Towards a new formulation of insulin: development of nanoparticles of insulin orally

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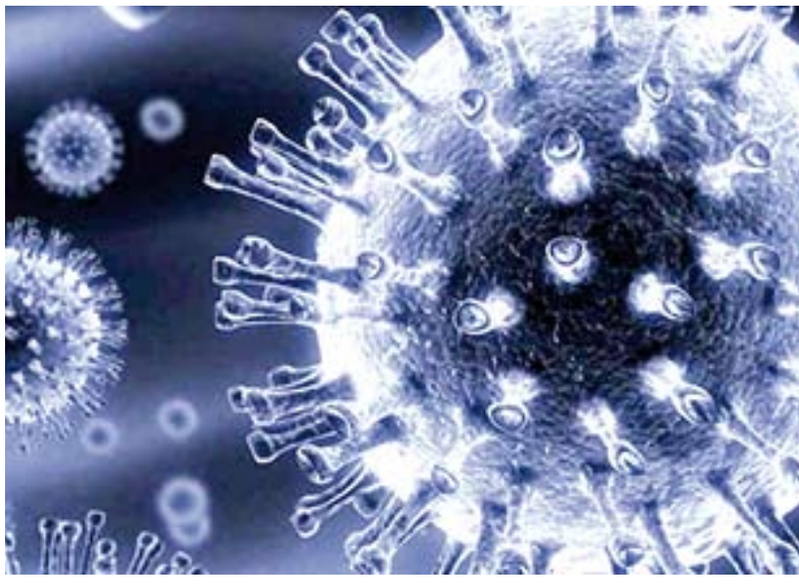
Abstract

The objective of this study was to develop alginate/chitosan nanoparticles to deliver a model protein drug insulin to protect it from gastric passage, using ionotropic pre-gelation method of an alginate core with calcium chloride followed by chitosan complexation. The characterization of different properties of prepared nanoparticles, such as particle size, zeta potential value, morphology, stability, structure, and drug loading was studied. The release of insulin from nanoparticles at acidic and alkaline environment was also evaluated. The results indicate that the insulin loaded mucoadhesive nanoparticles was a key factor in the improvement of its oral absorption.

Keywords — alginate nanoparticles, chitosan, insulin nanoparticles.

I. INTRODUCTION

THE development of biotechnology has led to new therapies based on peptides and proteins (hormone deficiencies, hepatitis C, multiple sclerosis ...).
Proteins are important therapeutic molecules because they have multiple biological functions; however, the development of a drugbased protein is the origin of many difficulties due to their specific properties and their sensitivity to environmental conditions [1].
Proteins are molecules of high molecular weight, with a complex structure. Their instability during preparation, storage and subsequent release is a major problem in the formulation and production of a drug. In addition, proteins have low oral and transdermal bioavailability, they are usually administered parenterally [2].
However, most proteins have a very short half-life, requiring frequent injections, which is not well tolerated by patients and may limit the extent of therapies based on protein.
Microencapsulation of peptides and proteins and the preparation of sustained-release forms have been widely studied in order to improve thetherapeutic efficacy of these bioactive molecules [3]-[4].
However, the difficulties mentioned above concerning the properties of the protein, reduce the choice of an encapsulation process and imposing a strict control of operating conditions to ensure the continued integrity of the protein during all stages of production.
To this end, we propose to use an encapsulation technique based on ionic gelation between two oppositely charged polymers at room temperature polymers at room temperature.
The ionotropic gelation method is very simple and mild.
In addition, reversible physical crosslinking by electrostatic interaction insteas of chemical crosslinking avoids the possible toxicity of reagents and other undesirable effects [5].



