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# $\alpha$-Zein nanoparticles as delivery systems for hydrophobic compounds: Effect of assembly parameters

# Nanopartículas de $\alpha$-zeína como sistemas de liberación de compuestos hidrofóbicos: Efecto de los parámetros de ensamblado

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## Abstract

In this study, protein nanoparticles were assembled from $\alpha$-zein using liquid antisolvent-precipitation methodology. The nanoparticles were loaded with krill oil, which is an important nutraceutical ingredient. The nanoparticles displayed different physicochemical characteristics depending on the assembly parameters chosen during their preparation. The fabrication process involved the use of biopolymer coatings (the protein $\beta$-lactoglobulin and the carbohydrate chitosan). Non-coated nanoparticles showed a particle size between 340-400 nm and surface charge of around -40 mV (pH 8.0). The protein copolymer $\beta$-lactoglobulin allowed the fabrication of smaller (d $\approx$ 200 nm; $\zeta$-potential $\approx$ -60 mV) and more stable nanoparticles against pH changes (from 3.0 to 7.0). Chitosan was the best biopolymer coating for improving the antioxidant activity of the particles. The apparent pI for the different nanoparticles was modified after krill oil nanoencapsulation. These results support the idea that controlling the solvent system is a means to control physicochemical characteristics of $\alpha$-zein nanoparticles.

*Keywords*: $\alpha$-zein, krill oil, astaxanthin, biopolymeric nanoparticles, hydrophobic interactions.

## Resumen

En este estudio, nanopartículas de proteína fueron ensambladas a partir de $\alpha$-zeína utilizando la metodología de precipitación antisolvente. Las nanopartículas fueron capaces de encapsular aceite de krill, el cual es considerado como un nutracéutico muy importante. Las nanopartículas mostraron diferentes características fisicoquímicas dependiendo de los parámetros utilizados durante su preparación. El proceso de fabricación involucró la utilización de un revestimiento o cobertura de biopolímeros (la proteína $\beta$-lactoglobulina y el carbohidrato quitosano). Las nanopartículas sin revestimiento presentaron un tamaño de partículas entre 340-400 nm y una carga superficial alrededor de -40 mV (pH 8.0). El revestimiento o cobertura de las nanopartículas con la proteína copolímero $\beta$-lactoglobulina permitió la fabricación de nanopartículas más pequeñas (d$\approx$ 200 nm; potencial $\zeta$ = -60 mV) y estables frente a los cambios de pH (de 3.0 a 7.0). El carbohidrato quitosano fue el mejor revestimiento polimérico para mejorar la actividad antioxidante de las partículas. El pI aparente para las diferentes nanopartículas se modificó después de la nanoencapsulación del aceite de krill. Estos resultados sustentan la idea de que controlando las condiciones del sistema de ensamblado es una manera de controlar las características fisicoquímicas de las nanopartículas formadas a partir de $\alpha$-zeína.

*Palabras clave*: $\alpha$-zeína, aceite de krill, astaxantina, nanopartículas biopoliméricas, interacciones hidrofóbicas.

## 1 Introduction

Food biopolymers have interesting properties for the fabrication of delivery systems with applications in food, pharmaceutical, health products, cosmetics, and other industries. These materials are used to encapsulate, protect and deliver bioactive compounds, such as nutraceuticals, vitamins, antimicrobials, antioxidants, etc. (Arroyo-Maya & McClements,

