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Morphology study of alginate micro/nano particles for the encapsulation of divalents Mg2+ and Zn2+ ions

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ABSTRACT

This research work aimed to promote the formation of alginic acid particles and the encapsulation of divalent ions, such as Zn^{2+} and Mg^{2+}; but using a combination of internal alginate gelation and micro-emulsification method. Both ions are essential elements of the human body, i.e., they are present in tissues and body fluids and participates in many bodily functions. The influence of different parameters was evaluated relate to the formation of the particles in micro/nano-scale, and their morphology was observed. The concentration of both ions used in the formulation was varied considering [0.075, 0.15 and 0.25] mol/L. It was found in general that the formation of particles in nanoscale, with a spherical shape and smooth surfaces (also by Atomic force microscopy AFM) after characterizing by electron microscopy (Scanning SEM and Transmission TEM) with energy-dispersed analysis of X-rays (SEM/EDX). The only evidence of formation of particles at higher concentrations of the ion ([0.25] mol/L) was found when the magnesium ion was used ($MgSO_4$) while the smallest particles (≤100nm) were formed when $ZnSO_4$ ([0.25] mol/L) was used. The results suggest that these particles can be used as a coat or carrier for essential nutrients for food fortification, for instance, for others applications in biomedicine or charge drugs in delivery systems.

Introduction

Zinc (Zn^{2+}) and Magnesium (Mg^{2+}) are between the essential elements for the human body since they are present in tissues and body fluids and they participate in many bodily functions linked to the metabolism of proteins, lipids, and carbohydrates, as well as to insulin synthesis, RNA, and DNA. Zinc deficiency affects cell growth, sexual maturation, regeneration, and repair of tissues, affects the functioning of the immune system[1,2] which is why intake and absorption are critical to a human being. Also, the intake of magnesium ion (Mg^{2+}) is very important because it is associated with the operation of several enzymes related to metabolism, protein synthesis, RNA, and DNA, as well as with the maintenance of the electrical potential of nervous tissues and cell membranes and calcium metabolism[3,4]. Magnesium deficiency produces malnutrition, vomiting, muscle weakness, inhibition of natural tissue regeneration, and a prolonged deficiency may conduct to great weight loss[5], and also has been suggested that maintaining low levels of magnesium may influence the onset of heart disease and hypertension[6]. For these reasons, the presence of these ions in the food is very important for being healthy, which is why is so important to study different the ways to incorporate them into our alimentation. Alginate is a biopolymer that has been used for a very long time in the food industry, and also it is a copolymer composed of polysaccharides (β -(1→4)-linked *D*-mannuronic acid (M) and α -(1→4)-linked *L*-guluronic acid (G). Guluronate groups (G) and Mannuronate groups (M) can rapidly cross-link in the presence of divalent cations[7]. Cations such as calcium have been widely used to induce gelation of the alginate[8-11]. They have also been employed for other divalent cations such as Zn^{+2}, Mn^{+2}, Co^{+2}, Sr^{+2}, Ba^{+2}, Cu^{+2} [12-14]. However, the Mg^{+2} had been considered not to induce the formation of alginate gelation for a long time[15,16]. This last idea is about to change because some researchers continue working in that, for example, a recent report from Topuz *et al.* (2012)[17] indicates through some rheological evidence that the gelation of alginate may occur in the presence of magnesium. Nanotechnology reaches an important role to incorporate these essential elements and their mixture with biopolymers. Then, in the studies related to determining the size of the polymers particles, these characteristics greatly depend on several parameters that can be modified during the preparation of these particles and which can reach sizes of micro or nanoscale. The type of ion used to influence the particle size, surfactant concentration, homogenizer speed, ion concentration, etc.[18-21] Because the ion binding is key to produce homogeneous particles, it could then ensure the nano size. Therefore, the effect of the encapsulation and concentration of cations used in the formulation of particles is one aspect that this research work considers, because understanding the encapsulation mechanism could lead to the delivery of these divalent cations.

