Microcapsules of alginate/chitosan containing magnetic nanoparticles for controlled release of insulin

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A B S T R A C T

The challenge of this work was to investigate the potential of alginate/chitosan beads containing magnetite nanoparticles as a drug delivery system. The insulin beads were prepared by dripping a solution of sodium alginate containing insulin into a CaCl₂ solution. Magnetite nanoparticles of 5 nm mean size were synthesized inside the alginate egg-box structure by co-precipitation of Fe(III) and Fe(II) in the presence of NH₄OH. Quantitative analysis revealed that insulin encapsulation depends on the initial protein content and 35% of insulin was entrapped by alginate beads for a protein concentration of 10 wt%. It was verified that approximately 50% of the insulin was released to Milli-Q water in 800 h release experiments. The application of oscillating magnetic field increased three fold the insulin release. The results suggest that the alginate/chitosan system containing magnetite nanoparticles is a promising system for clinical applications of controlled release of insulin in the presence of an oscillating magnetic field in a subcutaneous implant approach.

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1. Introduction

Since its discovery in the decade of 20, the insulin has been managed through injections and other invasive methods. The discomfort associated with this type of administration has led diabetic patients of type I to neglect and even to give up the therapy. For these reason alternative procedures of insulin administration using controlled release systems such as protein [1], lipids [2] and polysaccharides [3,4] have been proposed. Insulin encapsulation in polymeric matrices as ethylene vinyl acetate, chitosan, polylactic acid, ethyl cellulose, has been tested [5].

Biopolymers are chosen predominantly to produce microcapsules because of the advantages of biocompatibility and biodegradability. Beyond that, the biopolymer can be associated to a specific device which could control the drug release. Alginate has been considered one of the most suitable biopolymer for microcapsules production; its composition and sequential structure has a great importance for its function as encapsulation material [6].

It contains two uronic acids, β-(1-4)-linked d-mannuronic acid (M) and α-(1-4)-linked l-guluronic acid (G), and is composed of homopolymeric blocks M–M or G–G, and blocks with an alternating sequence of M–G blocks [7–9]. In addition, sodium alginate has a unique property of cross-linking in the presence of multivalent cations, such as calcium ions in aqueous media, which rather complex with G–G sequences in the polymer chain to form the ‘egg box junctions’. Alginate forms a reticulated structure in contact with calcium ions and this network can entrap proteins [10].

Chitosan is a linear copolymer polysaccharide consisting of β-(1–4)-linked 2-amino-2-deoxy-d-glucose (d-glucosamine) and 2-acetamido-2-deoxy-d-glucose (N-acetyl-d-glucosamine) units [10]. In general, it is produced commercially by deacetylation of chitin, which is the structural element in the exoskeleton of crustaceans. The strong electrostatic interaction of the amino groups of the chitosan with the carboxylic groups of the alginate leads to formation of the complex chitosan/alginate that becomes the microcapsule more resistant to the release of molecules [11].

The polyelectrolyte complex between chitosan and alginate has been widely used in order to obtain microcapsules for cell encapsulation and devices for the controlled release of drugs or other substances. In these systems the efficiency of encapsulation or drug...
release is directly dependent on pore dimension of the polymeric network. According to Papasakis and Bouropoulos coating of the calcium–alginate beads with chitosan caused significant reduction of micro/macropores and cracks observed on the surface and thus a decrease of its permeability [10].

Edelman and Langer defended that the release of biomolecules, from polymeric matrices, could be regulated by the movement of magnetic particles inside the matrix [12]. The magnitude of the release was directly connected to the intensity of the magnetic field, the magnetization of particles and the number of movements of particles in matrix for a specific period increases with frequency of the magnetic field. Sławska et al. produced beads of insulin in alginate/polysilysin matrix recovered with polyethyleneimine for a magnetic controlled release [13]. Particles of ferrite strontium in micrometer size were incorporated into alginate. The authors applied a magnetic field of 8900 G with the frequency of 4 Hz and verified that the release rate was 50 times bigger in the presence of the magnetic field, even though a fraction of insulin had been released also by passive diffusion. However, the mechanism responsible for the insulin release is not entirely clarified so far [13,14].