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2015; Sánchez-Juarez, 2019) due to their edible nature, stability, and processability (Lakkis, 2007). Nanoencapsulating systems can be fabricated from various food-grade materials. Among the many biomolecules that exist in nature, multi-polymeric nanoparticles can be assembled from proteins and polysaccharides using various preparation methods (Matalanis, Jones, & McClements, 2011; Rosales-Martínez, 2017). These methods include solvent extraction-evaporation (Freitas, Merkle, & Gander, 2005), coacervation/phase separation (Reza, 1990), and liquid antisolvent-precipitation (Joye, Davidov-Pardo, & McClements, 2015; Sánchez-Juarez, 2019). 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 to produce nanoparticles include dairy- and plant-based proteins, such as corn proteins (Lakkis, 2007). Zein, the prolamin of corn, is a mixture of alcohol-soluble proteins that comprises $\alpha$-, $\beta$-, $\gamma$-, and $\delta$-zein. According to Esen's classification (Esen, 1990), $\alpha$-zein bands appear at 19 and 22 kDa, $\beta$-zein at 17 kDa, $\gamma$-zein at 27 and 18 kDa, and $\delta$-zein at 10 kDa. Zein-based particles obtained by antisolvent precipitation methodology have already been used for the encapsulation and controlled delivery of bioactive compounds (Parris *et al*., 2005).

Recently, krill oil has gained importance as dietary supplement due to its biological functions as a nutraceutical (Massrieh, 2008). Krill oil is considered a rich source of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Castro-Gómez, Holgado, Rodríguez-Alcalá, Montero, & Fontecha, 2015). These fatty acids are almost exclusively bound to phospholipids such as phosphatidylcholine which improve their bioefficacy and bioavailability (Wijendran *et al*., 2002). In contrast, EPA and DHA in fish oil are present like triacylglycerol fatty acid esters. Another important distinction between fish and krill oil is that the last one contains astaxanthin, which is a carotenoid pigment with important biological functions (Guerin, Huntley, & Olaizola, 2003). For this reason, astaxanthin can protect EPA/DHA from oxidative damage by being preferentially oxidized. (Choubert, Dentella, Atgié, & Baccaunaud, 2005). However, the limited water solubility of astaxanthin has lowered its bioavailability and hampered its technological applications (Anarjan, Mirhosseini, Baharin, & Tan, 2010).

The aim of the present research was to fabricate zein nanoparticles capable of nanoencapsulate krill oil. The nanoparticles are based on a fraction of hydrophobic zein proteins ($\alpha$-zein), which can bind hydrophobic molecules, and two different hydrophilic copolymers: $\beta$-lactoglobulin and chitosan. Another important aim was to study the effect of the nanoencapsulation on the antioxidant functionality of krill oil. This study is needed to develop nanodelivery systems capable to stabilize and preserve the antioxidant properties of entrapped hydrophobic bioactives. Also, it was desired to characterize the nanoparticles formed and study their aggregation behavior against pH destabilization. The nanoparticles were fabricated using antisolvent precipitation methodology (Joye *et al*., 2015; Sánchez-Juarez, 2019).

## 2 Methods and materials

### 2.1 Materials

Alpha-Zein and low molecular weight chitosan (M.W. 50 - 190 kDa) were purchased from Sigma-Aldrich (St. Louis, MO). $\beta$-lactoglobulin ($\beta$-lac with a purity of 95%) was obtained from Agropur Inc. (Eden Prairie, MN). Krill oil was obtained from Nano-nutrition (Naucalpan Edo. de Mexico, Mexico). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO.

### 2.2 Preparation of non-loaded zein nanoparticles

Nanoparticles were fabricated following the procedure described by Joye *et al*., (2015) and Sánchez-Juárez *et al*., (2019) with some modifications. Briefly, 6.0 g of $\alpha$-zein were dissolved in 95% (v/v) ethanol aqueous solution. The protein solution was subsequently added to the aqueous antisolvent at 1:5 (solvent/antisolvent) under continuous stirring. Ethanol was removed from the mixture using a rotary evaporator at 40 ºC. For the production of coated particles, the titration of the solvent solution was carried out in the antisolvent phase containing $\beta$-lac (0.5% w/v) or chitosan (1% w/v). The pH of the antisolvent phase containing the copolymers was adjusted to 4.8 for chitosan, while the pH of the $\beta$-lac was brought to 6.5.

