luo, Zhang, Whent, Yu, & Wang, 2011). The diameter of the particles produced in this study was in agreement with these authors (from 120 to 900 nm). After fabrication, the ζ-potential of zein nanoparticles was between -40 and -45 mV (at pH 8.0) for the three nanoparticles samples (non-coated, and coated with caseinate and pectin), which are in agreement with previously reported values (Davidov-Pardo, Joye, & McClements, 2015; Joye *et al*., 2015). Based on these results, 1:5 solvent/antisolvent ratio was used in further studies carried out in this work.

ii) *Effect of ethanol concentration in solvent solution and zein/krill oil ratio on physical properties (size, ζ-potential and astaxanthin content) of zein-based nanospheres*

α-zein does not dissolve in anhydrous ethanol or pure water. It can be dissolved in binary solvents including mixtures of alcohol and water (Manley & Evans, 1943). Zein is normally dissolved in 60-95% aqueous ethanol.

Table 1. Effect of solvent/antisolvent ratio on the size and ζ-potential of zein nanospheres.

Solvent/antisolvent ratio	Stabilizing agent	Diameter (nm)	PDI	ζ-Potential (mV)
1:03	Non-coated NPs	3764 ± 272.5	0.99 ± 0.023	-41.97 ± 0.603
	Caseinate	247.5 ± 2.160	0.196 ± 0.00152	-42.23 ± 1.95
	Pectin	6073 ± 630.1	1	-40.03 ± 1.331
1:5	Non-coated NPs	340.23 ± 3.52	0.264 ± 0.007	-39.7 ± 0.435
	Caseinate	294.5 ± 4.25	0.307 ± 0.015	-46.73 ± 0.513
	Pectin	275.17 ± 7.76	0.265 ± 0.029	-44.4 ± 0.2

Table 2. Effect of ethanol concentration (solvent solution) on the size, ζ-potential and loading efficiency of zein nanospheres loaded with 3% krill oil.

Solvent solution concentration (% w/v)	Stabilizing agent	Mean Particle Diameter (nm)	PDI	ζ-Potential (mV)	Loading efficiency (%)
80	Caseinate	203.90 ± 2.65	0.27 ± 0.01	-53.50 ± 1.31	75.28 ± 1.81
85		197.60 ± 2.41	0.16 ± 0.01	-51.30 ± 1.20	90.52 ± 1.89
90		190.00 ± 1.31	0.25 ± 0.03	-56.63 ± 1.01	93.18 ± 3.44
95		175.80 ± 2.50	0.17 ± 0.03	-50.80 ± 0.82	89.08 ± 1.39

In order to find the best solubilization capability of zein solutions, the effect of ethanol concentration (in solvent solution) on the final properties of zein-based nanoparticles was analyzed. 6% w/v of zein in aqueous ethanol solutions (80, 85, 90 and 95% v/v) were used to assembly the nanoparticles by the mentioned precipitation-antisolvent method (Joye *et al*., 2015). All the experiments were carried out with 0.1% (w/v) sodium caseinate as electrosteric stabilizer because casein-coated nanoparticles showed a consistent small particle size (Davidov-Pardo *et al*., 2015).

The experimental results are shown in Table 2. The data show a decrease in the mean particle diameter as the ethanol concentration increases from 80 to 95% v/v. These measurements indicated a mean particle diameter of ca. 200 and 175 nm at 80 and 95% v/v ethanol content, respectively. However, the ζ-potential remained around -50 mV for all the four aqueous ethanol concentrations used. Moreover, the astaxanthin content (loading efficiency) augmented from ca. 75 to 90% as ethanol increased from 80 to 90% v/v. However, there was no increase in the loading efficiency above 90% v/v ethanol. These results indicate that the ethanol content in the initial zein solution affects both the size and loading efficiency of zein nanoparticles.

As is clearly shown in Table 2, there is a decrease in the size of nanoparticles at ethanol content greater than 90%. On the other hand, as the ethanol concentration in the solvent solution was increased from 80 to 95%, the zein solutions became less turbid (data not shown), indicating that the zein aggregates formed colloidal suspensions with smaller particle size at higher ethanol content. This led to the formation of smaller particles when the solvent solution was titrated into the antisolvent solution. This can be somewhat explained by the fact that during particle formation ethanol evaporates faster than water whereby the ethanol content within the antisolvent solution decreases faster than the water content.

