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Nanostructured polymeric system based of cashew gum for oral admnistration of insulin

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ABSTRACT

The subcutaneous administration of insulin has been the treatment of millions of diabetics in the world. However, for such via insulin is invasive and not mimics the physiological action causing side effects. The oral route would be the most physiological and comfortable option, but the oral bioavailability of insulin is low by proteolytic activity and reduced permeability of the gastrointestinal tract. The aim of the study was to develop a nanostructured system integrating biomaterials for oral insulin delivery. Cashew gum (CG) is a polysaccharide extracted from the exudate of the plant *Anacardium occidentale*. It is a biopolymer composed of simple sugars and glucuronic acid and it can be used in nanostructured systems for the incorporation of molecules. The exudate was isolated, dissolved in water, filtered, precipitated in ethanol and purified. The CG was characterized by infrared spectroscopy and molecular weight by size exclusion chromatography. Nanoparticles were prepared through ionotropic gelation integrating cashew gum, dextran sulfate and poloxamer containing insulin stabilized with chitosan, poly(ethyleneglycol) and coated with albumin. The particles were analyzed for particle size, zeta potential and insulin entrapment efficiency. The FTIR spectrum for CG showed a band at 3395 cm^{-1} due to the stretching vibration of O-H, a band at 2926 cm^{-1} of C-H vibrations; absorption at 1639 cm^{-1} of O-H type from bound water molecules and bands at 1143, 1073 and 1024 cm^{-1} due vibrations of the C-O-C from glycosidic bonds and O-H of alcohols. The peak molar mass of GC was 2.35 × 10^4 g/mol. The particles had a size of 156 nm and after coating, size of 5387 nm with 92% insulin entrapment efficiency and zeta potential of -51 mV indicating electrostatic stabilization. The results suggest an innovative cashew gum base system for oral insulin administration.

Keywords: Nanostructures, biomaterials, cashew gum, insulin, oral delivery.

1. INTRODUCTION

Diabetes mellitus is a metabolic disease that requires strict glycemic control to reduce its progression and complications; the therapy is based on the exogenous administration of insulin performed subcutaneously, however, in addition to being invasive and requiring several daily injections, administered insulin by this way does not mimic the physiological action of insulin causing side effects [1,2]. The oral route would be the most physiological and convenient for diabetics; however, because of this, insulin has low bioavailability due to high proteolytic activity, as well as reduced permeability of the fatty-intestinal tract [3,4]. Among the most promising approaches to oral insulin therapy are nanostructured biopolymers based on the incorporation of insulin into biocompatible, biodegradable and mucoadhesive nanoparticles that protect and promote the absorption of insulin into the gastrointestinal tract [5].

Cashew gum (CG) characterized as a polysaccharide, is a biopolymer from the *Anacardium occidentale* (cashew tree), with varied applications in the food, medical and pharmaceutical industries, and can be used as an encapsulating agent in active ingredient release systems. GC is composed of several simple sugars and guluronic acid anionic chains [6]. This polycrystalline block is capable of interacting selectively with multivalent cations forming small nuclei that are stabilized in nanoparticles after polyolefin poly-complexing

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with polyamines [7]. Chitosan is a cationic polymer that has amine groups in the structure responsible for conferring positive charge on the molecule, besides being biodegradable and biocompatible, it has mucoadhesive properties because it is able to interact ionically with negative charges present in the intestinal mucus and on the surface of the cells [8]. This interaction increases the time of permanence of the nanoparticles in the place favoring, consequently, greater absorption of the drug [9].

The incorporation of polyanions in nanostructured systems containing insulin, allows an increase of the electrostatic interactions, improving the efficiency of incorporation of the drug in the nanoparticles [10]. Dextran sulfate is a biodegradable, biocompatible, anionic polymer with a branched chain structure of anhydrous units grafted with free sulfate groups that interact with isolated ions such as calcium or with other cationic polymers promoting stability to interactions [7].

In addition to the use of polymers in the nanostructured system, stabilizers have also been used in this work. Poloxamer, a nonionic copolymer, is capable of conferring steric stabilization to the system by its amphiphilic nature [11], and poly(ethyleneglycol) (PEG), a non-toxic polyether capable of reducing the interaction between the particles conferring steric stabilization [12], besides it being mucoadhesive contributing to improve the transport of large proteins through the intestinal mucosa favoring the absorption [13].

In addition, insulin denaturation has been indicated as one of the causes of failure in the preparation of insulin delivery systems [14]. Therefore, albumin coating has been indicated to avoid degradation of insulin by proteases, preventing they can reach the insulin inside the nanoparticles, giving stability to the system in the stomach [15, 16].