Thus the purpose of this research is to provide positive results that were achieved by encapsulating Zn^{2+} and Mg^{2+} ions using sodium alginate through an ionic gelation process combined with the microemulsion methodology. It is expected that the final application of these nanoparticles would help to improve the nutritional value for several food industry applications mentioned above, and also opens the door for this methodology to be used in the preparation of nanoparticles containing these ions as well as to serve as a vehicle for encapsulating drugs or other biomolecules[22].

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Materials and methods

Materials. A Sodium Alginate, with structural relation = 0.95 Mannuronic/Guluronic acid (M/G Groups) determined by NMR analysis, was used from Sigma Aldrich. Zinc Sulfate ($ZnSO_4$), Magnesium Sulfate ($MgSO_4$) as a precursor of Mg^{+2} and Zn^{+2} ions. As surfactant Polyvinylpyrrolidone (PVP) with a molecular weight Mw 10.000 g/mol and Tween 80 as a non-ionic surfactant. All these reactive were purchased from Sigma-Aldrich. Also, deionized water was employed.

Alginate Purification

There was used the same method described previously[23].

Preparation of Alginic Acid Solution. It was prepared a solution of alginic acid [1% w/v] and blended with Tween 80 [0,05% w/v] in deionized water.

Preparation of Zinc and Magnesium Sulfate Solutions

Solutions of $ZnSO_4$ and $MgSO_4$ ([0.075], [0.15] and [0.25] mol/L) and PVP [2% w/v] were prepared in deionized water and added to isopropyl alcohol in proportion 80/20 v/v. The emulsion was prepared by using a high-speed homogenizer (IKA T-10 Ultra-Turrax) at 10.000 r.p.m during 15 min.

Preparation of Nanoparticles

Alginate particles were produced by dropwise the alginate aqueous solution into the $ZnSO_4$-PVP or $MgSO_4$-PVP solutions under continuous stirring during 30 min and stop. After this time, the particle suspension was kept at room temperature for 24 hours.

Nanoparticles Recuperation

After 24 hours, the resulting nanoparticles were purified via washing with deionized water by four centrifugation cycles (15 minutes each). The samples were frozen at -4°C for 24 h and then lyophilized during 24 hours, in a Labconco freeze dryer. The PVP used as surfactant has a cryoprotectant action[24] during lyophilization process at –45°C and 0.075 Torr.

Morphological Analysis

Scanning Electron Microscopy with energy-dispersed analysis of X-rays (SEM/EDX) has been used to characterize the size, shape, surface texture and elemental composition of nanoparticles. All samples were gold coated using a Sputter-coater Balzers-SCD-030 unit and then analyzed under a JEOL JSM 6460 microscope at 15 kV. Elemental compositions (semi-quantitative) are reported as weight percents for all tested compounds. A JEOL equipment was used for Transmission Electron Microscopy (TEM), JEM 2100, 200 kV accelerating voltage and filament lanthanum hexaboride (LaB_6). In this case, only the sample with 0,15 M of Zn^{+2} was observed and prepared by suspending wet and deposited on a copper grid of 200 mesh and coated with carbon.

Also, the topography of the alginate obtained particles were analyzed using an Agilent 5500 AFM equipment, Atomic Force Microscope (AFM). Small sections of the particles were introduced and gelled into the resin and then cut using an ultramicrotome. The samples were digitized in an acoustic mode with a resonance frequency of 157.070 kHz. The observation in the AFM was carried out through scanning areas of dimensions (2μm × 2μm).

Particle Size Distribution

This parameter was measured in a Dynamic Light Scattering equipment Zetatrac (from Microtrac, Inc) with zeta potential measurement. For that 20 mg of nanoparticles were suspended in 10 mL of 1:1 ethyl alcohol: deionized water solution. The samples were first sonicated for thirty seconds in a bath-type sonicator Hielscher UP400S, 400 W and 24 kHz (70% frequency) at room temperature to reduce agglomerates between particles and obtaining better results. Each formulation was performed and recorded three times to get the average zeta potential.