### *2.3 Preparation of krill oil-loaded zein nanoparticles*

The preparation of krill oil-loaded nanoparticles was carried out as previously mentioned with the only difference that krill oil (3.0 % w/v) was added to the solvent phase and the solution was stirred for additional 20 min.

### *2.4 Characterization of krill oil-loaded nanoparticles*

#### *2.4.1 Particle size and ζ-potential*

The particle size distribution and surface charge of the $\alpha$-zein nanoparticles were measured using a dynamic light scattering (DLS) instrument (Zetasizer Nano-ZS, Malvern Instruments Ltd., Malvern, UK). Biopolymer particle suspensions were diluted 1:100 with deionized water (adjusted to the pH of study) immediately before analysis. Samples were placed in the measurement cell and equilibrated to 25 ºC.

#### *2.4.2 Scanning electron microscopy (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 $\mu$L) was drop-casted onto a carbon-coated surface. The sample was then air-dried at room temperature before loading into microscope.

#### *2.4.3 Determination of the total krill oil content by UV analyses*

The total krill oil content (in equivalents of astaxanthin) within zein nanoparticles was determined by a spectrophotometric method. Briefly, an aliquot of particle 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 ($E^{1\%}_{1cm}$ = 2198) (Chen & Meyers, 1984).

#### *2.4.4 Chemical stability of krill oil extract: Antioxidant activity*

Antioxidant activity was determined by the 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical-scavenging assay. (Re *et al*., 1999). The ABTS radical cation was produced by mixing a 7 mM ABTS solution with 2.45 mM potassium persulfate and allowing the mixture to stand protected from light for 12 to 16 h. The radical solution was diluted with water to an absorbance of 0.70 ± 0.02 and 1.9 mL of this solution was mixed with 100 $\mu$L of zein nanoparticles suspension, and the absorbance at 734 nm was measured 1 min after initial mixing. The results are expressed relative to the % of antioxidant activity.

#### *2.4.5 Stability against pH*

The nanoparticle size and electrical characteristics of the nanoparticle dispersions were determined at various pH values by means of dynamic light scattering. The effect of pH on the stability of the zein nanoparticles was determined by mixing equal volumes of 20 mM buffer with pH values ranging from 3.0 to 9.0. The samples (when needed) were then adjusted to the desired pH values with 0.1 M HCl or NaOH.

#### *2.4.6 Statistical analysis*

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

## 3 Results and discussion

### *3.1 Production of coated nanoparticles*

$\alpha$-zein allows the production of encapsulated food products based on its ability to self-assemble when the solvent solution polarity changes towards a more hydrophilic environment. Electrosteric biopolymers such as proteins and polysaccharides have been reported to increase the loading capacity, stability, and redispersibility of the particles (Joye *et al*., 2015; Luo, Zhang, Whent, Yu, & Wang, 2011). In this study, $\beta$-lactoglobulin ($\beta$-lac) and chitosan were used as coatings for zein particles. $\beta$-lac and chitosan are used in food products due to their functional properties. Different methods can be used to associate these copolymers to the nanoparticles such as electrostatic deposition after particle formation (Joye *et al*., 2015) or co-precipitation during particle fabrication (Arroyo-Maya & McClements, 2015). In this study, the second method was used. Initially, the influence of $\beta$-lac and chitosan on the dimensions and stability of the

zein/copolymer particles was measured using dynamic light scattering (Fig. 1).

*i) β-lac as copolymer*

In the absence of coating, the mean diameter of the zein particles was 400 and 250 nm for non-loaded and loaded zein nanoparticles, respectively. Smaller nanoparticles (non-loaded and loaded) were formed (Fig. 1) when these nanoparticles were mixed with $\beta$-lac (0.5% w/v). The zeta-potential for the non-coated nanoparticles (pH 8.0) was from around -20 (non-loaded particles) to -40 mV (loaded particles). In the case of zein/$\beta$-lac particles, the non-loaded particles showed an average $\zeta$-potential of -60 mV while the loaded ones about -30mV.

