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# DESIGN OF INSULIN–LOADED ALGINATE NANOPARTICLES: INFLUENCE OF THE CALCIUM ION ON POLYMER GEL MATRIX PROPERTIES

*Alginate–based nanoparticles were produced by dispersing alginate aqueous solution containing an insoluble calcium salt within mineral oil forming a water–in–oil emulsion. Subsequently, alginate gelled upon contact with the calcium ions due to the physical cross–linking between the carboxylate anions of the alginate and the calcium ions. The influence of the calcium salt, added in varying amounts, on gel integrity and on particle size was investigated. The efficiency of encapsulating active biological compounds by nanoparticles was also assayed. The calcium concentration was seen to be a crucial parameter in particle production, influencing the particle size, the viscosity of the solutions at different stages of the emulsification/gelation process and, finally, the encapsulation efficiency. The most appropriate mass relation between calcium and alginate was 7% (w/w). Under this condition, the smallest mean diameter obtained was 2.604 ± 2.141 μm combined with the narrowest range of particle sizes. The encapsulation efficiency of insulin was over 71%. These previous characteristics appear to be best suited for producing small, well–dispersed and stable nanoparticles with high encapsulation of insulin. This particulate system may be considered as a promising carrier for the oral delivery of insulin.*

*Key words: Alginate, Emulsification/internal gelation, Insulin, Nanoparticles.*

In humans, blood glucose and insulin are maintained within a narrow range despite wide variations in physical activity and dietary intake. At present, reproducing this pattern is an impossible task in diabetes mellitus type 1 and extremely difficult in diabetes mellitus type 2 [1]. Though many approaches for treating diabetes type 1 have arisen throughout the years, insulin therapy remains the mainstay of managing the disease. Insulin therapy is subject to the many liabilities associated with products the active ingredient of which is a chemically labile molecule. To improve patient compliance and disease management, insulin has been incorporated and studied in conjunction with a variety of drug delivery approaches and alternative routes of administration. The oral route takes advantage over the portal–hepatic route of absorption [1] and it is the preferred route for patients on chronic therapy; however, oral insulin remains an unresolved challenge mainly due to its physicochemical characteristics. The use of colloidal carriers such as particles with size less than 10 μm made of hydrophilic polysaccharides, e.g. alginate, has arisen as a promising alternative for improving the bioavailability of insulin [2]. Alginates are a family of linear unbranched polysaccharides which contain varying amounts of 1, 4–linked –D–mannuronic acid (M) and α–L–guluronic acid residues (G) [3]. Alginate is a naturally occurring copolymer widely used in biomedical applications and is capable of being processed under mild conditions [4].

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Alginate nanoparticles can be produced by several methods, one being the emulsification/internal gelation protocol [2]. This method has been used to prepare larger microspheres or beads [5,6] and is actually an alternative to conventional external gelation in the microencapsulation of peptidic drugs. In this study the method was significantly changed and a new protocol was applied and extended toward the production of nanoparticles. The internal gelation method uses fine, insoluble calcium carbonate microcrystals as an internal calcium source for gelation. Calcium is released from the calcium complex upon pH reduction from 7.5 to 6.5 by the addition of an oil–soluble acid [6]. The mass relation (w/w) between calcium and alginate is an important factor to the resultant structure of the alginate gel network. According to previous literature [6], a calcium–alginate monomer ratio of 1/4 (25 mM calcium/ 100 mM alginate monomer) appears to be sufficient to ensure strong bead formation. The main purpose was to investigate the most appropriate calcium–alginate mass relation (Ca–Alg, 2.5–16.6%, w/w) in terms of particle size distribution, alginate solution viscosity, insulin encapsulation efficiency (E.E.) and finally insulin release at pH 1.2. Due to the importance of the acid–calcium molar ratio (Ac–Ca, 1.5–3), the influence of this ratio on the same parameters was also studied.

## EXPERIMENTAL

### Materials

Low viscosity sodium alginate (supplier's specifications: viscosity of 2% solution at 25°C, 250 cps) and dextran sulfate (approx. MW 5 kDa) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Setacarb 06 calcium carbonate was obtained from Omya (Orgon, France). Paraffin oil was supplied from

Vaz Pereira (Lisbon, Portugal). The emulsifier, Span 80, was purchased from Fluka, Chemie GmbH (Buchs, Switzerland). Insulin was kindly donated by *Hospitais da Universidade de Coimbra* (Actrapid Insulin® from Novo Nordisk, Bagsvaerd, Denmark). All other chemicals and reagents were of reagent grade or equivalent.