Determination of the amount of encapsulated ion

Atomic absorption spectroscopy (Perkin Elmer equipment, model 3300) was used to determine the quantity of the ion encapsulated in the nanoparticles. (10 ± 1) mg of sample was placed with (10 ± 1) mL of concentrated hydrochloric acid in a test tube. The mixture was stirred for 15 min and then left to stand for 24 hours. After that

time, the solution was placed in a 50 mL graduated balloon and leveled. The amount of ion present in the sample was determined from this solution. Knowing the quantity of the encapsulated ion/mass of particles and the total amount of ion that was added in each formulation, it was possible to calculate the efficiency of the encapsulation (% EE) that was reached with the method employed, through the following equation:

$$\%E = \frac{m_{(total-ion)} - m_{(non-encapsulated-ion)}}{m_{(total-ion)}} \times 100$$

Tests for the release of the encapsulated ion. The release of the encapsulated ion (according to USP XXXI 2008 protocol) was evaluated by simulating in vitro gastric pH conditions and pH conditions of the human intestine, both tests carried out at 37 ° C, without considering the presence of enzymes. All samples were analyzed in triplicate, using atomic absorption spectroscopy (Perkin Elmer equipment, model 3300). Protocol was follow: *Release assay in a simulated gastric medium (Part 1)*. 10 mg of particles were placed in graduated and sterile plastic tubes to which 25 mL of HCl solution at pH 1.75 and stirring. Subsequently, a volume of 1.2 mL of the supernatant of the particles was taken every 30 min for a total of 120 min. Each of these aliquots was placed in a 50 mL volumetric flask and filled with deionized water for later analysis by atomic absorption spectroscopy, to determine the amount of ion released as a function of time to that conditions. *Release assay in a simulated intestinal medium (Part 2).* Once the release in the gastric medium was studied, the test was carried out in the intestinal medium. In this case, the test residue in the gastric medium was centrifuged and used for the next test to simulate the passage of the particles through the gastrointestinal tract. The same procedure was used above, but using a phosphate buffer solution at a pH of 6.56. The results obtained under these two gastric and intestinal conditions were then gathered in a single figure to know the in vitro simulated release process.

Results and discussion

The method of preparation is very important to determine the properties, stability and final application of nanoparticles[18-21] and one of the important parameters to evaluate its the effect of ion concentration on internal gelation of alginate. The only process of crosslinking by the ionic gelation process of the alginate is not a guarantee that structures are formed at the micro and nanometric level, because the simple use of ionic gelation allows the formation of a continuous gel. But when doing the formation of this gel in an emulsified system, it is where particles are guaranteed to form on this scale and to be maintained stable once they have precipitated.

The microemulsion method and ZnCl2 were evaluated in a previous research work 23. In this case, $ZnSO_4$ and $MgSO_4$ were used for crosslinking the alginate, and the formation of drops allows obtaining the particles into a micro emulsification system. Fig. 1 and Fig. 2 shows SEM micrographs and EDX analysis of samples prepared with Zn^{2+} and Mg^{2+} respectively.

As shown in Fig 1, all assays tested with $Zn^{2+,}$ produced particles at the nanoscale with narrow particle distribution (as evidenced in Fig 3); the TEM results will verify these dimensions. Fig. 1 shows the spherical morphology of the particles, including some agglomeration, and also verifies the presence of the zinc element using EDX analysis, where results showed that zinc values are proportional with the [$ZnSO_4$] concentration. As has been reported in the scientific literature, researchers have concluded that the M/G ratio of alginate has a major influence on the degree of shrinkage as it affects the gelation mechanism (i.e. 'egg-box' formation). A higher concentration of group G residues in the molecular chain of alginate guarantees the formation of more stable structures[25], which is the case of the alginate that this research utilizes. The formation of the particles is not only the result of the ionic gelation process but also the micro-emulsion system used here for the formation of the particles.

In cases where the magnesium ion was used one can observe at low ion concentrations [0.075] mol/L structures like fibers (see Fig. 2(a)). At [0.15] mol/L a mixture of fiber and particles seems to appear (Fig. 2(b)); but only particles were observed when the maximum concentration of Mg2+ [0.25] mol/L was employed, as shown in Fig. 2(c).