In order to control the release several nanostructured devices has been used as modulating agents in open-loop delivery systems. In this work nanoparticles of iron oxide were used to generate mechanical impulses to modulate the rate of protein delivery from alginate/chitosan matrix. The synthesis of biocompatible superparamagnetic materials has been used in biomedical applications including magnetic resonance imaging for clinical diagnosis, tissue engineering, magnetic drug targeting, hyperthermia anti-cancer strategy, drug delivery and enzyme immobilization [15,16]. Iron oxides such as magnetite (Fe₃O₄) and maghemite (γ-Fe₂O₃) have been suggested for biotechnological applications because of their biocompatibility and magnetic properties. In our previous work we synthesized and characterized these nanoparticles, therefore the combination of magnetic properties of iron oxide nanoparticles with the biocompatibility of calcium-alginate suggests that these materials have a great potential to be used as drug delivery system [17]. In the current study, maghemite nanoparticles were synthesized inside alginate/chitosan matrix in order to obtain a system of controlled release of insulin through interaction with an applied oscillating magnetic field.

2. Materials and methods

2.1. Materials

Sodium alginate was purchased from Keltoine LV. Sodium alginate solutions (2%, w/v) had viscosity at 25 °C and 60 rpm (No. 2 spindle) of 100–300 mPa s, as determined with LV model of the Brookfield viscosimeter. The ratio of mannnuronic acid to guluronic acid residues (M/G) was between 0.4 and 1.9. The ferric chloride (FeCl₃·6H₂O) was obtained from Nuclear Inc. The solution of ferrous chloride (FeCl₂) was prepared by a reaction between FeSO₄·7H₂O (Química Moderna) and CaCl₂·2H₂O (Isofar) in a stoichiometric ratio. The methanol and ammonium hydroxide were obtained from Synth and Merck, respectively.

Insulin (Mw 6000 Da) was purchased from Lilly; this insulin is manufactured using genetic engineering techniques from human DNA. High molecular weight chitosan (Mw 474 kDa) was purchased from Sigma; calcium chloride and all other chemicals were obtained from Merck.

2.2. Preparation of alginate beads containing insulin

The beads containing insulin were prepared in triplicate by extrusion method (dripping method). Sodium alginate was dissolved in distilled water at a concentration of 3% (w/v) and a pre-calculated quantity of insulin was added (10%, w/v). The solution was stirred thoroughly to ensure complete mixing of drug. The alginate/insulin mixture was dropped into calcium chloride solution (2%, w/v) under constant stirring at 300 K. After gelation the beads were maintained for a period of 10 min in the CaCl₂ solution. The beads were then removed from the CaCl₂ solution and washed several times with Milli-Q water.

2.3. Preparation of alginate/chitosan beads containing insulin

Firstly the insulin was entrapped into alginate by extrusion as described above. Then for the recovering with chitosan the beads were placed in the 3 mg/mL chitosan solution under stirring for 60 min at 25 °C. In the literature, this method is generally called two step method. The chitosan was previously dissolved in acetate buffer pH 5.0 (0.02 mol/L of sodium acetate/acetate acid 1%). Lastly the beads were taken out and washed several times with Milli-Q water. Beads containing different contents of insulin were prepared by the same described procedure.

2.4. Efficiency of entrapment of insulin in beads

The insulin content of the beads was determined spectrophotometrically (λ = 266 nm; HACH DR/4000V). The alginate and alginate/chitosan beads loaded with insulin were completely dissolved in phosphate buffer pH 7.4 and measured. Preliminary UV studies showed that the presence of dissolved polymers did not interfere with the absorbance of the drug at 266 nm. The efficiency (%) of entrapment was calculated using the following formula:

\[
efficiency (\%) = \frac{mass \ of \ insulin \ present \ in \ beads}{mass \ of \ insulin \ in \ the \ formulation} \times 100
\]