In this method polysaccharides (alginate, gellan and pectin) are dissolved in water or in weak acidic medium (chitosan).
These solutions are then added dropwise under constant stirring to the solutions containing other counterions.
Due to the complexation between oppositely charged species, polysaccharides undergo ionic gelation and precipitate to form spherical particles.
The use of polysaccharides and especially natural biopolymers has attracted particular interest due to their desirable biocompatible,biodegradable, hydrophilic and protective properties. The interaction between cationic and anionic biopolymers leads to the formation of the polyelectrolyte complex, which has demonstrated favorable characteristics for drug entrapment and delivery [6]-[7].
In this study, two polysaccharides, alginate and chitosan were used as polyionic polymers to associate with the model protein drug insulin.
The use of chitosan in complex with alginate is a promising strategy if we consider the biocompatibility of two polyelectrolytes [8].
Chitosan, (1.4) –[2amino-2-deoxy-β-D glucan], is naturally derived from chitin, obtained by the partial deacetylation. Chitosan has an amino group which gives it a character of polycation and the ability to form complexes with acids or polyanions.
It is abiocompatible and biodegradable polymer; it has antimicrobial activity and a mucoadhesive property, and is

used in many biomedical applications, especially in the preparation of microparticles and microspheres containing drugs [9]-[10].

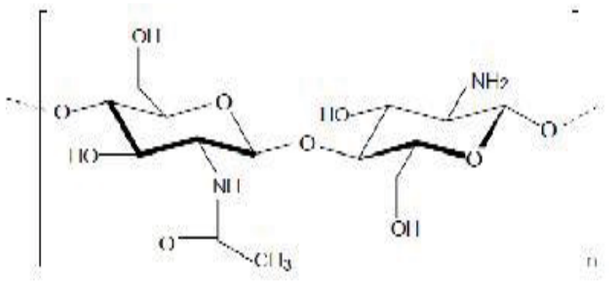


Fig.1: *Chemical Structure of Chitosan*

Sodium alginate is a polysaccharide found in all brown algae, it is water soluble, biodegradable and biocompatible. Alginate is found in numerous pharmaceutical and biomedical applications such as drug delivery and cell encapsulation.
It consists of an anionic chain of (1–4)-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) in different arrangements and proportions. In the presence of calcium ions (Ca), interaction between the bivalentions and guluronic acid residues causes the alginate to form a gel. The gelling properties depend on the composition and extent of the G and M sequence, molecular weight of the polymer and concentration of the counter ions during gelation. The solubility characteristics of the alginate network are also affected by the pH of the medium.
The pKa values of mannuronic acid and guluronic acids are 3.4 and 3.6, respectively [11].

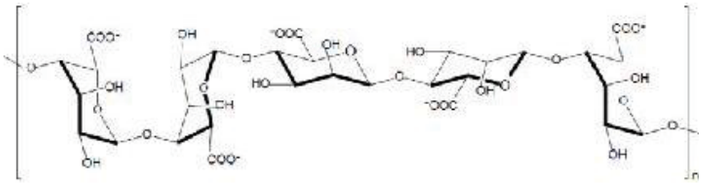


Fig.2:.*Chemical Structure of alginate Na*

II. Materials and methods

A.Materials

algae of low viscosity (M~48000), η = 250 cps at Sigma and was used without further purification.
Human insulin in the form of crystals (no excipient) provided by Sometha. The anhydrous calcium chloride (CaCl2) 99.9%, was supplied by Aldrich Chitosan with a molecular weight intermediate (M=450KD), acetylation levels of 74%, was supplied by Fluka. Sodium alginate from brown

B.Preparation of insulin loaded alginate/chitosan nanoparticles

Alginate/chitosan nanoparticles were prepared through an adapted protocol initially described by Rajaonarivony et al [12], but motified according to ideal pre-gelation stoichiometric ratio under gel point and time of drug association. Unless noted otherwise, formulation was as follows: Sodium alginate was dissolved at a concentration of 0.063% (w/v) in ultrapure water. A common solution of chitosan was prepared by dissolving a concentration of 0.05% in 1% acetic acid. The pH of polyelectrolytes solutions was adjusted to 4.9 and 4.6 respectively with 1N HCl solution and a concentrated solution of NaOH.
The solutions of sodium alginate and chitosan were then placed under vigorous stirring for 48 hours.
The solutions are then filtered on a medium swixness 25 equipped with a filter paper (0.22µm) to remove undisso ved materials. The particles of alginate are crosslinked with 18mM Calcium chloride to provide an alginate pre-gel, then the chitosan solution was added dropwise to the solution cross-linked under 800rpm over 60 min. For the encapsulation of insulin: 7mg of human insulin (equivalent to 200UI) is added to the alginate solution before the addition of sodium chloride. After chitosan addition, nanoparticles were maintained with additional stirring for 30minutes to improve curing [8].