### Preparation of nanoparticles

The preparation method of alginate nanoparticles was adopted from the emulsification/internal gelation technique described by *Poncelet et al.* [5]. By controlling the conditions [2] under which the water–in–oil emulsion is produced, the size can be controlled from nanometers to a few microns in mean diameter. Briefly, a 2% (w/v) aqueous solution of sodium alginate and dextran sulfate (0.75 %, w/v) was prepared by suspending the polymer and adjuvant in distilled water. This solution was stirred overnight on an orbital shaker. The solution stood for at least one hour to allow deaeration. Insulin (35 mg) was added to the previous solution. An aqueous suspension of calcium carbonate (5%, w/v) was sonicated for 30 min and then added to the previous solution (Ca–Alg mass relation ranging from 2.5 to 16.6%, w/w). This dispersion was emulsified within paraffin oil containing an emulsifier (1.5% v/v, Span 80). This water–in–oil emulsion was prepared by using a mixing impeller (1600 rpm/15 min). After 15 min of emulsification and continued agitation, gelation was induced by the addition of 20 mL of paraffin oil containing different amounts of glacial acetic acid (Ac–Ca molar ratio, ranging from 1.5 to 3). After 60 min, the oil–particle suspension was added with gentle mixing to an acetate buffer solution (pH 4.5, United States Pharmacopeia, USP XXVIII) with dehydrating solvents (100 mL) followed by centrifugation (10000 rpm during 10 min). The nanoparticles were frozen in an alcohol bath –50°C and lyophilized (Lyph–lock 6 apparatus®, Labconco, Kansas City, MS, USA) at 0°C for at least 48 h.

### Size distribution

Size distribution analysis was performed by laser diffraction spectrometry using a Coulter LS130® granulometer (Beckman Coulter Inc., Fullerton, CA). The mean diameters of the aqueous suspension of hydrated nanoparticles were calculated in triplicate. The size distribution was estimated with the SPAN factor, which was defined by the ratio $[(D_{90}–D_{10})/D_{50}$Ć [7], where $D_{90\%}$, $D_{50\%}$ and $D_{10\%}$ are the mean diameters at which 90, 50 and 10% (cumulative volume %) of the nanoparticles are counted and calculated. A high SPAN indicates a wide distribution in size, whereas a low value indicates a narrow size distribution.

### Alginate solution viscosity during the gelation step

To simulate the gelation process, 100 mL of aqueous solution of alginate (2%, w/v), designated *Alg*, were stirred at 200 rpm. For a specific Ca–Alg mass relation, different amounts of calcium carbonate (5%, w/v) were added to the pre–formed solution of alginate and this dispersion was designated *Alg–Ca*. Finally, gelation was induced upon pH reduction by the addition of glacial acetic acid. The resulting gel was designated *Alg–Ca–gel*. The viscosity was measured using a rotational viscometer (Visco Star plus®, Fungilab, S.A., Barcelona, Spain) in triplicate at 200 rpm for all states *Alg, Alg–Ca* and *Alg–Ca–gel*. All the measurements were carried out at 25 ± 0.1 °C. The pH values of the previous states were also determined using a pH meter. The determinations were carried out in duplicate and the results averaged.

### Insulin E.E. and release at pH 1.2

Assuming that all the encapsulated insulin was released from the alginate nanoparticles after incubation under simulated gastrointestinal conditions, the encapsulation efficiency of insulin (E.E.) was determined upon complete dissolution of the alginate matrix. Ten milligrams of lyophilized insulin–loaded alginate nanoparticles prepared as described above were incubated in 10 mL of hydrochloric acid buffer at pH 1.2 (United States Pharmacopeia, USP XXVIII). Samples were collected and centrifuged (10000 rpm/15 min). An aliquot of each supernatant was collected to be quantified. A pellet of nanoparticles was re–suspended and transferred into phosphate buffer at pH 6.8 (United States Pharmacopeia, USP XXVIII). Similarly, samples were collected and centrifuged (10000 rpm/15 min). The supernatant was collected and the protein was quantified spectrophotometrically at 595 nm using the Bradford method. The insulin E.E. and release at pH 1.2 (%) were then calculated according to equations (2) and (3), respectively: (2) E.E. (%) = [Insulin mass released/Initial mass of insulin used] x 100 and (3) Insulin release pH 1.2 (%) = [Mass of insulin released at pH 1.2/Total mass of insulin released (pH 1.2 + pH 6.8)] x 100.