2.5. Synthesis of iron oxide nanoparticles into alginate/chitosan beads

The iron oxide nanoparticles were synthesized using the procedure described by Morales et al. [17]. A solution of Fe(III) and Fe(II) ions in an equal molar ratio was prepared from FeCl₃·6H₂O, FeCl₂, and deionized water. Then, the iron solution was mixed with the alginate/chitosan beads containing insulin and kept at a constant temperature of 60 °C for 15 min. Next, ammonium hydroxide solution 25% (v/v) was added dropwise to the iron–polymer mixture to maintain the pH in the range of 11–12, while the mixture was kept at 60 °C for another 15 min, while being stirred vigorously. The beads containing the iron oxide nanoparticles were then washed several times in methanol solution (50%, v/v) and dried in the oven at 35 °C for about 24 h. The characterization of iron oxide nanoparticles were detailed in a previous work [17].

2.6. Beads microstructure and magnetic characterization

The microstructures of the magnetic alginate beads were studied by scanning electron microscopy (SEM). Randomly selected dried beads were deposited on double-coated carbon conductive tape previously adhered to SEM aluminum stubs. The beads samples were then sputter coated with a thin gold layer using a coating unit (Balzers Union model FL 9496), and analysed in a JEOL JSM 5310 operated at 15 or 20 kV. In order to assess the fine structure of the alginate/chitosan–magnetite composite, at the nanometer level, the beads were embedded in epoxy resin, ultrathin-sectioned using a RMC ultramicrotome, and analysed by transmission electron microscopy (TEM). Conventional TEM was performed in a JEOL
1200 EX, while elemental mapping was done in a Zeiss CEM-902. Iron image (Fe-L$_{2,3}$ edge) was obtained using inelastically scattered electrons (two window method with the following parameters: pos edge, 720 eV; pre-edge, 690 eV; energy-slit width, 20 eV; operating voltage, 80 kV). The iron map was obtained by subtracting the pre-edge image from the pos-edge one after normalizing the background intensity in both images (for details of the method, see [18]).

Magnetic characterization was performed using a Quantum Design MPMS-5S SQUID magnetometer at a magnetic field of 100 Oe.

### 2.7. In vitro insulin release studies

The in vitro insulin release profiles of beads were followed in 20 mL of Milli-Q water for 800 h under mechanical stirring rotation of 100 rpm at 37 °C. At predetermined time intervals agitation was stopped, 1.5 mL of the samples were withdrawn and replaced with fresh medium. The insulin content was determined spectrophotometrically at 266 nm.

In the experiment of controlled release, the magnetic field was generated from a prototype designed specifically for application in beads as shown in Fig. 1. It consists of a disc with two cylindrical neodymium permanent magnet (NdFeB, 10 × 10 mm) fixed radially on its ends. The disc with the magnets was fixed in a motor which could rotate at 33 Hz inducing an oscillating magnetic field. The magnetic field of was 1800 G for samples located at 0.5 cm from the rotating disk. The release tests were carried out using eppendorf containing 1.5 mL of Milli-Q water and the magnetic beads. At predetermined time intervals the oscillating magnetic field was stopped, all the volume of the samples were withdrawn and replaced with fresh medium. The insulin content was determined spectrophotometrically at 266 nm.

### 2.8. Preliminary in vivo studies

Swiss male mice (10 weeks) were divided into two groups: control microcapsules ($n = 3$) and insulin microcapsules ($n = 3$). For the subcutaneous microcapsules implantation, the animals in a fed state were sedated with ethyl ether but not placed under general anesthesia. The area of microcapsules implantations were shaved and surgically prepared under sterile conditions in laminar flow equipment. Subcutaneous implantations of microcapsules were performed in a longitudinal incision of approximately 1.5 cm on the dorsal region, followed by creation of a small cavity that received the material. Control group received 10 mg of microcapsules without insulin and the other group received 10 mg of insulin microcapsules. The material was inserted into the subcutaneous incision with a glass Pasteur pipette and the incision closed with sutures. Blood glucose concentration was determined using a glucometer (Accu-Chek Active – Roche) at time zero after the subcutaneous implantation of the control microcapsules or insulin microcapsules. After the chirurgical process mice were starved and glycemia was monitored at 2 and 6 h. Eight hours later, mice were refeed and glycemia monitored again at time of 22 h. During the experiments, mice were housed in a temperature-controlled room with a 12-h light and dark cycle and given free access to water. The animal protocol was approved by the internal institutional animal care and use committee [21].