As shown in Table 2, this change in the quality of the solvent induces a higher degree of nucleation and the subsequent particle formation. In all cases monomodal size distributions with small polydispersity index were obtained, suggesting that the balance between zein-zein interactions and zein-solvent interactions allowed zein to form thermodynamically stable particles around 180 nm. This behavior of zein aggregates can be explained by a structural inversion at a given solvent composition. In other words, the amphiphilic nature of zein allows the zein molecules to form a macromolecular micelle (globular aggregates with the hydrophilic moiety exposed to the surface and hydrophobic moieties clumped together) in the solution (Kim & Xu, 2008). As the solvent medium turns hydrophobic, the orientation of each molecule will be reversed. In other words, micellar inversion is expected depending on the condition of the solvent medium (Q. Wang, Geil, & Padua, 2004). Govor *et al*. (2009), proposed that the microstructure of polymer aggregates formed during evaporation of binary solutions can be controlled by varying the concentrations of solutes, the type of solvents, and the volume ratio of solvents.

The fabrication of krill oil-loaded nanoparticles by the antisolvent-precipitation method was found to be an effective and rapid approach to entrap hydrophobic compounds. This method relies on the decreased solubility of zein when the ethanol/water ratio of the solvent is lowered. This reduced ethanol concentration causes the precipitation of zein that encloses krill oil *in situ*. This process continues until the formation of solid particles composed of a continuous zein matrix with interdispersed krill oil. Because the principal components in krill oil are EPA and DHA, as well as the carotenoid astaxanthin, this carotenoid was used to determine the encapsulation efficiency due to its simple determination by a spectrophotometric method (λ = 477 nm) (Nobre *et al*., 2006). Typically, the yield of encapsulation efficiency ranged between 75 and 90% (Table 3, see Supplementary data).

Table 3. Effect of krill oil/zein ratio on the size, ζ-potential and encapsulation efficiency of zein nanospheres.

Krill oil/zein ratio	Stabilizing agent	Mean Particle Diameter (nm)	PDI	ζ-Potential (mV)	Loading efficiency (%)
1:02		189.20 ± 2.21	0.23 ± 0.002	-42.33 ± 1.27	89.92 ± 1.34
1:04	Caseinate	150 ± 2.51	0.176 ± 0.02	-36.6 ± 3.12	75.71 ± 9.20
1:06		223.80 ± 12.02	0.41 ± 0.016	- 42.33 ± 1.30	80.80 ± 4.06

The higher encapsulation efficiency (89.9% ± 1.3%) was reached using 1:2 krill oil-to-zein ratio, while 75.71 ± 9.20 and 80.80 ± 4.06 were obtained from 1:4 and 1:6 ratios, respectively. As for zein, this renewable biopolymer has been studied extensively as material for biodegradable films with good barrier properties against moisture, oxygen and carbon dioxide permeation (Rakotonirainy & Padua, 2001). The barrier properties of zein are attractive for encapsulation of krill oil, which can be easily degraded at environmental conditions during food processing and storage. The antisolvent precipitation method provides an alternative process for the application of zein as a carrier for bioactive oil-based compounds.

For example, DLS measurements of nanoparticles suspension (Table 3, see Supplementary data) indicated that the particles had a smaller mean diameter when the krill oil/zein ratio used was 1:4 (150 ± 2.51 nm). Levels of krill oil under and above this proportion yielded larger particles, around 190 and 224 nm for 1:2 and 1:6 krill oil/zein ratios, respectively. Moreover, DLS data showed a difference in mean particle size between non-loaded and loaded particles (Table 1 and 3), indicating a decrease in particle size when hydrophobic compounds (to be encapsulated) interacted with α-zein before the formation of nanospheres. These results indicated that the average particle size could be controlled through the solvent system and the ratio of zein to krill oil. After de production of zein nanospheres, the ζ-potential was about -40 mV (pH 8.0), which is in agreement with previously reported values (Joye *et al*., 2015).