*ii) Chitosan as copolymer*

The particle size decreased when chitosan (1% w/v) was added as copolymer, from 900 for the non-loaded to 250 nm when the particles were loaded. Moreover, the $\zeta$-potential values for chitosan-coated particles were from -10 (non-loaded particles) to -40 mV for loaded particles. These results indicated a clear effect of the load on the dimensions and surface characteristics of the particles. The above-mentioned was in agreement with previous research (Li, Yin, Yang, Tang, & Wei, 2012) where zein particles exhibited a change in electric characteristics after loading with thymol.

During particle formation, zein molecules self-assembled into particles upon solvent evaporation; this process is driven by hydrophobic protein-protein interactions, which allow the hydrophobic protein molecules to form the core of the nanoparticle and the copolymer to form the shell (Joye *et al.*, 2015). In this study, it is proposed that zein nanoparticles formed have a protein core and a copolymer shell structure. Although, there is no clear evidence to demonstrate the mentioned structure, previous work reported that encapsulated plant essential oils, prepared by a similar methodology, were gradually released from zein particles, upon enzymatic hydrolysis (Parris *et al.*, 2005). Thus, the gradual release of essential oils indicated a homogeneous distribution of oil within the particles. Similarly, the idea for core-shell particles obtained in this study comes from the $\zeta$-potential measurements on the zein particles. The $\zeta$-potential versus pH profile of the zein particles resembled that of the copolymers (data not shown), which suggests that the particles presented a protein core while copolymers were found on the surface of the particles.

## 3.2 *Effect of pH on the particle aggregation stability*

The assembly of zein/copolymer nanoparticles was carried out at two specific pH values because of the different physicochemical characteristics of the copolymers. Zein nanoparticles coated with $\beta$-lac (pI≈ 5.1) were produced at pH 6.5 because $\beta$-lac could present extensive aggregation at pH values close to 4.5. The particles containing chitosan were prepared at pH 4.8. At this pH, the hydrophobic protein nanoparticles were positively charged (Fig. 2) and so the interaction with chitosan could be attributed to hydrogen bonds, since both biopolymers were positively charged at this pH value (Luo *et al.*, 2011).

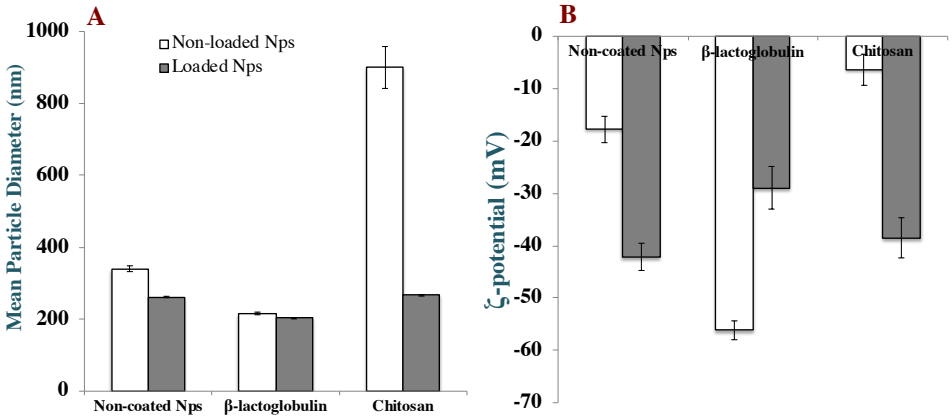


Fig. 1. Mean particle diameter (A) and zeta-potential (B) of zein nanoparticles prepared with two different electrostatic stabilizers (coatings): 0.5 % (w/v) $\beta$-lactoglobulin and 1% (w/v) chitosan.