## RESULTS AND DISCUSSION

Alginate is a multi–functional biopolymer and has been the focus of much research in biomedical and pharmaceutical applications because of its biocompatibility and biodegradability, and by the fact that it is non–toxic and abundant in naturally occurring raw materials. As commonly described, alginate is a water–soluble polymer, but gels in the presence of cations such as barium, strontium and calcium [8]. Calcium is the most popular cross–linking agent for alginate [8]. This study involved the preparation and characterization of alginate nanoparticles for drug delivery systems. Sodium alginate is formed into a gel when contacted with calcium ions in solution by cross–linking between the carboxylate anions of the alginate guluronate units and the calcium ions [9]. The

*Table 1. Size distribution with mean and SPAN values for different Ca–Alg mass relations and Ac–Ca molar ratios*

| Parameter | | Mean Size (μm) ± SD | SPAN |
|---|---|---|---|
| Calcium–Alginate Mass Relation (%) | 2.5 | 4.147 ± 2.659 | 1.92 |
| | 5 | 4.855 ± 3.578 | 2.35 |
| | 7 | 2.604 ± 2.141 | 1.52 |
| | 10 | 3.306 ± 3.394 | 1.51 |
| | 16.6 | 3.409 ± 2.604 | 2.51 |
| Acid–Calcium Molar Ratio (moles acid per moles calcium) | 1.5 | 1.651 ± 1.062 | 1.85 |
| | 2 | 1 ± 1.299 | 2.38 |
| | 3 | 1 ± 0.979 | 1.53 |
| | 3.5 | 2.421 ± 2.604 | 2.51 |

* SD means standard deviation.

concentration of calcium ions is then crucial in influencing the particle size distribution, the alginate solution viscosity during the gelation process, the pH variation during the dispersion/gelation process, the insulin E.E. and the release at pH 1.2.

### Size distribution

The calcium salt concentration demonstrated a significant influence on particle size, as seen in Table 1 and Figure 1. All the mass relations for which calcium–alginate was formulated were in the desired range of less than 10 um. The smallest size and narrowest size distribution was observed with a Ca–Alg mass relation of 7% (2.604 ± 2.141 μm). For lower mass relations, the nanoparticles agglomerated and caused a slight increase in the mean particle size. For higher mass ratios, the mean particle size increased, probably due to some residual calcium carbonate grains that might not have been completely solubilized. With increasing calcium salt concentration, the mass of calcium ions per unit volume inside the liquid core increases and more calcium ions bind to the alginate chains [10]. Thus, it is reasonable that the mean diameter increases with calcium salt concentration. Relative to the Ac–Ca molar ratio, it did not demonstrate an influence on the mean particle size. However, an Ac–Ca molar ratio of 3.5 showed the highest value of the mean size and an increase of the SPAN value, probably due to some agglomeration. The SPAN values varied from 1.51 to 2.51 which might suggest that both parameters had a considerable influence on size polydispersity.

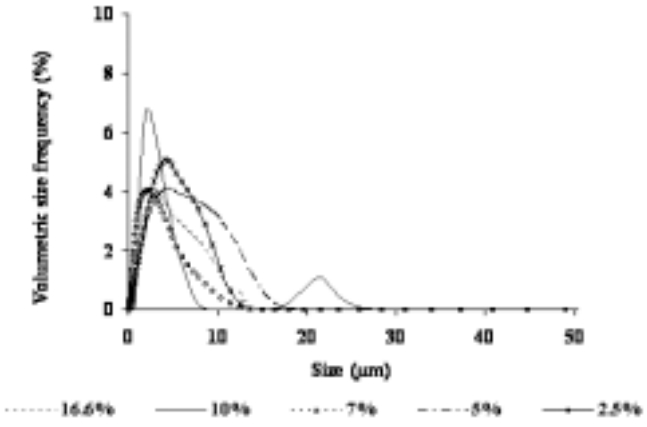


*Figure 1. Example of a narrow and a monodisperse volumetric size distribution of alginate nanoparticles for different calcium–alginate mass relations*

### Alginate solution viscosity during the gelation process

Concerning the matrix properties, the most stable gel and uniform matrix was verified with a Ca–Alg mass relation of 7% (Figure 2). When no calcium was present, *Alg* exhibited a Newtonian flow pattern. This flow behaviour was attributed to a low alginate concentration (2%, w/v) and low shear stress. Adding calcium to the alginate resulted in a viscosity decrease. After acidification, the viscosity was significantly increased due to alginate gelation. Above or below a Ca–Alg mass relation of 7%, poor quality gels with complex flow patterns were produced. The resultant gels were inhomogeneous (Figures 3 a, b, d and e) and led to a complex measurement of their viscosity.