3.2 Effect of different electrosteric stabilizing agents on the stability against pH of α-zein nanospheres

3.2.1 Self-assembly of coated nanospheres

Zein nanospheres can be highly susceptible to aggregation when they are prepared in the absence of a stabilizer, effect that can be attributed to hydrophobic attraction between non-polar patches on the particles surface. Besides, the stability of non-coated zein particles is often limited due to their sensitivity to environmental conditions, such as changes in pH. For these reasons, copolymers are often used to improve the stability of protein particles. Copolymers such as proteins and polysaccharides (Joye *et al*., 2015; Luo *et al*., 2011), have been reported to increase the loading capacity, stability, and redispersibility of the particles. In the first part of this study, sodium caseinate and pectin were used as copolymers for non-loaded and loaded zein particles. Sodium caseinate and pectin were chosen as an example of widely used food ingredients.

Different methods can be used to associate these copolymers to the nanoparticles such as electrostatic deposition after particle formation. In this study, the copolymers are dissolved in the antisolvent (water or buffer) prior to particle assembly, was used.

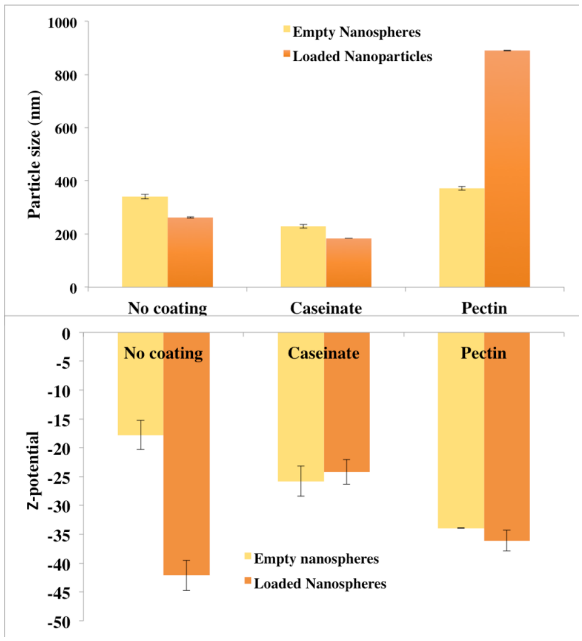

Fig. 1. Particle size (A) and zeta-potential (B) of zein nanospheres prepared with different electrostatic stabilizers (coatings): 1% (w/v) sodium caseinate and 0.1% (w/v) pectin.

Initially, the influence of a copolymer coating on the physicochemical properties of the zein nanoparticles was investigated. The influence of sodium caseinate and pectin on the dimensions and stability of the biopolymer particles was therefore measured using dynamic light scattering (Fig. 1).

i) *Caseinate-coated nanospheres*

In the absence of a stabilizing copolymer, the mean diameter of the zein particles was around 400 and 250 nm for non-loaded and loaded zein nanoparticles, respectively. And the suspension of particles had a milky appearance confirming that colloidal particles were formed. When zein nanoparticles were mixed with sodium caseinate (1%w/v) smaller nanoparticles were formed for non-loaded as well as loaded particles. The zeta-potential of the non-coated nanoparticles (pH 8.0) went from around -20 (non-loaded particles) to -40 mV (loaded particles). In the case of caseinate-coated particles, the zeta potential was about -20mV.

i) *Pectin-coated nanospheres*

When pectin (0.1% w/v) was added as copolymer, the mean particle diameter for pectin-coated particles increased from 400 nm for non-loaded to 900 nm for loaded particles. Moreover, pectin-coated (non-loaded and loaded) particles had an average surface charge of ca. -40mV. These results indicated a clear effect of the load on the surface characteristics of the particles.