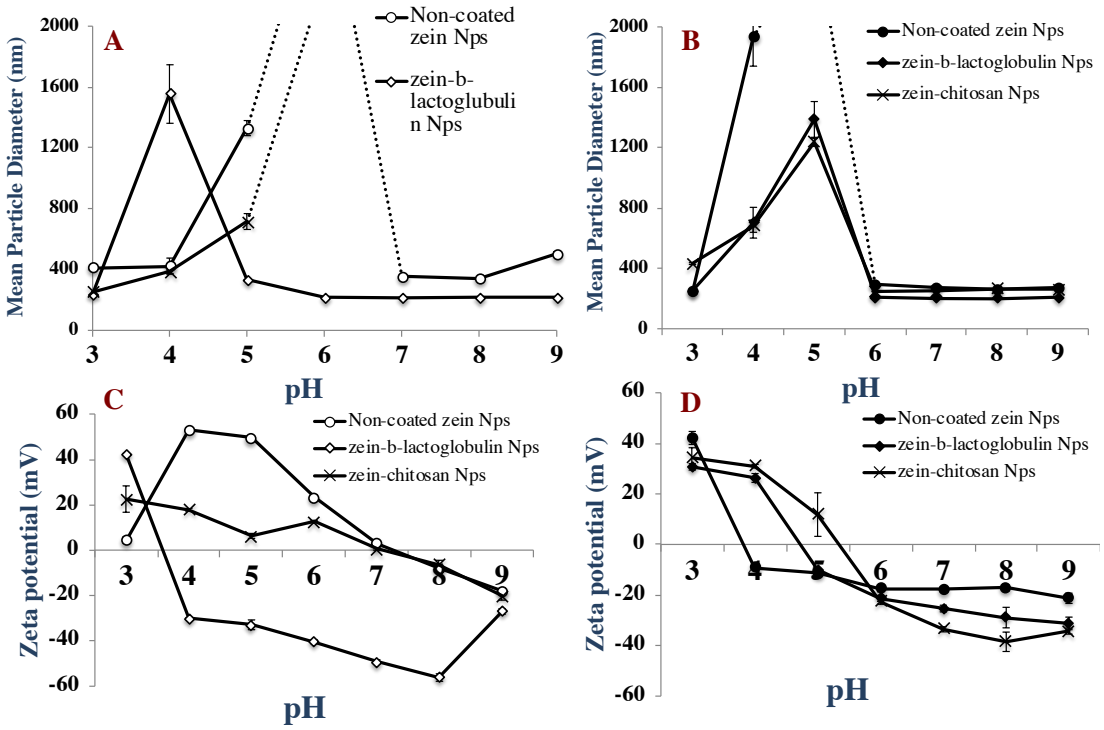


Fig. 2. Mean particle diameter and zeta-potential of non-loaded (A and C) and krill oil-loaded (B and D) zein nanoparticles (NPs). The concentration of $\alpha$-zein and krill oil used to prepare the nanoparticles was 6 and 3% (w/v), respectively.

In contrast, at pH 6.5 the hydrophobic protein nanoparticles exhibited neutral surface charge (Fig. 2), while $\beta$-lac was negatively charged. In this case the interaction of the zein particles with $\beta$-lac may have been due to hydrophobic interactions or hydrogen bonds.

The effect of these two different stabilizers on particle size of zein nanoparticles can be seen in Fig. 2. As can be observed, the size of zein nanoparticles decreased when krill oil was encapsulated within the particles; as both krill oil and zein are hydrophobic compounds, the hydrophobic interactions could be another force involved during the formation of krill oil-loaded zein particles. The results showed that empty nanoparticles had a larger particle size in a range between pH 4.0 - 7.0 for zein/chitosan particles, whereas zein/$\beta$-lac particles showed a consistent particle size through almost the entire pH range studied. In addition, the size of non-coated particles augmented in a pH range between 3.0 and 7.0. This could be due to the proximity to the zein isoelectric point ($\approx$ 6.2) (Shukla & Cheryan, 2001) allowing particle aggregation due to a reduced electrostatic repulsion between particles.