C. Experimental Design

The characteristics of the nanoparticles including size distribution, zeta potential, morphology, and the efficiency of encapsulation were optimized by means of a full factorial experimental design over the influence of four different independent variable.
We chose a D-optimal design [13]. Xi factors selected and their levels are summarized in Table 1.

Table.1 Experimental Factors and Levels

Factors	Levels	
	(-1)	(+1)
mass ratio (Alg/Ch)	0.7	1.26
pH	4	5.2
Stirring speed during the addition of cationic polyelectrolyte (rpm)	800	2000
Duration of agitation during the addition of cationic polyelectrolyte (min)	30	120

D. Particle size and zeta potential determination

Size distribution analysis was performed by laser diffraction spectrometry using a granulometer (MASTERSIZER 2000, Malvern Instruments).
The mean diameters of the aqueous suspension of hydrated nanoparticles were calculated in triplicate.
The zeta potential of nanoparticles was obtained by Malvern Zetasizer 2000 (Malvern Instruments).
The samples are nearly transparent, so do not require dilution prior.

E. Nanoparticle morphology

The morphologie of nanoparticles were determined by scanning electron microscopy (SEM, Philips XL30 ESEMto a complete EDSX microanalysis by energy dispersion); an aqueous droplet of nanoparticles suspension was immobilized on copper grids coated with carbon film.
After adsorption for 1 minute, the grid is dried at room temperature.

F.Determination of encapsulation efficiency

The encapsulation efficiency is calculated after centrifugtion of dispersion formulated at 4 ° C for 45min.
The concentration of insulin was determined by High

Performed Liquid Chromatography (HPLC, Perkin Elmer, Series 200, detector: UV / Vis).) (Column: stationary phase: silica gel octadécylsilyé C18 for chromatography R, dimensions: L = 250m, d = 4 mm, USA).
Mobile phase consisted of acetonitrile and 0.1% TFA aqueous solution initially set in the ratio 30:70 (v/v), which was linearly changed to 40:60 (v/v) over 5min. From 5 to 10min the ratio 40:60 (v/v) was kept constant.
Samples and standards (20µl) were injected and eluted at a flow rate of 1mL/min. The absorbance of insulin was determined using the UV trace at 214nm.
The drug encapsulation efficiency (EE) was calculated by formulas:
Encapsulation efficiency (%) = [(Total amount of insulin insulin in supernatant)/Total amount of insulin] 100 [19].

G.Characterization of insulin-loaded alginate/chitosan nanoparticles through Fourier Transform Infrared (FTIR) study

In order to confirm alginate-chitosan- protein interactions, samples were analysed by Fourier transform Infrared Spectroscopy (brand: Nicolet 380 FT-IR). The samples were scanned from 400 to 4000cm-1. The optimal complex formed was first lyophilized with a freeze dryer (brand name: Martin Christ Alpha 1-4 LD-2).

H. In vitro insulin release study

In order to determine the kinetics of release of insulin encapsulated in alginate/ chitosan nanoparticles. The lyophilized nanoparticles were previously studied in simulated physiological media.

The insulin loaded nanoparticles were dispersed in simulated gastric fluid (SFF. HCl 0.1N, pH 1.2) and simulated intestinal fluid (phosphate buffer, pH = 6.8) without enzymes and shaken at 100rpm at 37°C. for 4 hours and 24 hours respectively. At specified time intervals (0, 30, 60, 120,240 and 480min).
Release experiments were performed using a dissolution test system (Dissolutest apparatus brand: ERWEKA).
The supernatant was collected by centrifugation.
The concentrations of insulin in the supernatant were determined by UV-visible spectroscopy (SAFAS DES 190) at 275nm in the simulated gastric fluid, and 262nm in the simulated intestinal fluid.
Each experiment was carried out in triplicate. The total amount of insulin released from the nanoparticles was calculated.