The first step that occurs in the gelation process of alginate is the metal ion complexation with the carboxylate group present in the polysaccharide structure. The affinity of the alginate for the multivalent cations depends exclusively on a guluronic acid fraction (G) present in the polysaccharide because mannuronic acid (M) presents almost no ion selectivity 16. Had been reported that the affinity of the guluronic alginate fraction cross-linked with calcium ions (II) compared to other metals ions, and it is increasing in the following order: Ca+2<Zn+2<Sr+2<Ba+2 [12,15,16]. That is, as the atomic radius of the element in question increases the affinity increases 12-14. As seen in these previous investigations, no magnesium ion comparisons were established, likely because this ion did not produce immediate gelation when used with this polysaccharide. But new interest in this aspect has been presented, and further efforts are being conducted in this particular. There is recent evidence propose for Topuz *et al.* 17, that indeed the crosslinking occurs in the presence of Mg^{+2}, their results support our results shown in Fig. 2. In this work, Topuz *et al.* 17, evaluate the gelation using dynamic rheological studies in the oscillatory mode of alginate with magnesium (at a concentration range of 10-40 mM). Their results were shown in a Sol-Gel graphic and SEM morphology, and this further justifies the fact that a high concentration of magnesium is necessary for the gelation can be facilitated between guluronic acid and this Mg^{+2} ion. These results open a window to use different technologies for sample

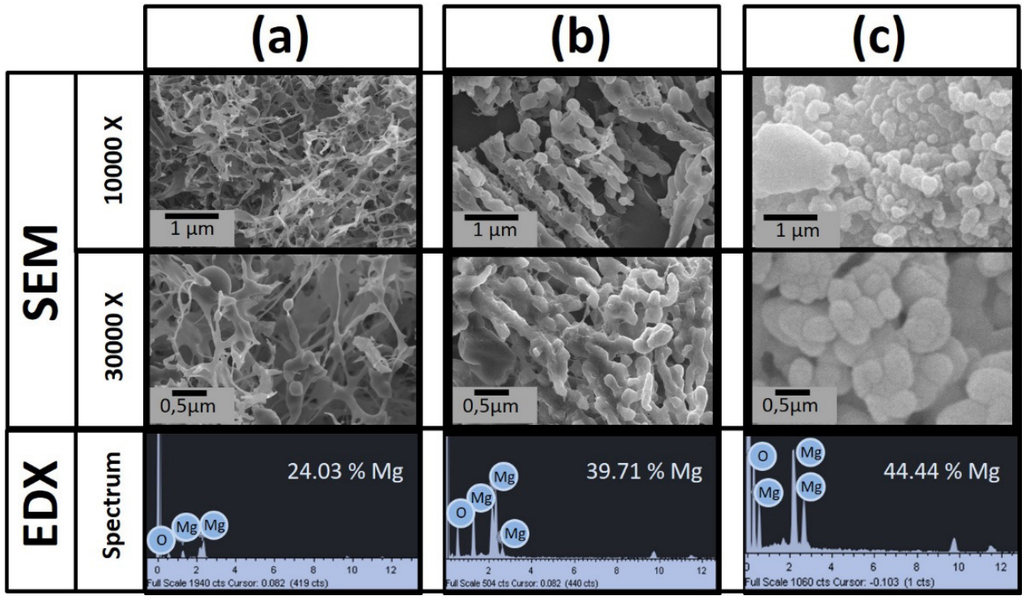

Figure 1 - Scanning electron micrographs, EDX spectrum and elemental composition of nanoparticles prepared with three Zn^{2+} concentrations: (a) 0.075 mol/L, (b) 0.15 mol/L and (c) 0.25 mol/L.