The aim of the study was to develop a nanostructured system using cashew gum integrating biomaterials for oral insulin delivery, making the adhesion to the treatment more efficient and significantly improving the quality of life of the patients.

2. MATERIALS AND METHODS

2.1 Materials

The crude cashew gum was obtained from the natural exudate from trees in Parnaíba, Piauí, Brazil and the samples were purified by the method described by [17]. Low molecular weight chitosan (50 kDa), bovine serum albumin (BSA) and Poly(ethyleneglycol) 35000 (PEG 35000) were purchased from Sigma-Aldrich Chemie (France); Dextran sulfate sodium salt from *Leuconostoc ssp*., Poloxamer 188 (Lutrol® F68, BASF, Ludwigshafen, Germany); Poly(ethyleneglycol) 4000 (PEG 4000) purchased from Fisher Scientific (UK); Calcium chloride (Riedel-de-Haë¨n, Germany); 90% lactic acid purchased from VWR BDH Prolabo (France); 99% trifluoroacetic acid (TFA) and acetonitrile (LiChrosolv) were obtained from Sigma-Aldrich Co. (St Louis, MO, USA); Insulin 100 IU/ml (Actrapid®, Novo Nordisk A/S, Bagsværd, Denmark).

The chitosan was dissolved in 0.5% (v/v) of lactic acid solution. The solutions were filtered through, Whatman, qualitative 1, filter paper under vacuum.

2.2 Purification and characterization of cashew gum

CG was isolated from exudate from trees of the genus *Anacardium occidentale* L. and purified with sodium salt and precipitated in ethyl alcohol by the method described by Paula et al., 1998.

2.2.1 FTIR spectroscopy analysis

The polysaccharide obtained (CG) was characterized by infrared spectroscopy on an FT-IR PerkinElmer, spectrum 400, in the ATR module, in the range of 4000 to 700 cm^{-1}.

2.2.2 Proton nuclear magnetic resonance (^{1}H NMR)

The CG sample was dissolved in deuterium oxide (D_2O). The spectra were obtained in the Varian 400-vnmrs400 model, with temperature control at 50° C.

2.2.3 Size Exclusion Chromatography (SEC)

To determine the molecular weight, the polymer was analyzed by a size exclusion chromatography (SEC) system equipped with an on-line degasser, a refractive index (RI) detector and a set of columns including a Shodex OHpak SB-G column protector and the OHpak SB-SB-802.5HQ and OHpak SB-804HQ columns.

The polymers were eluted with a flow rate of 0.5 mL/min with 0.1 M Na2 SO4 (aq); 1 wt% acetic acid; 0.02% NaN3 at 40 ° C. Prior to injection (50 µL), the samples were filtered through a polytetrafluoroethylene (PTFE) membrane with a pore size of 0.45 µm. The system was calibrated with five narrow PEG standards and the molecular weight of polymers (MnSEC) and Đ (Mw/Mn) were determined by conventional calibration using software clarity version 2.8.2.648.

2.3 Preparation and characterization of the nanoparticles

Nanoparticles were developed based on the methodology described by [7], with some modifications. Nanoparticles were prepared by complexation of biomaterials carrying opposite charges under controlled pH conditions. An aqueous solution of 0.2% (w/v) cashew gum was prepared by stirring overnight. 0.02% (w/v) dextran sulfate, 0.04% (w/v) poloxamer 188 and 0.006% (w/v) insulin was added and dissolved. Complexation involved the dropwise addition of aqueous solution of 0.2% (w/v) calcium chloride, followed by a solution at pH 4.6 containing 0.07% (w/v) chitosan and 0.35% (w/v) PEG 4000 for stabilization of nanoparticles. Followed by dropwise addition of 1% (w/v) albumin solution. Nanoparticles were concentrated by dialysis using regenerated cellulose membrane with nominal dry weight of 100K MWCO (SnakeSkin Pleated Dialysis Tubing-Thermo Fisher Scientific Inc., USA) and dialysis solution of 10% poly(ethyleneglycol) 35000 for 24 hours at 4 °C. A formulation was prepared at room temperature under magnetic stirring at 800 rpm for 40 min.

2.3.1 Particle size and zeta potential analysis

Particle size and zeta potential measurements were performed on Zetasizer Nano ZS (Malvern Instruments Ltd.). Surface charge was determined by laser doppler electrophoresis and measurements were carried out in a folded capillary electrophoresis cell. The size distribution was measured by dynamic light scattering (DLS) and represented by normalized intensity distribution. The measurements were performed in triplicate at 25ºC, with a detection angle of 90º. The results were presented as mean particle size distribution and zeta potential.