It is possible that the structure of the composite biopolymer particles is highly dependent on the kinetics of two competing processes that occur during particle formation: precipitation of hydrophobic zein, and association of the hydrophilic copolymer with the hydrophobic protein (Davidov-Pardo *et al*., 2015). In this case, the hydrophobic protein would form the core, and the hydrophilic biopolymer the shell. On the other hand, if particle formation occurs more slowly than the association of the hydrophobic protein core, it is expected a mixed internal structure to be formed. In this case, the hydrophobic protein and hydrophilic copolymer would be homogeneous distributed throughout the particle. Thus, the core-shell structure is the result of rapid precipitation of zein followed by a layer of copolymer on the particle surfaces. In this research, it is proposed that the particles formed are more likely to have a protein core covered by the copolymer.

i) *Effect of pH on the particle stability*

The production of the mixed biopolymer particles was carried out at different pH values because of the different charge and stability characteristics of the hydrophilic copolymers. The biopolymer particles containing sodium caseinate (pI ≈ 4.6) were produced at pH 6.5 because caseinate have a negative charge at this pH (an extensive aggregation occurred around pH 4.5). The particles containing pectin were prepared at pH 4.8. At this pH, the hydrophobic protein nanospheres presented a positively charge (Fig. 2) and so the interaction with the negatively charged pectin would be mainly driven through electrostatic attraction.

The effect of sodium caseinate and pectin on particle size of non-loaded and loaded zein nanoparticles can be seen in Fig. 2. As can be observed in Fig. 2A & 2B, the particle size of zein nanoparticles decreased when krill oil was encapsulated within the particle matrix. The results showed that empty nanoparticles had a larger particle size in a range between pH 4.0 and 6.0 for non-coated and pectin-coated nanospheres, whereas sodium caseinate showed a uniform size over the entire pH range.

As mentioned above, the particle size of krill oil/zein particles decreased when compared with the empty ones. Extensive particle aggregation could be observed when the low net charge on the particles reduced the electrostatic repulsion between them, i.e. in proximity to their pI.

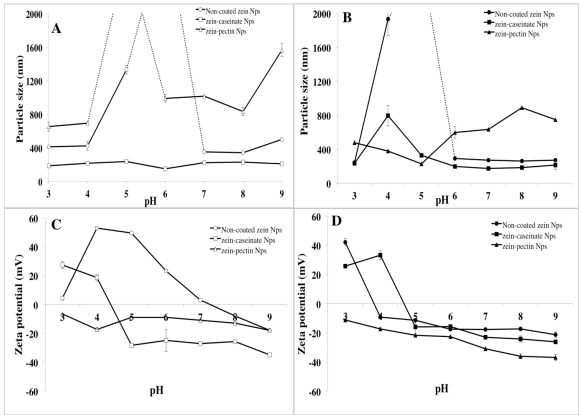

Fig. 2. pH stability: Particle size and zeta-potential of empty (A and C) and krill oil-loaded (B and D) zein nanoparticles (NPs). The concentration of α-zein and krill oil used to prepare the nanospheres was 6 and 3% (w/v), respectively.

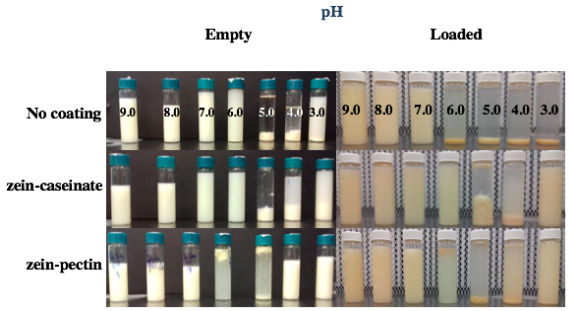

Fig. 3. Effect of pH on the stability of zein nanoparticles: visual inspection of dispersions of empty and loaded zein nanoparticles. The protein nanoparticles were loaded with krill oil [3% (w/v)]. Caseinate and pectin (electrosteric stabilizers) concentrations in the antisolvent solutions were 1 and 0.5% (w/v), respectively. The pH was adjusted with HCl and NaOH.

At adequate biopolymer (coatings) concentrations, the particles surfaces are completely covered with a layer of coating molecules, leading to the formation of coated zein nanoparticles. These coated particles would be stabilized against aggregation by a combination of electrostatic and steric repulsion due to their biopolymer coating.