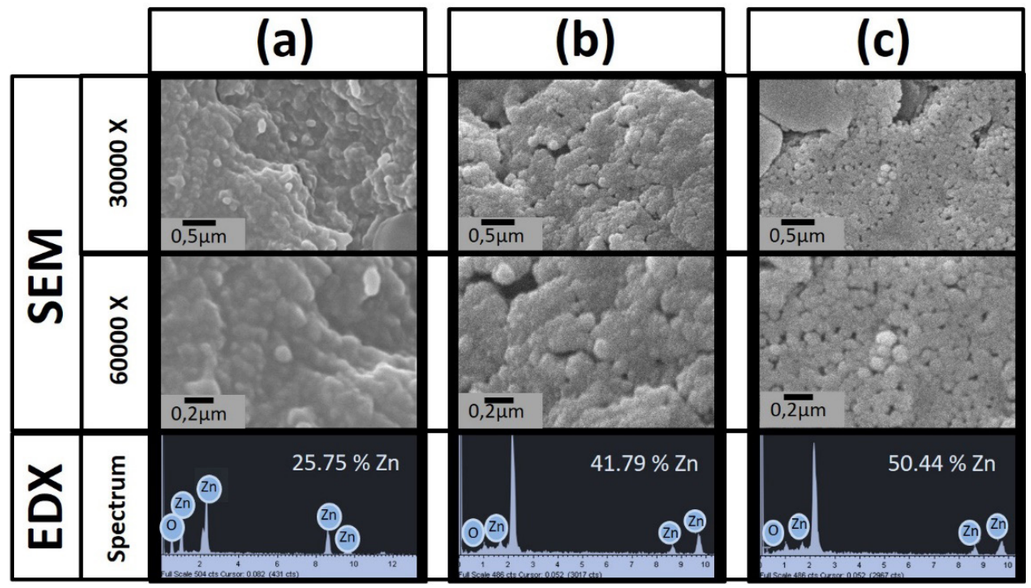

Figure 2 - Scanning electron micrographs, EDX spectrum and elemental composition of nanoparticles prepared with three Mg^{2+} concentrations: (a) 0.075 mol/L, (b) 0.15 mol/L and (c) 0.25 mol/L

preparation, mixing injection, in situ cross-linking, which makes these gels promising candidate for bioengineering and biomedical applications.

Relate to the gelation process, the most widely accepted model in which the divalent cation is bound to two G groups (Guluronics groups) into contiguous alginate chains is known as “egg box” [11,26]. This process would explain the observed in Fig 1(a) wherein the formulation of lower Zn+2 concentration the number of particles formed is low, and these appear to be more agglomerated (which can also be associated to the result reported in Fig 3 (a)). Graphs of figure 3, represent the behavior of Intensity and accumulated frequency vs. particle size.

The difficulty of formation of particles could be further related to the different components of the formulation during the emulsification process. For example, (i) the quantity of surfactant used in the formulation (about the amount of salt added) and (ii) part of the alginate molecule does not form part of the nanoparticles, and it can be formed as a continuous alginate film.

Another hypothesis is that at higher ion concentrations, the ions can saturate all guluronic groups (G), and then, starting to interact with mannuronic groups (M), so the stereochemistry of the molecule appears not to be favorable 26,27. Thus, it could form the more substantial amount of nuclei which will produce particles with better dispersion in size (as shown in Fig 2(b) and 2(c)). Also, a slight change in shape and the surface topography of the particles could appear. The aforementioned is shown in Fig 1(c), where the particles are more uniform in size and its surface seems smoother when compared with Fig 1(a) and Fig 1(b). Additionally, EDX results show that the concentration of Zinc and Magnesium increases quantitatively in the particles as expected.

Although it has been mentioned, others parameters should determine the properties and morphology of the particles and guarantee its size into nanoscale. Between these parameters are: the viscosity generate for the gelation ion-alginate and also for alginate molecular weight, surface area, density and encapsulating capacity of substances (absorption rate and release profile) [18,20,29]. Figure 3 shows a Gaussian distribution for each of the formulations (a) [0.075], (b) [0.15] and (c) [0.25] mol/L, being resulted that increasing the ion concentration will reduce the dispersion and size. Thus the Fig. 3(a) shows sizes range from 100-600 nm, having a quasi-bimodal distribution. In Fig. 3(b) the particle size was among 50-1200 nm, concentrating the largest number of particles with sizes around 480-500 nm and a monomodal distribution. And finally, for the highest concentration of salt, it can reduce the particle size and size dispersion resulting in a monomodal curve with a range of 150-500 nm, with a media around 345 nm.