2.3.2 Insulin entrapment efficiency

Insulin entrapment efficiency was determined by the difference between the total amount of insulin used to prepare nanoparticles and the amount of free insulin per total amount of insulin. Nanoparticles containing insulin were separated from aqueous supernatant containing free insulin by centrifugation (10.000 rpm for 10 min at 4 ºC, and the amount of free insulin was determined in triplicate by high-performance liquid chromatography (HPLC).

2.3.3 Insulin quantification

Insulin was determinated according to the methodology validated by [10]. Insulin was analyzed by high-performance liquid chromatography (HPLC) using an LC-2010 HT HPLC system (Shimadzu, Japan) equipped with a quaternary pump, a UV detector set at 214 nm, a reversed-phase X-Terra RP 18 column, 5 µm, 4.6 x 250 mm (Waters, USA) and Purospher STAR RP-18 precolumn 5 µm, 4 x 4 mm (Merck KGa, Germany). The mobile phase consists of acetonotrile (A) and 0.1% trifluoroacetic acid (TFA) aqueous solution (B) operated in gradient mode at flow rate of 1.0 ml min^{-1} set to 30:70 (A:B), changed to 40:60 (A:B) in 5 min for elution over 5 min, and changed to 30:70 (A:B) in 1 min for elution over 1 min. The chromatograms were recorded and the peak area responses were measured using an automatic integrator.

3. RESULTS AND DISCUSSION

3.1 Purification and characterization of cashew gum

The cashew gum was characterized by infrared spectroscopy and the spectrum showed a band at 3395 cm^{-1} by O-H stretching vibration, a band at 2926 cm^{-1} by C-H vibrations; absorption at 1639 cm^{-1} of O-H type from bound water molecules and bands at 1143, 1073 and 1024 cm^{-1} due to the presence of C-O-C vibrations from glycosidic bonds and O-H of alcohols (Fig. 1) [18].

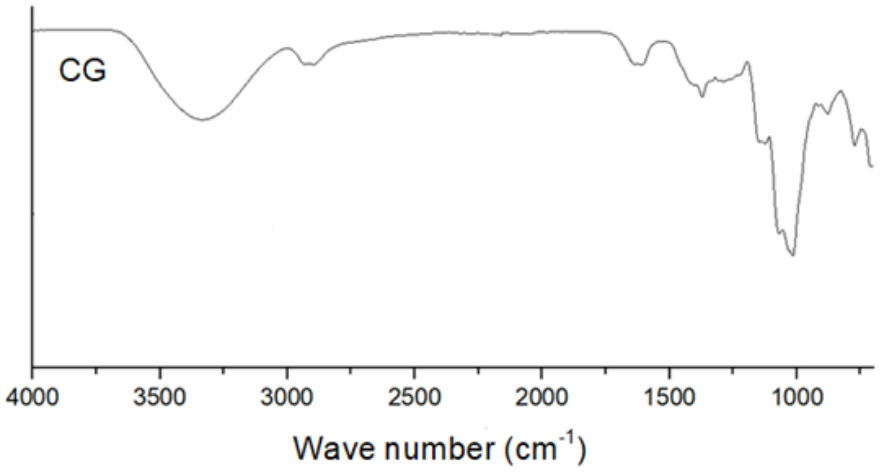

Figura 1: FTIR spectra for cashew gum.

The ^{1}H NMR spectrum for CG is shown in Fig. 2. The CG spectrum showed a signal at 1.1 ppm due to the presence of CH3 of rhamnose and signals at 3.0 - 4.5 ppm due to the presence of OH and H-1 to H-6 protons present in the polysaccharide [18, 19].

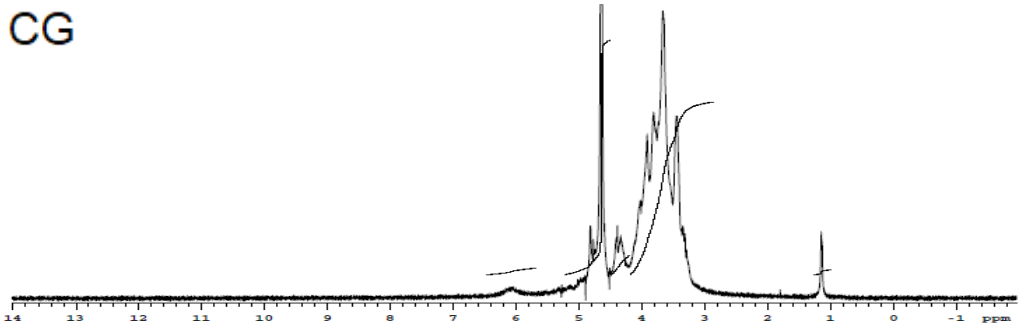

Figura 2: ^{1}H NMR spectra for the cashew gum.