### 3. Results and discussion

The dripping technique produced spherical droplets that, after falling down in the CaCl$_2$ solution, resulted in spherical thermostable gel particles due to the ionic interactions between guluronate blocks from alginate and Ca$^{2+}$ ions. Insulin-loaded alginate/chitosan beads containing iron oxide nanoparticles showed a spherical geometry ($1–1.5$ cm diameter) and a compact structure as evidenced by the SEM analysis reported in Fig. 2a. The beads surface exhibited a non-homogeneous microstructure constituted by polyhedral particles of variable sizes (Fig. 2b). The particles arrangement originates a surface porosity with pores dimension of few micrometers. Fig. 2c shows a section just below the bead surface. The image reveals the existence of an external shell constituted by elongated particles radially oriented. This external shell may be associated to the first bead gelation layer. The bead interior in Fig. 2d presented a distinct morphology from the external bead layer. It is composed by smaller particles in a more compact arrangement than the external shell. The existence of two different morphologies is a consequence of the external gelation process. There was observed any influence of insulin on that morphology.

In order to assess the fine structure of the alginate/chitosan–magnetite composite, at the nanometer level, a transmission electron microscopy (TEM) was used. The small beads, produced as previously described (see Section 2), were embedded in epoxy resin and ultrathin-sectioned using a RMC ultramicrotome. The analysed area was the bulk region of the bead, near the surface. It was observed that the magnetite nanoparticles presented a narrow distribution of diameters and were homogeneously distributed in the whole area (Fig. 3 and inserts). This fact means that the organization pattern of the inner structure of the composite was reproducible allowing a correlation between structure and behavior of the whole ensemble of synthesized spheres.

At 300 K the M(H) loop showed negligible coercivity field (Fig. 4). The loop was fit to a Langevin function weighted with a lognormal distribution of particle size. The mean size and the standard deviation was 5.8 nm. Others results about characterization can be found in our previous work [17].
The insulin encapsulation efficiency was 33.3 ± 5.2% and 34.0 ± 5.0% for alginate and alginate/chitosan beads, for insulin concentration of 10 wt%, respectively. This result indicated that insulin was not lost from beads during the procedure of alginate beads coating with chitosan. The low insulin entrapment was due to insufficient cross-linking of alginate network which permitted the diffusion of insulin out from beads. The strength of the polymer cross-linking is related to the G/M ratio. Moreover, we must consider the insulin hydrophilic in nature and the small molecule size (6000 Da) which contribute to its diffusion out of the polymer structure. Gaumann et al. demonstrated the molecular weight cut-off in alginate capsules should be in the range of 60 000–200 000 Da [19].

The beads prepared with chitosan showed a tendency to agglomerate due to the adhesive properties of chitosan. Therefore constantly stirring was employed during the process to maintain the beads deagglomerated.
Beads containing different insulin contents had been prepared in order to understand the effect of protein concentration on encapsulation efficiency. Fig. 5 shows the relation between the entrapment efficiency and insulin initial concentration in beads. Increasing the insulin content from 1.7 to 7.5 mg/g of beads, the entrapment efficiency decreases from 65% to 28%. However, the total amount of entrapped insulin in the beads increased almost linearly with the protein concentration.

The purpose of these drug delivery systems is to implant the microcapsules subcutaneously, for which reason, the water release medium was chosen, instead of simulated gastric fluid or even a phosphate buffer, which present affinity to calcium and could influence the insulin release by alginate erosion. The release of insulin from alginate beads in Milli-Q water was monitored periodically until its concentration in the solution reached a constant value. It was verified that the insulin release occurs at least in three steps: a very fast release of about 18% in the first hour of the assay, which correspond to insulin physically entrapped to bead’s external layer. Afterwards the insulin release keeps constant up to 48 h. In the third stage (48–140 h) released insulin reached its maximum value, which corresponds to insulin physically entrapped to bead’s external layer. After 140 h of assay, the insulin concentration was constant until the end of the experiment (~800 h).