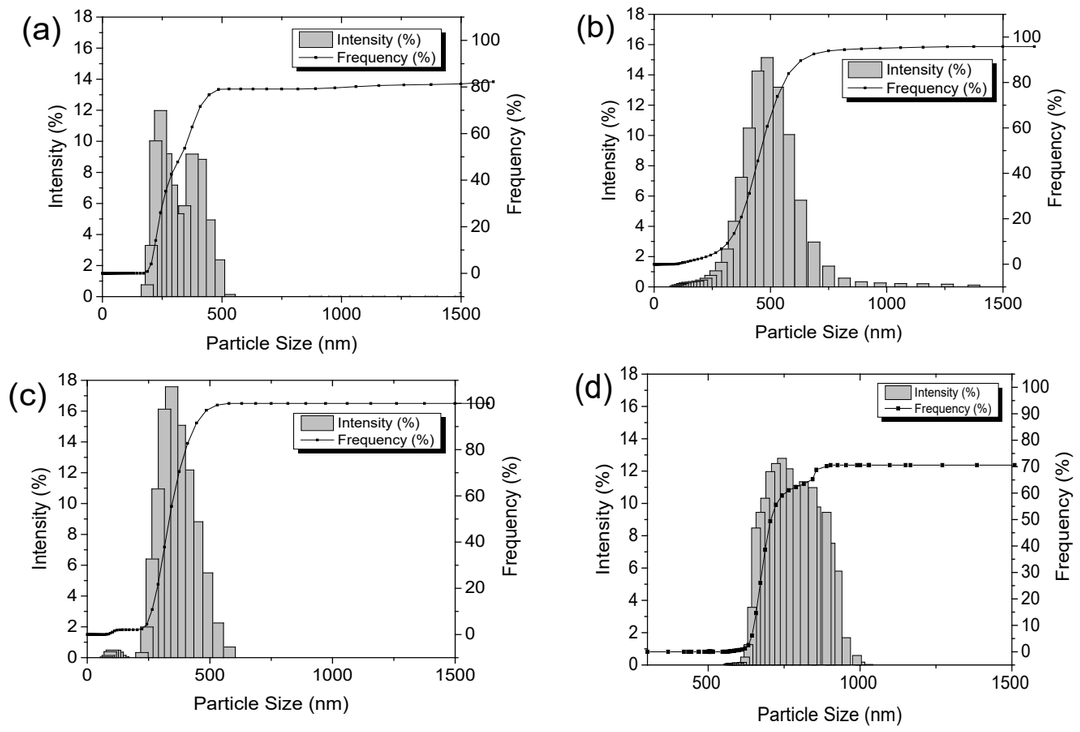

Figure 3 - Particle size distributions of Zn+2 concentrations (a) 0.0075 (b) 0.15 and (c) 0.25 mol/L and Mg+2 concentration (d) 0.25 mol/L.

The particles obtained with Mg^{+2} concentration [0.25] mol/L are shown in Fig. 3(d), because it was the only concentration that showed the formation of particles, as has been mentioned. The particle size distribution, in this case, is such where the unimodal particle size remains in the nanoscale (600 to 1000 nm), these being larger than those obtained with Zn^{+2} at the same concentration. It is possible to think that in the case of Mg^{+2} ion, these larger sizes of particles are obtained due to its ionic radius. Because these Mg^{+2} ions serve as nuclei for gelation, and it can be assumed that they occupy the most space to interact with the biopolymer chains during the formation of particles. It is for this fact that can be assumed that better packing is induced into the structure called box egg and thus generate larger and more compact particles [28,29].

The morphology analysis follows using TEM, is shown in Fig. 4. It is possible to verify the nanoscale reach of the nanoparticles forming using Zn^{+2} ions, and also that they are spherical and appear to be compact (are not nanocapsule) confirming what was appreciated in Fig. 1 and Fig. 3(b). All nanoparticles prepared uniform exhibit size, and it must be pointed out that the largest % of particles present diameters below submicrometer range (< 1 µm).

A detail of the smaller nanoparticles (≤ 100 nm) can be seen at higher magnifications in Fig. 4, and it is clear that there are compact particles. However, it seems that around their surface a thicker wall is formed, which could be defining surface characteristics of these nanoparticles. Also, TEM micrographs demonstrate that the density of crosslinking into the particles is different from their surface radially towards their interior.