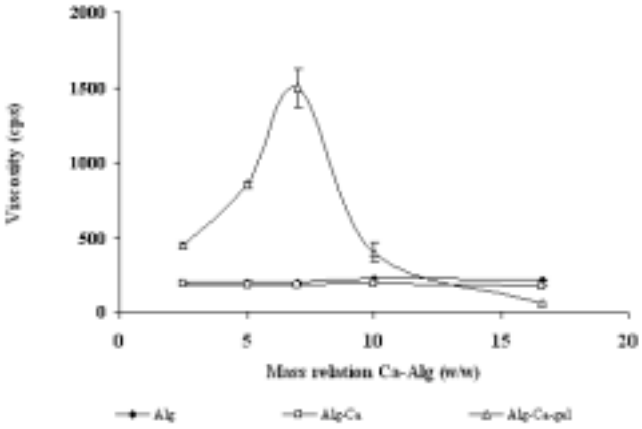


*Figure 2. Variation of alginate solution viscosity during the dispersion–gelation process*

### Influence of the acid–calcium molar ratio on the pH during the dispersion/gelation process

The pH variation during the process was also assayed. Alginate solutions are stable in a wide pH range of 4–10 at room temperature. At a pH of approx. 3.5 or lower, alginate precipitates in the form of alginic acid which is insoluble in water. At pH 4.5, alginate nanoparticles demonstrated high–quality gel properties. According to Figure 4, the final pH for all the

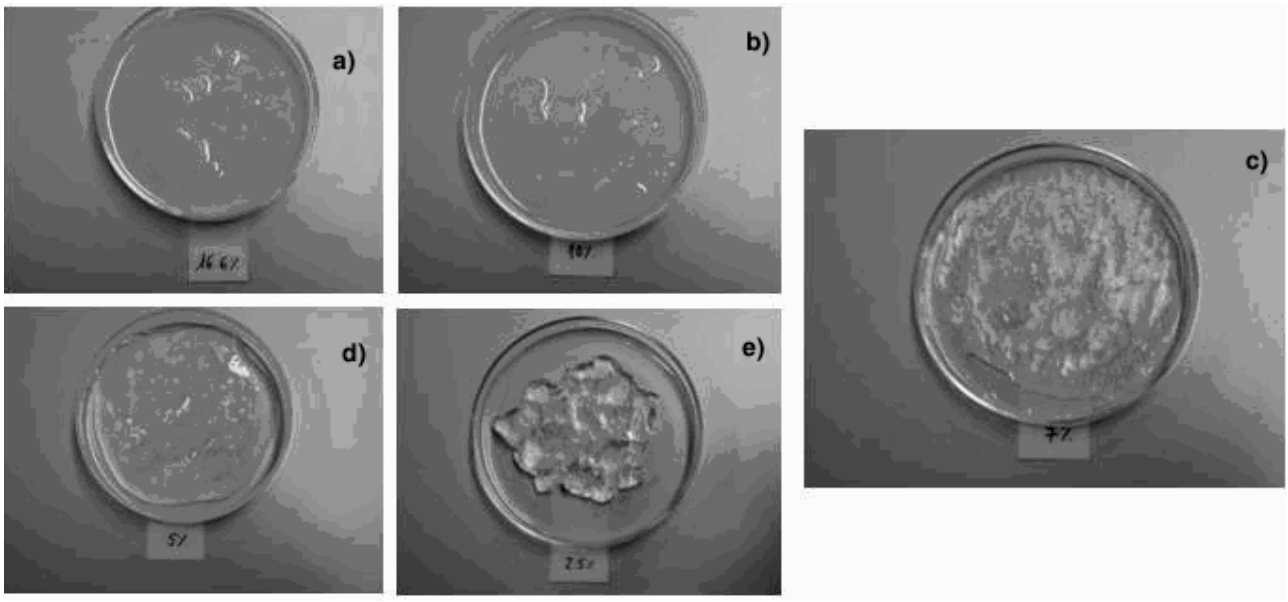


*Figure 3. Different Ca–Alg mass relations and their physical characteristics: a) 16.6%, b) 10%, c) 7%, d) 5% and e) 2.5%*