The cashew gum presented molar mass of 2.35 x 10^{4} g/mol. The molecular weight of CG was determined by the size exclusion chromatography technique using the molecular weight distribution characterization markers, *Mn* (mean molecular weight), *Mw* (mean molecular weight) and *Đ* (polydispersity) calculated by the quotient *Mw/Mn* (Table 1). The chromatogram from which these values were exported is presented in figure 7. During the process of purification of the material, it is of extreme importance that the treatments applied to the polysaccharide do not discharacterize it, guaranteeing the intact maintenance of its structure and/or average molar mass.

Tabela 2: Mean molecular weights (Mn and Mw) and polydispersity (*Đ*) of cashew gum.

M_n	M_w	*Đ*
19700	23500	1,20

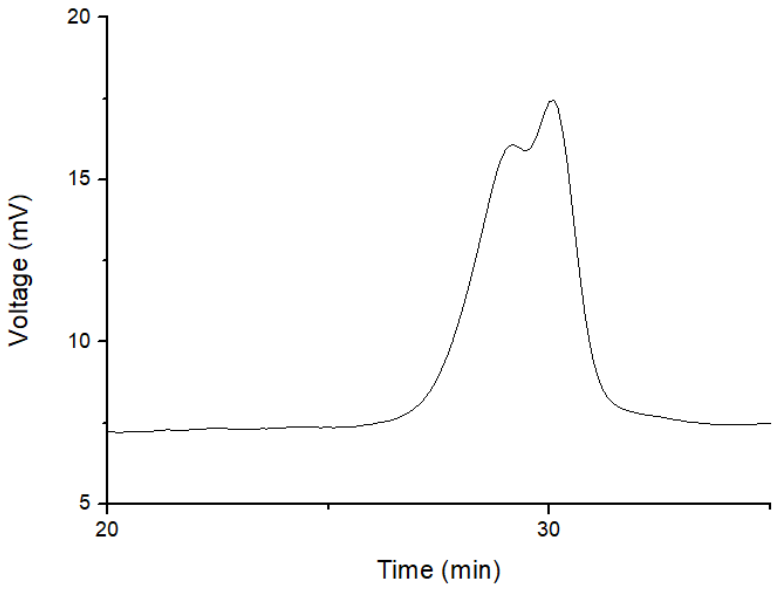

Figura 3: Chromatogram of the molecular mass of cashew gum.

Similar molar mass value was also observed by [20], where cashew gum showed a peak molar mass of 2.3 x 10^4 g/mol, corroborating the results.

3.2 Preparation and characterization of nanoparticles

The prepared nanoparticles formed a multilayer complex with the insulin protected and retained, containing the outermost layer of albumin. Polyelectrolyte complex nanoparticles were formed by interaction of opposite charges of biopolymers.

The mean uncoated particle size containing insulin determined by the dynamic light scattering was 156 nm. After coating the particles had an average size of 522 nm and 5387 nm, respectively, for chitosan and albumin coating.

The particle size is important for the assessment of gastrointestinal absorption [21, 22], because it influences the body distribution [23], mucoadhesion [24, 25] and drug release profile [26]. The particles had a particle size lower than the critical value reported in the literature indicating that they can be absorbed orally. The polydispersive index of coated and finished particles was 0.2 indicating good particle size distribution. The confirmation of the formation of nanoparticles was given by the presence of Tyndall effect, by the visualization of the opalescent suspension. The granulometric distribution of the nanospheres was unimodal as shown in figure 4.

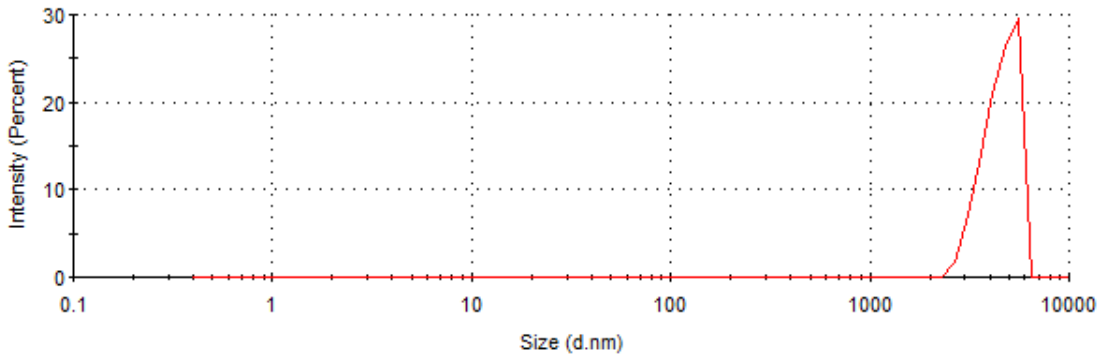

Figura 4: Particle size distribution (determined by DLS) of nanoparticles with insulin.