Functional ingredients can be incorporated in a range of food products with widely different pH conditions. Moreover, the pH of a food product often changes during production, storage, or after consumption. In this regard, assessing the pH stability of the nanoparticles is important to predict their performance in commercial food products. Previously, it has been reported that a minimal ζ-potential is needed to ensure good particle stability through electrostatic repulsion (Arroyo-Maya & McClements, 2015). In this study the isoelectric point of the non-coated (and non-loaded) zein particles was around pH 7, while pectin-coated zein particles showed isoelectric point around 4.4 (Fig. 2C & 2D). Extensive aggregation of non-coated zein particles occurred between pH 3.0-6.0 (Fig. 2 & 3). Particle aggregation may have occurred over this pH range because the electrical charge on the particles was relatively low, which only led to a weak electrostatic repulsion between the particles. The presence of electrosteric stabilizers reduced the amount of aggregation of the zein nanoparticles at pH values from around 6.0 to 9.0, although a greater amount of aggregation occurred at lower pH values (Fig. 3).

The improvement in zein particle stability in the presence of electrosteric stabilizers at high pH values can be attributed to the copolymer outer layer that would have increased the electrostatic repulsion between the nanospheres. The increased aggregation of the coated nanoparticles at low pH values can be attributed to the reduction in electrical charge on the coated zein particles, which would lead to a reduced electrostatic repulsion between them.

Loaded zein particles were destabilized at pH values lower than pH 6.0 (Fig. 3), which can be attributed to the large decrease in the magnitude of the electrical charge on the particles near the isoelectric point. Coating the zein nanoparticles with caseinate and pectin greatly improved their stability to aggregations at low pH (< 3.5) and higher pH (> 6.0) conditions, which can be attributed to an increase in the electrostatic and steric repulsion between the particles in the presence of a copolymer coating.

3.3 Loading efficiency of krill oil

An important attribute of any delivery system is the fraction of active compound that is actually entrapped within the particles. As previously described, three different concentration of krill oil extract (1.0, 1.5 and 3.0% w/v) were added to the zein solutions prior particle formation to determine the maximum amount of krill oil to be entrapped into the protein nanoparticles. The final krill oil-to-protein ratio used was 1:2 (final concentration of 3.0% w/v). The loading efficiency (LE) of krill oil in copolymer-coated zein nanoparticles was relatively high and remained constant (between 60 and 80%) for the different copolymers (Fig. 4B). For this study, it could be seen that the solubility of krill oil in ethanolic solutions is low.

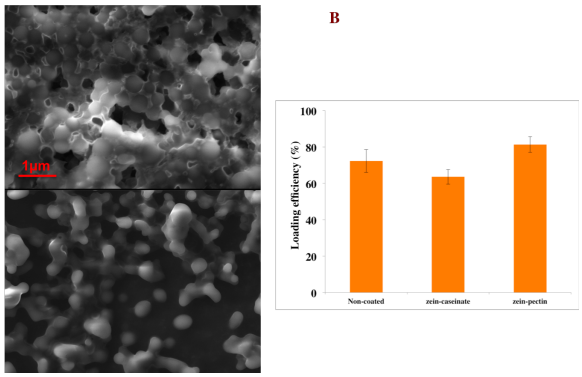

Fig. 4. SEM images of empty zein nanoparticles coated with pectin (A); Impact of biopolymer coating on the loading efficiency of krill oil-loaded zein nanoparticles (B).

However, the solubility of krill oil was improved in presence of zein. The fact that zein improves the krill oil solubility can be related to the hydrophobicity of the protein. It is well established that one of the main driven forces for binding of hydrophobic compounds to proteins is hydrophobic attraction (Acharya, Sanguansri, & Augustin, 2013). It is well known that the primary structure of zein contains more hydrophobic amino acids than other proteins (Esen, 1990). The higher hydrophobicity of zein is also reflected in the high percentage of krill oil encapsulation efficiency.

Coating with pectin increased the LE of krill oil in about 9% compared with non-coated particles (Fig. 4B), and in the case of caseinate, the LE increased was slightly reduced. The increase in LE for pectin-coated particles could be explained by the large size of mixed biopolymer particles resulting in more space for krill oil to be entrapped. The addition of krill oil did not affect the diameter of the caseinate-coated zein nanoparticles, but it did cause an appreciable increase in the diameter of the pectin-coated particles (Fig. 1). An increase in the amount of hydrophobic material on the surface of the particles could lead to an increase in the hydrophobic attraction between particles, which would lead to an increase in particle size (Zou, Li, Percival, Bonard, & Gu, 2012).