In Fig. 5, the entire surface of the sample under study can be seen in a scan of 2µm x 2µm, where a high presence of semi-spherical particles and agglomeration of the same are observed. In Fig. 5b we have a 3D micrograph of the entire surface studied and detailed the valleys formed by the differences in height between the particles. Fig. 5c corresponds to an extracted profile measured horizontally on a group of particles. Through these AFM micrographs show that the particles do not exceed 20 nm in height and a width of between 100-250 nm so that we can confirm the sizes observed by both SEM and TEM.

Encapsulated and release. Two formulations were chosen to perform release assays. Since it was desired to study the release of the ion under controlled conditions, the formulations chosen to study were those prepared with the highest concentration of the ion (0.25 mol / L), both for Zinc and for Magnesium. The sample identified as Zn1 corresponds to the SEM micrographs shown in Fig. 1(c), while the sample identified as Mg1, corresponds to the SEM micrographs shown in Fig. 2(c). These results are summarized in Table 1.

The tests were made with the same formulations Zn1 and Mg1 to which the percentage of encapsulation was determined. Such as shown in fig. 6, particles prepared with Zn^{+2} show a release below 30% in gastric medium, while that of the Mg^{+2} exhibit a release around 80% of the ion in this medium at 120 min. The fact above may be because the interaction of the alginate with the Mg^{+2} ion is weaker as already discussed previously[16,17,30,31] and therefore the bonds could be broken, allowing the release of Mg^{+2} more rapidly than in the case of Zn^{+2}. The results of these tests indicate that indeed, the greater amount of the encapsulated Zn^{+2} ions are released in intestinal conditions.

Our results support other research about alginate because it is resistant to acid hydrolysis and soluble in alkaline solutions[15]; which is very important for the encapsulation of nutrients in alginate is a viable procedure. That means the alginate protects the nutrients during its passage through the upper digestive tract and allows its release in the intestine, which increases the level of usage of a compound encapsulated in alginate particles[32].

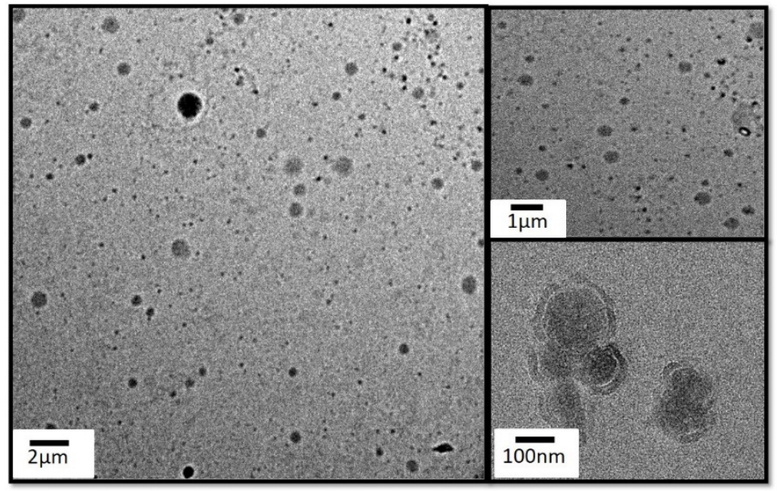

Figure 4 - TEM micrographs of nanoparticles prepared with Zn^{2+} ([0.15] mol/L).