III. Results and discussions

As a result of the process described above, the optimized Alg/chi nanoparticles had a mean particle size of 80±4.5nm with a positive zeta potential 42.9±1.4mV; showing a spherical shape and uniform size (Fig.4).
The EE of the nanoparticles were 73.7%.
The particles of unencapsulated insulin are in the form of homogeneous crystals, however spherical particles were observed with a large polydispersity with an average size nanometer (80nm) in the complex formulation obtained.

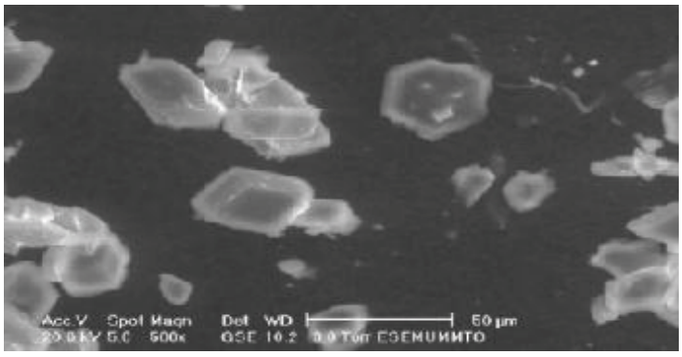


Fig.3: *Morphology of unloaded insulin standard according to SEM micrograph*

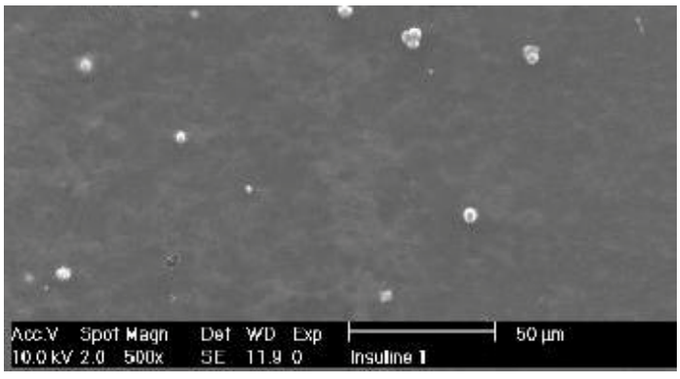


Fig.4: *Morphology and size of insulin-loaded alginate/chitosan nanoparticles according to SEM micrograph (magnification 1000×) prepared by using the standard conditions.*

FTIR spectra showed that both chitosan peaks were similarly changed after complexation with alginate.
Observed changes in the absorption bands of the amino groups, carboxyl groups, and amide bonds can be attributed to an ionic interaction between the carbonyl group of alginate and the amino group of chitosan [14].The obtained complex spectra revealed two additional shoulders on the insulin-loaded nanoparticles absorption bands in the Amide I (~1650cm-1) and Amide II (~1650cm- 1 These peaks are characteristic of protein spectrum, and possibly demonstrative of the entrapment of insulin into alginate/-chitosan nanoparticles.).
The FTIR spectra confirm the formation of polymer-drug complexes occurs by electrostatic interactions between ionized groups of the polymer and insulin [8].The different spectra obtained are represented by the following figures:

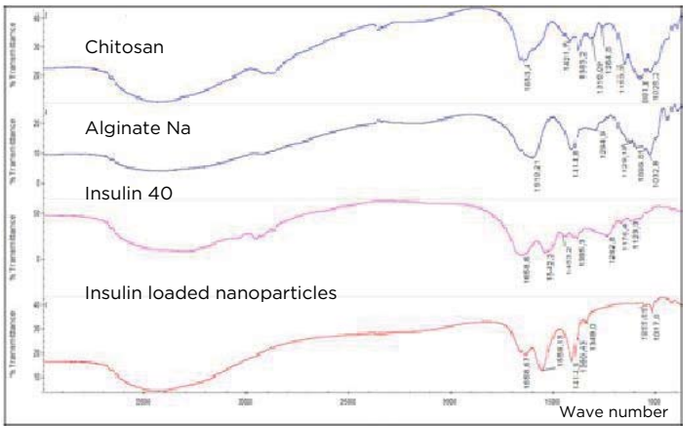


Fig.5: *FTIR spectra of pure insulin, alginate, chitosan and insulin loaded alginate/chitosan nanoparticles.*

Insulin release profile from nanoparticles, in gastrointestinal simulated pH conditions, is plotted in Fig.6-7: Plain alginate/chitosan nanoparticles showed an initial burst effect, atpH1.2, about 15% to 20% of the initial amount of insulin (from the first measurement) in gastric pH, followed by a release of 40.09% ± 0.023% after 4 hours. This low rate of release could be due to protection seen in the polymer matrix while protecting insulin aggressive gastric acid pH and enzymatic degradation [15].