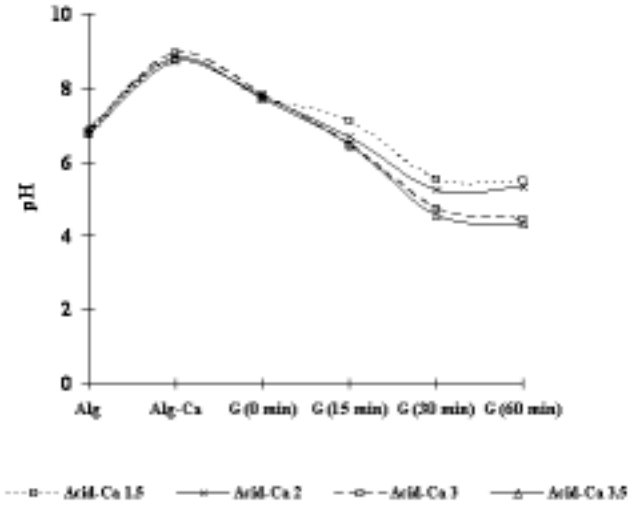


*Figure 4. Influence of the acid–calcium molar ratio on the pH during the dispersion–gelation process for different states: Alg, Alg–Ca, Alg–Ca gel at 0, 15, 30 and 60 min of the gelation process (G (0 min), G (15 min), G (30 min) and G (60 min))*

formulations was lower than 6. In the literature [11], a certain buffer property of alginate has been reported, likely explaining the maintenance of the final pH. Moreover, an Ac–Ca molar ratio of 3 demonstrated the same pH as the pH of storing the buffer solution, which can be accepted as a technological advantage for insulin stability inside the alginate nanoparticles.

### Insulin E.E. and release at pH 1.2

The insulin E.E. improved with an increase of the Ca–Alg mass relation (Figure 5 a). A higher calcium concentration means more free calcium ions available to react with M and, especially, with the G monomer of alginate. As the level of calcium increases, the number

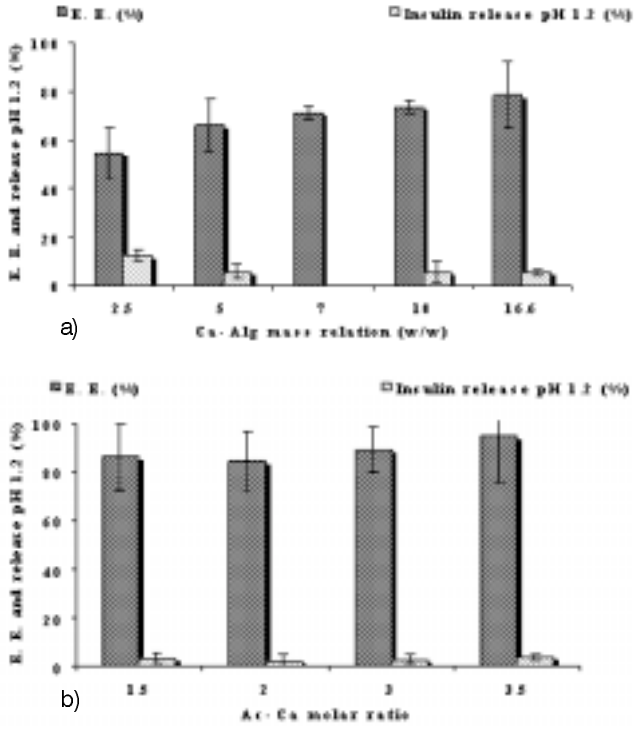


*Figure 5. Insulin E.E. (%) and its release at pH 1.2 (%) for Ca–Alg (a) and Ac–Ca (b)*

of cross–links increases for a given sodium alginate concentration until an insoluble calcium–alginate gel is formed, probably due to a decrease in the flexibility of the polymer chains, which were partially held together by the calcium ions, reducing the subsequent interaction with the calcium ions and resulting in a more permeable matrix [12]. Consequently, a lower drug encapsulation efficiency was observed. It was thought

that a more orderly arrangement of the polymer chains would give rise to a stronger and less permeable matrix capable of sustaining drug release [12].