Protein nanoparticles can be incorporated (as functional ingredients) in food products in a range of pH conditions. In this concern, evaluating the pH stability of the zein nanoparticles is important to predict their performance in food products. In this study, the isoelectric point (pI) of the non-coated (non-loaded) zein particles was calculated from $\zeta$-potential measurements to be around pH 6.8, while coated nanoparticles showed a pI around 3.1 and 6.9 for $\beta$-lac and chitosan, respectively (Fig. 2C, 2D & 3). Aggregation of non-coated zein particles occurred between pH 3.0-5.0, as determined by a large increase in size (Fig. 2) and particle sedimentation (Fig. 4). Most food proteins have a pI in the range of 4.0 to 7.0 and aggregation may occur around this pH range. $\beta$-lac reduced the amount of aggregation of the zein nanoparticles when compared with non-coated and coated with chitosan (Fig. 4). In addition, $\beta$-lac/zein particles exhibited homogeneous nanoparticle suspensions (except at pH 5.0). Zein/chitosan particles irreversibly aggregated, even at pH conditions well above their isoelectric point. It might be occurred due to strong hydrophobic attractions among particles that surpassed electrostatic

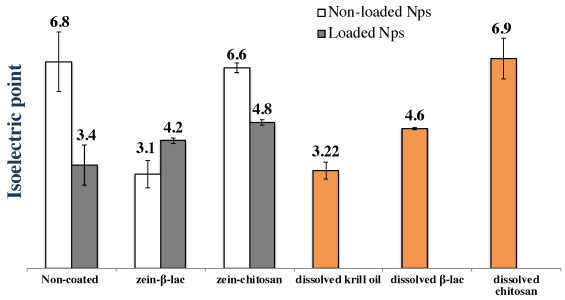


Fig. 3. Isoelectric point of non-loaded and loaded zein nanoparticles, and dissolved 0.5% (w/v) $\beta$-lac, 1% (w/v) chitosan, and 3% (w/v) krill oil.

repulsion and induced destabilization of nanoparticles. The improved stability of zein/$\beta$-lac nanoparticles can be attributed to an increase in electrosteric repulsion due to the adsorption of $\beta$-lac around the particles. Our results indicated that the pI values for the zein/copolymer nanoparticles resembled those for the dissolved copolymers (in the absence of zein) (Fig. 3), suggesting the presence of copolymer layers covering the nanoparticles. Also, copolymers may form a hydrated polymeric coating that will improve the steric repulsion between particles.

Loaded (non-coated) zein particles were destabilized at acidic pH values (Fig. 4), which may be attributed to the decrease in the magnitude of the electrical charge on the particles near their pI (~3.4) and the presence of krill oil. Because loaded (non-coated) particles also precipitate in a wider pH interval than the non-loaded particles, it seems that loading nanoparticles with krill oil could affect the electrostatic repulsion through decreasing their apparent pI from ~ 6.8 to ~ 3.5 which is the value of the krill oil mixture. The later was found to be the maximum shift in pI obtained for loaded nanoparticles and corresponding to -3.4 pH units. The suspension stability of loaded zein/$\beta$-lac particles was similar to the non-loaded system, this was demonstrated by the small shift amplitude of the pI from ~3.1 (non-loaded particles) to ~4.2 (loaded particles) compared to the non-coated system (Fig. 3), this difference in pI shift was around +1.1 pH units. In contrast, aggregation was evident in a pH range from 3.0 - 7.0 for zein/chitosan particles (Fig. 4), which can be due to the low electrical charge near its pI. The point of zero charge for the zein/chitosan particles was similar (~7) for the dissolved chitosan (in the absence of zein) and for the non-coated particles as well, which suggests that the outer protein layer might contribute to the electrical characteristics of these nanoparticles. Loading zein/chitosan nanoparticles with krill oil induced a decrease of 2.1 pH units in the pI of the particles. Interestingly, the apparent pI for the different nanoparticles was modified after loading the particles with krill oil, indicating a possible interaction between the components involved. In addition, self-assembly of $\alpha$-zein modified the surface charge and the pI of the