This many-stage release pattern is certainly due to the complexity of beads microstructure as presented before.

The kinetics of insulin release from alginate/chitosan bead showed a similar profile as the alginate beads without chitosan (Fig. 6). However, in this case, a significant reduction in the concentration of insulin released to solution was observed in all the steps of the process. The addition of chitosan provided a coating on the alginate beads surface increasing cross-linking density and decreasing pore size due to the polycationic property of chitosan. The chitosan is bound to alginate through strong ionic interactions between the carboxylate groups of alginate and the protonated amine groups in the chitosan. The consequence of this process was that less insulin was released from alginate/chitosan beads.

Electron paramagnetic resonance were performed previously and indicated that an isolated paramagnetic Fe$^{3+}$ ions may be in a structural site of polymer network probably substituting Ca$^{2+}$. A similar result was previously observed in alginate polymer cross-linked with Fe$^{3+}$ [20]. The relative fraction of this specie is small because the method used to prepare the samples prioritizes the crosslink of alginate units by Ca$^{2+}$; therefore there is no significance influence in insulin release.

Fig. 7 shows the release of insulin from alginate/chitosan beads containing iron oxide nanoparticles, with and without the application of a magnetic field. As mentioned before the application of an oscillating magnetic field induces an oscillatory movement of magnetite nanoparticles causing a widening of the chains in the structure of the polymer and promoting the insulin release. In the first 6 h the insulin release occurred independently of the application of magnetic field. This behavior corresponds to the protein fraction released from bead external layer (first stage of kinetics). For longer periods a significant increase of insulin release was evidenced in beads subjected to oscillating magnetic field, in comparison to control samples. This result proves the efficiency of the proposed drug delivery system based on the magnetic nanoparticles agitation induced by oscillating magnetic field in an alginate/chitosan matrix.

In order to prove that the insulin was not affected by the process of encapsulation and magnetic nanoparticle synthesis, preliminary in vivo studies were carried out. The aim was investigate if the insulin maintained its in vivo activity after release, independently of oscillating magnetic field application. Thus, the Fig. 8 shows the glycemia control during 24 h after the microcapsules implant.

Swiss mice were divided into two groups where one group received an implant of microcapsules without insulin (control) and the other group received an implant of microcapsules containing insulin. During 6h before the surgery, the animals were fed (ad libitum). At time zero, the animals were starved, thus the glycemia were reduced in both groups. After 8 h, an increase of glycemia...
Fig. 8. Blood glucose concentration of control microcapsules (light beads) and insulin microcapsules (black beads) after implantation. Data represent the mean ± SEM for three mice from each group (n = 3). *P < 0.05 compared with time zero and #P < 0.05 compared with control (Student’s t-test).

can be observed because the animals were fed (ad libitum) once more, however, this occurred only in the control group. Insulin group maintained a high insulinemia, therefore the glycemia did not rise despite feeding at least for 22 days. Furthermore, this fact confirmed that the protein conformation were not affected by all process involved in the production of magnetic microcapsules.

4. Conclusion

The alginate showed a low efficiency of encapsulation of insulin and this is attributed to the size of pores on the surface, allowing the release of the protein during the gelation and the washing of microcapsules. After treatment with chitosan, there was no reduction in the efficiency of encapsulation.

The alginate beads allowed more release of insulin than alginate/chitosan beads. Thus, the association of these two polymers reinforced the structure of beads and improved their impermeability, decreasing the release of insulin. The association of magnetic nanoparticles to the beads promoted a more intense release profile, when the oscillating magnetic field was applied. In addition, in vivo preliminaries tests established that the insulin released maintained its activity.

The results presented here encourage us to evaluate in deep the influence of the intensity and frequency of the applied field as well as the concentrations of the magnetic nanoparticles, in the release of insulin. This will be carried out by us in the near future.

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