The increase of the nanoparticles resulting from the coating with chitosan was also described in algi-

nate nanoparticles prepared by [27], where the coating with the chitosan contributed to the increase of the size of the alginate nucleus in relation to its initial diameter. The result of the albumin coating on particle size may be related to changes in the electrical state causing the particles to increase or shrink in size, depending on whether the electrical repulsions increase or decrease within the particles [28].

The zeta potential characterizes the global electric charge of the surface of a particle, determining the electrophoretic mobility of the particles that is measured by their velocity per unit of electric field that is applied on the dispersion of ions in the diluent with ionic force. The result of the zeta potential indicates the electrostatic stability of the NP with values greater than 30 mV or less than 30 mV, related to nanoparticles with higher suspension stability and low tendency of aggregate formation [29].

The results show that the nanoparticles are negatively charged with zeta potential values of -27 mV for the uncoated nanoparticles. After coating with chitosan the particles had a surface charge of -6.8 and the coated and finished particles after the albumin coating presented a -51 mV loading indicating stable system [30]. Suspension stability, allows less formation of aggregate formation due to the high repulsive force between the particles [31]. The changes in the surface charges of the particles observed after the coatings confirm that the polyelectrolyte complexation occurred. The chitosan coating altered the surface charge of the particles, making it less negative, evidencing that the interaction with the chitosan occurred in fact and the change in the load after the albumin coating also confirms the efficiency of the interaction.

The albumin coating was added for protection of insulin against proteolytic enzymes from the gastric environment, improving the resistance of the nanoparticles in this medium. In alginate and dextran sulfate nanospheres containing insulin coated with chitosan and albumin, the second coating with albumin worked as a target for pepsin degradation by keeping the encapsulated insulin protected from proteolytic attack in the stomach and exposing the underlying layer of the coating with chitosan, allowing the nanospheres to exert mucoadhesive properties in the intestinal environment [32].

3.2.1 Insulin entrapment efficiency

The efficiency of insulin encapsulation is a measure of the amount of insulin entrapped and retained by the nanoparticle formulation. The value of insulin encapsulation efficiency was 92%, higher than previously reported (90%) by WOITISKI *et al*. [7], in alginate and dextran sulfate nanoespheres coated with chitosan and albumin.

Such results of high level of encapsulation indicate that there was an effective result in the formulation of the nanospheres with the cashew gum under the proposed conditions. The effect of pH on the components of the formulation may have contributed to the good result. Insulin has an isoelectric point at pH about 5.3, when the pH is above the isoelectric point, insulin has a negative charge and when it is below the point it has a positive charge. In the isoelectric point the hormone presents minimal solubility, since the lack of its total charge indicates that the molecules no longer repel, tending to agglutinate and precipitate [33].

In addition, the cashew gum, at pH less than 5.0, presents the carboxylic groups more ionizable, besides showing better rheological characteristics by the high viscosity [34,35]. Thus, strong electrostatic attractiveness can occur at the final pH of 4.6, providing high encapsulation efficiency of the oppositely charged insulin.

Calcium ions interacting with the glucuronic residues of cashew gum can establish ionic bridges of the negatively charged carboxylic residues with insulin, strengthening the interaction [27]. The inclusion of poloxamer 188 may also favor the efficiency of insulin encapsulation due to promoted steric stabilization [7].

4. CONCLUSIONS

In conclusion, cashew gum was successfully purified and characterized with results corroborating with the literature, with a molar mass of 2.35 x 10^4 g/mol. The particles shows a mean final size of 5387 nm, after the coatings, it being below the critical value required for instestinal absorption and could then be absorbed by the oral route. The final zeta potential of the formulation (-51 mV) indicated nanoparticles with high electrostatic stability. The polyelectrolyte complexation methodology applied with mild conditions for insulin and easy to perform procedures allowed the preparation of particles with 92% retention of insulin, being promising as a formulation for oral insulin administration using biopolymers.

5. ACKNOWLEDGEMENTS

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