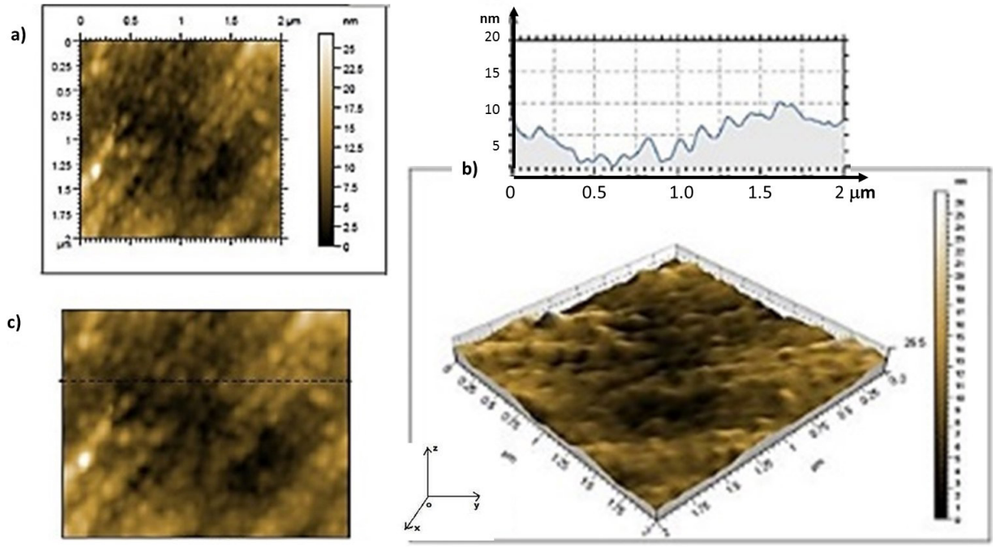

Figure 5 - Atomic force micrographs of alginate particles prepared with 0.25 mol/L of Zn^{+2} (a) Micrograph of an area corresponding to 2µmx2µm; (b) 3D image of the observed surface; c) Profile extracted in horizontal line

Table 1 - Encapsulation efficiency of the ions into each particles.

Sample	%EE
Zn1	71,99
Mg1	61,35

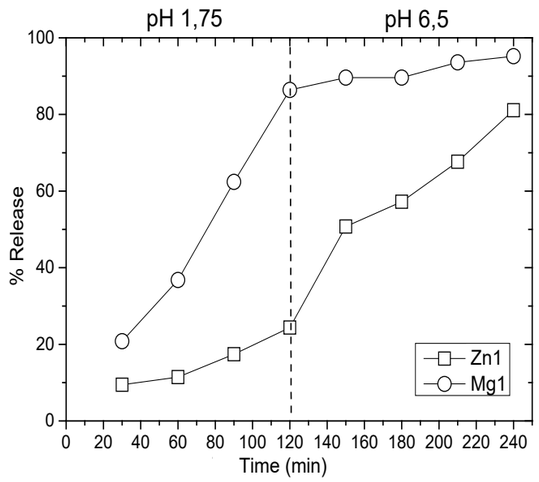

Figure 6 - *In vitro* release of Mg^{+2} and Zn^{+2} contained in the particles in digestive gastric and intestinal medium without enzymes

Conclusions

The ion concentration and its chemical characteristic are parameters with the greatest effects on morphology and size of the nanoparticles, which were prepared with higher Zn^{2+} concentrations show smoother surface morphologies and unimodal distribution with low polydispersity index as compared with lowest Zn^{2+} concentration. Higher Zn^{2+} concentration could saturate the guluronic (G) nucleus, and it is proposed that the ions could be binding both to G and M units of the alginate molecule for forming particles at the nanoscale and increasing their amount, as was shown by SEM and TEM images. Typically, and until now, has been reported that Mg^{+2} ion could not cause gelation of the alginic acid. However, this research demonstrates that gelation occurs. In the case of Mg^{+2} ion, an increase of ion concentration is necessary to obtain particles and additionally considering that ionic crosslinking relies on a micro-emulsion process to guarantee the stabilization of the particles. This result was evident after TEM and AFM microscopy, which also allowed verifying that these particles have sizes in the nanometer scale. The latter suggests that these micro/nanoparticles can be used as a carrier for essential nutrients for food fortification, as well as for others applications in biomedicine or charge drugs in delivery systems. Also, the results of this publication related to the use of Mg^{+2} as an ionizing and nucleating agent for the formation of alginate particles is a significant contribution and expansion of the possible use of this biopolymer, and they also weaken a belief that has been considered for several years that magnesium could not gel alginate.

The experiments carried out in this research, where gastrointestinal media without enzymes was simulated, represent a key tool for the evaluation of future applications of the nanoparticles obtained considering that the utilization of the nutrients occurs to a greater extent in the intestinal tract. Also, the particles obtained can be used successfully to fortify foods, or even to release other types of biomolecules that can be ingested orally.

Acknowledgments

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