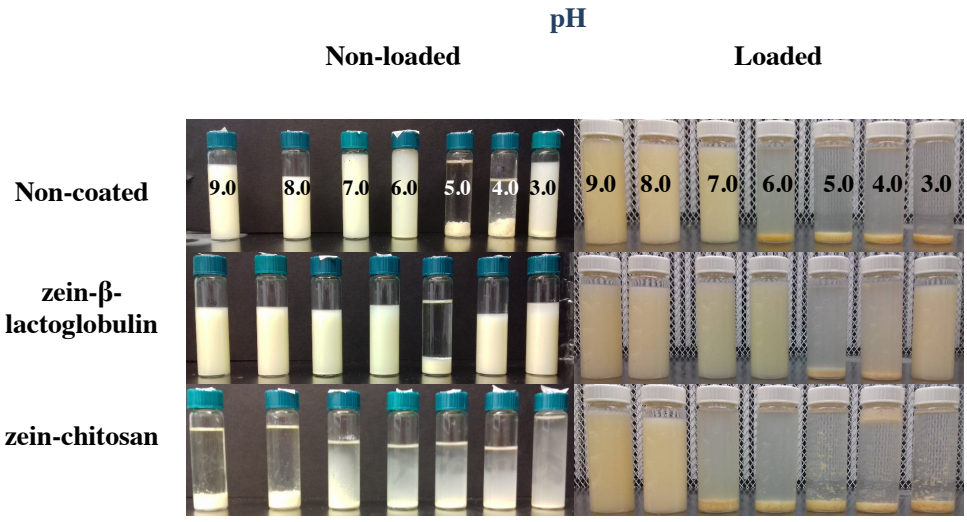


Fig. 4. Effect of pH on the stability of zein nanoparticles: visual inspection of dispersions of non-loaded and loaded zein nanoparticles. The protein nanoparticles were loaded with krill oil [3% (w/v)]. $\beta$-lactoglobulin and chitosan (electrosteric stabilizers) concentrations in the antisolvent solutions were 0.5 and 1% (w/v), respectively. The pH was adjusted with HCl and NaOH.

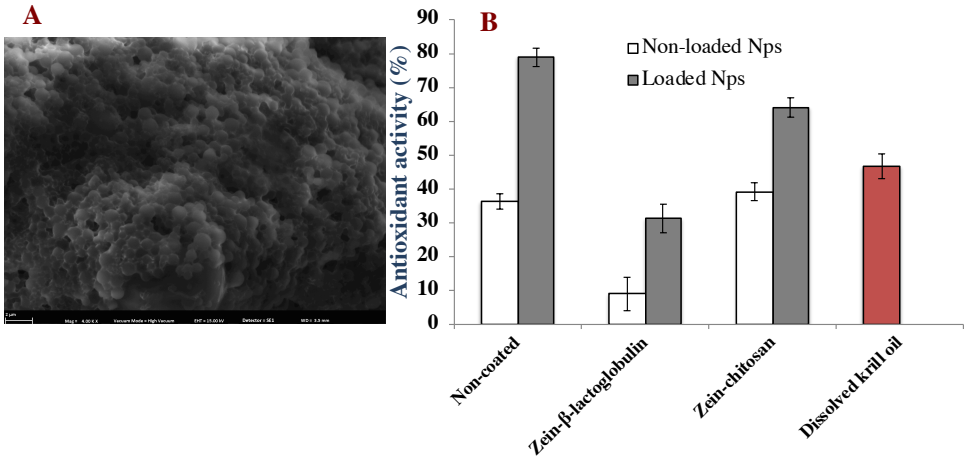


Fig. 5. SEM images of non-loaded and non-coated zein nanoparticles (A); Comparison between the antioxidant activity of non-coated and coated zein nanoparticles (non-loaded and loaded with krill oil) (B).