Concerning the release of insulin in phosphate buffer medium at pH 6.8, approximately 20% of insulin was released immediately. The observation of this burst effect could be related to the diffusion of the active ingredient in close proximity to the surface of the nanoparticles and the low resistance of nanoparticles in intestinal conditions [16]. The release was continuous and reached 75% ± 0.019% after 24 hours.

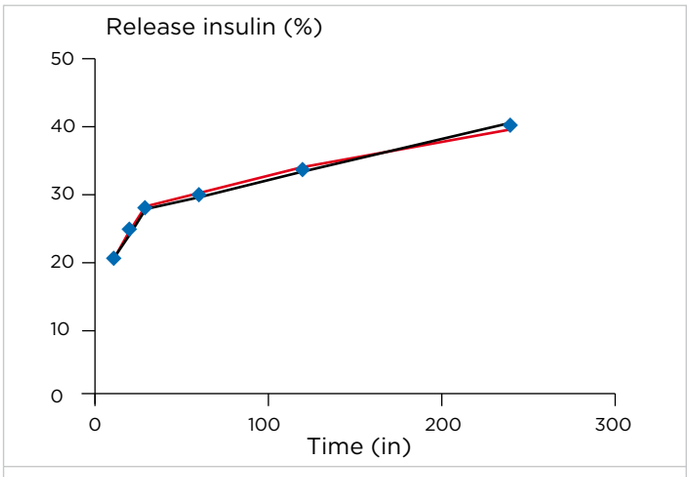


Fig.6: *Dissolution profile of the optimized formulation in HCl0.1N,pH 1.2 medium. Data are the mean of three determinations ±SE.*

V. CONCLUSION

To avoid the use of solvents and additives, we chose to use the technique of ionotropic gelation to the formation of polyelectrolyte complexes in aqueous conditions, nondenaturing for active protein as the insulin; with the design of experiments, we were able to define the area of a formula corresponding to the optimal best compromise, namely nanoparticles: Obtained from natural and bioavailable polyelectrolytes: chitosan and alginate Sodium; using a process that does not require solvents or additives: the ionotropic gelation, having a size less than 500 nm, allowing the passage of the intestinal barrier, With their surface charged groups, allowing them to enable their subsequent functionalization, and have a maximum encapsulation efficiency.
After the invitro study in simulated environments, we can now confirm that the obtained nanoparticles containing insulin have shown their ability to protect insulin from the gastrointestinal tract with a gastric resistance (low release) and a crossing of theintestinalepithelium.

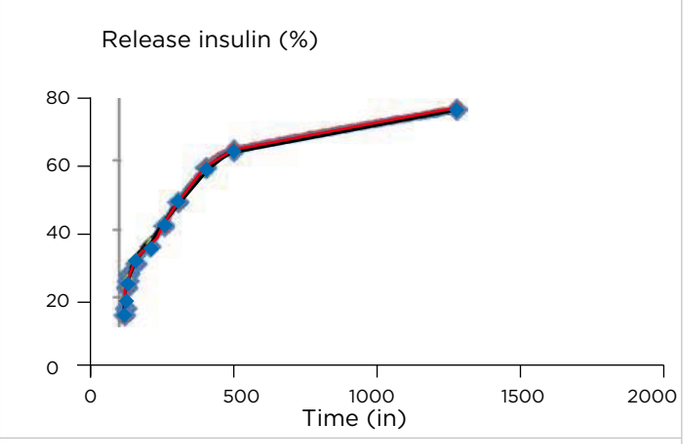


Fig.6: *Dissolution profile of the optimized formulation in phosphate buffer medium. Data are the mean of three determinations ±SE.*

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