Generally, drug release from alginate matrices is modulated by a swelling–dissolution–erosion process. Under acidic conditions, alginate matrices do not swell or erode, due mainly to alginic acid precipitation. Nevertheless, under neutral conditions, the matrix tends to swell, influencing drug release. The swelling process is further enhanced by the presence of phosphate ions, which act as a calcium sequestrant [13]. When immersed in phosphate buffer pH 6.8, the alginate matrix immediately begins to swell, recovering its initial production spherical shape, and begins to erode.

Relative to insulin release at pH 1.2, a Ca–Alg mass relation of 7% demonstrated an absence of insulin release probably due to the same previous explanation and to gel stability demonstrated in the preceding study. Considering the reaction between glacial acetic acid and the calcium carbonate, each mole of the calcium complex reacts with two moles of acid [14]. However, different ratios were studied including lower and higher acid–calcium ratios.

$$2H^+ + CaCO_3 \longrightarrow Ca^{2+} + H_2O + CO_2$$

$$Ca^{2+} + 2Na^+Alg^- \longrightarrow Ca^{2+}(ALG^-)_2 + 2Na^+$$

Higher molar ratios (corresponding to an excess of acid, 3:1 or 3.5:1) increased the insulin E.E. (Figure 5 b), probably due to complete gelation of the alginate. In addition, the final pH after acidification was approximately 4.5. We hypothesized that the pH decrease reinforced the electrostatic interactions between the alginate (negatively charged due to its pKa values of 3.38 and 3.65 for M and G residues, respectively [3]) and insulin (positively charged due to its isoelectric point of 5.3 [15]). This may explain the negligible increase of the E.E. with increasing Ac–Ca molar ratio. In the case of insulin release at pH 1.2, the lowest value was observed with an Ac–Ca molar ratio of 2 followed by an Ac–Ca molar ratio of 3. As in previous studies, [16], this could be initiated by intense gelation due to more efficient calcium release from the insoluble calcium carbonate complex. When glacial acetic acid was insufficient (Ac–Ca molar ratio of 1.5) to dissolve all the calcium complexes, higher insulin losses during manufacture were observed, probably due to incomplete alginate gelation.

## CONCLUSIONS

The alginate degree of gelation is essential for the retention of protein inside the nanoparticles. In general, an optimal calcium concentration for the design of a particulate carrier system must be chosen based on several influencing factors. The calcium concentration and acid required to dissolve the calcium complex seem to be critical factors in the production of alginate nanoparticles. Small particles with a narrow size distribution, high encapsulation efficiency and low insulin release at pH 1.2 were obtained with a Ca–Alg mass relation of 7% and an Ac–Ca molar ratio of 3. There is an optimal level of calcium for maximum gel strength. In this case, a Ca–Alg mass relation of 7% was demonstrated to be the most adequate mass relation. The results indicate that an insulin–loaded alginate formulation was obtained, with high encapsulation efficiency and produced by a mild encapsulation technique, presenting promising characteristics which may be particularly well suited for the oral administration of insulin.

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## IZVOD

METODA ZA INKAPSULACIJU INSULINA U ALGINATNE NANOKAPSULE:
UTICAJ KALCIJUM JONA NA PONAŠANJE DOBIJENIH KAPSULA

(Naučni rad)

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Alginatne nanočestice su proizvedene metodom emulsifikacije vodenog rastvora alginata u ulju. Mehanizam geliranja dolazi neposredno posle kontakta alginata I kalcijum jona. Ispitivan je uticaj koncentracije kalcijum jona na dobijene mikro kapsule. Koncentracija kalcijuma, kako je pokazano eksperimentima, ključni je parametar za formiranje veličine mikročestice i stepena inkapsulacije, a optimalna vrednost potvrđena eksperimentima bila je oko 7% (maseni odnos kalcijum/alginat, w/w). Pod tim uslovima srednja veličina čestica je iznosila 2.64 + 2.141 μm, uz usku raspodelu (jednomodalnu), sa stepenom inkapsulacije insulina od oko 70%. Ovako planiran eksperiment je obećavajući jer se dobijanjem čestica nanometarskih veličina može u budućnosti očekivati njihova primena za oralno doziranje insulina.

Ključne reči: Alginat, Emulzifikacija, Geliranje, Insulin, Nanočestice.