nanoparticles. These changes are expected because self-assembly involves conformational changes that may affect the microenvironment of acid and basic side chains within zein molecules, leading to shifts in their pKa values. Also, coatings may also screen some ionizable groups on protein surface contributing to further variations in the pI. Overall, the interaction between zein and copolymers will depend on several physicochemical parameters. Through its role in the ionization of $\alpha$-zein and copolymers molecules, pH is an important factor affecting electrostatic zein/copolymer interactions.

### *3.3 Influence of encapsulation in astaxanthin antioxidant activity*

The antioxidant activity of krill oil was monitored by its ability to scavenge the $ABTS^{\bullet+}$ radical cation, which was measured based on the loss of green color of $ABTS^{\bullet+}$ radical in the presence of antioxidants. The properties underlying the activities of carotenoids such as astaxanthin (contained in the krill oil mixture) towards free radicals relates particularly to their abilities to donate electrons or hydrogen atoms (Miller, Sampson, Candeias, Bramley, & Rice-Evans, 1996). As most carotenoids, astaxanthin is a highly unsaturated molecule and can be degraded by heat, light and oxygen (Christophersen, Jun, Jorgensen, & Skibsted, 1991). Since many physicochemical factors may occur during nanoparticles fabrication, the effect of the nanoencapsulation on the antioxidant activity of the krill oil was measured.

In the absence of krill oil, zein particles had moderate antioxidant capacity (Fig. 5B), indicating that the particles can act as antioxidants. It is known that zeins present antioxidant activity (Diaz-Gomez, Ortiz-Martinez, Aguilar, Garcia-Lara, & Castorena-Torres, 2018). This bioactivity is due to the presence of amino acids such as leucine and proline, which are related to the antioxidant activity of zein (Peña-Ramos, Xiong Youling, & Arteaga Guillermo, 2004). The particles with entrapped krill oil had higher antioxidant capacity (i.e. 40%) than the non-entrapped krill oil subjected to similar nanoparticle fabrication conditions, suggesting that nanoencapsulation of krill oil might improve its physicochemical stability and/or biological activity. For zein/chitosan nanoparticles the antioxidant activity was around 75%, indicating that this carbohydrate is a suitable copolymer to protect loaded zein particles. In contrast, particles coated with $\beta$-lac, (non-loaded and loaded) exhibited an antioxidant activity of 40 % (even with a loading efficiency for astaxanthin of ca. 60%). This reduction in activity may be the result of degradation of the krill oil during nanoparticle formation because of environment exposure or binding of the astaxanthin to zein and $\beta$-lac within the particles. Overall these experiments suggest that the nanoencapsulation of krill oil may improve its physicochemical stability, but the encapsulation method itself could decrease their antioxidant capacity.

## Conclusions

This study showed that antisolvent precipitation is a suitable procedure for the nanoencapsulation of krill oil within zein particles. The use of zein/$\beta$-lac allowed the fabrication of smaller ($\approx$ 200 nm) and more stable nanoparticles, which allowed the solubilization of appreciably amounts ($\approx$ 60% loading efficiency) of krill oil in aqueous systems. In addition, zein/chitosan particles displayed the best potential to enhance antioxidant activity as could be demonstrated by the 40% increase in this property. The entrapment of bioactive compounds within nanoparticles provided some protection against pH destabilization. In this study, it was evident that the assembly process, coating, and loading of zein particles is a means to modify the surface charge and pI of the particles, consequently modifying their colloidal stability against aggregation. In near future, additional studies (through different environmental conditions) to evaluate the physicochemical stability of this important class of natural nutraceuticals will be considered in order to complement the results obtained in this research but to the best of our knowledge this is the first evidence of successful application of the developed nanoencapsulation methodology and could represent an advantage over conventional emulsification methods.

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