3.4 Influence of encapsulation in astaxanthin antioxidant activity

The antioxidant activity of astaxanthin (one of the main components of the krill oil) was monitored by its ability to scavenge the free radical DPPH. This radical absorbs at 515 nm, but upon reduction by an antioxidant compound its absorption decreases. The ability of natural zein to have antioxidant properties should be considered in this assay. Due to its molecular characteristics (highly saturated molecule) astaxanthin is easily degraded by heat, light and oxygen during the manufacturing and storage (Christophersen, Jun, Jorgensen, & Skibsted, 1991). Empty particles exhibited minimal antioxidant activity (except for zein/pectin particles) compared with loaded particles (Fig. 5). In the presence of krill oil, the better performance of the non-coated and casein-coated particles could result from a combination of several factors, i.e., the higher EE, the stabilization of astaxanthin within the particles, and the presence of a protective caseinate-coating which could conferred some extra protection against environmental degradation.

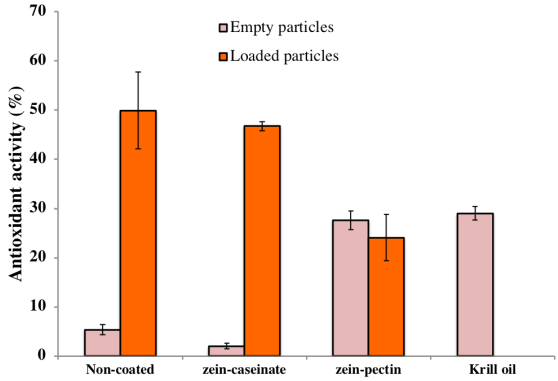

Fig. 5. Antioxidant activity of krill oil-loaded zein nanospheres.

However, the particles with higher EE showed moderate antioxidant activity, as is the case of pectin-coated zein nanoparticles. It could be possible that pectin formed a thick layer (due to the significantly higher pectin content 0.1% w/v of this sample) around the particles avoiding the accurate determination of antioxidant capacity. In general, the hydrophobic interactions of pectin with hydrophobic/lipophilic compounds are know to increase with the degree of esterification of pectin molecules (by increasing its hydrophobicity) (Dongowski, 1997) and the molecular weight (viscosity) (Pfeffer, Doner, Hoagland, & McDonald, 1981) of the pectin molecules. The effect of pectin coating can be attributed to the fact that the surfaces of the nanoparticles were saturated with polysaccharide molecules forming an electrosteric barrier against degradation, and also leading to a hidden antioxidant capacity. The data obtained in this study revealed that the encapsulation of krill oil within zein particles could be an adequate strategy to protect these compounds. Additionally, these results clearly showed that the type of copolymer added to coat the particles has an influence on the determination of the antioxidant capacity.

Conclusions

The use of different assembly parameters in the fabrication process resulted in the formation of zein nanospheres with a wide variety of properties (i.e., mean particle diameter, ζ-potential, PDI, and pH stability). The smallest particles (< 200 nm) were obtained using a solvent/antisolvent ratio of 1:5, an ethanol concentration of 95% (v/v) and with sodium caseinate as electrosteric stabilizer. The smallest

nanoparticles were also the most stable according to ζ-potential and visual analysis. On the other hand, the use of pectin produced the largest particles, indicating that an increase in hydrophobic interactions allows the formation of bigger assemblies. Pectin-coated nanoparticles increased the LE of krill oil in about 9% compared with non-coated particles. Moreover, the krill oil/zein ratio was found to be a suitable way to increase the LE (the particles were able to nano-entrap appreciable amounts of a hydrophobic krill oil extract from ≈ 60 to 90%). The improved antioxidant activity of some nanoparticles (non-coated and casein-coated) suggested that these nanoparticles could thus be used to protect and deliver easily oxidizable compounds such as bioactive lipids. In summary, these results suggest that the zein nanoparticles obtained in this study were particularly suitable for encapsulation and protection of hydrophobic compounds such as krill oil. Zein nanospheres were physically unstable at acidic conditions, so stabilizing coatings that better perform at low pH range might be necessary if nanoparticles are to be used in acidic food systems.

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