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Physical and chemical characterization insulin-loaded chitosan-TPP nanoparticles

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Abstract The purpose of this study was to develop and characterize insulin nanoparticles systems using chitosan. Insulin-loaded nanoparticles were prepared by ionic gelation of chitosan with tripolyphosphate anions (TPP). The interactions between insulin and chitosan were evaluated by differential scanning calorimetry (DSC), thermogravimetry/derivative thermogravimetry (TG/DTG), and Fourier-transform infrared (FTIR) spectroscopy. Besides, particle size distribution, polydispersity index (PDI), zeta potential, and association efficiency (AE%) of the nanoparticles were evaluated. In general, inert nanoparticles and insulin-loaded nanoparticles showed an average size of 260.56 nm (PDI 0.502) and 312.80 nm (PDI 0.481), respectively. Both nanoparticles showed positive charge, but after insulin incorporation the zeta potential was reduced, evidencing its incorporation. Nanoparticles obtained also showed AE% around 70%, measured by high-performance liquid chromatography (HPLC). The results of FTIR, DSC, and TG/DTG corroborated the data presented suggesting that insulin was successfully encapsulated. However, drug incorporation seems to be related not only to electrostatic interactions, but also to physical process and/or adsorption phenomena.

Keywords Nanoparticles · Chitosan · Insulin · Thermal analysis · FTIR

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Introduction

Chitosan (β(1–4) linked 2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-D-glucopyranose) is a cationic polymer extensively explored due to their characteristics such as hydrophilicity and biocompatibility. These properties make it able to interact with negatively charged polymers, macromolecules and also with certain polyanions in aqueous solution [1–3]. This interaction usually occurs under relatively easy conditions and low cost, when compared with other crosslink agents [4]. These features make it an interesting target in drug delivery systems.

Chitosan/tripolyphosphate (TPP) nanoparticles prepared by ionotropic gelation method have been increasingly studied for controlled-release applications [2, 3, 5]. Nanoparticles formation by ionotropic gelation method is based on the presence of electrostatic interaction between chitosan and TPP [1–4]. The TPP ($Na_5P_3O_{10}$) is a multivalent polyanion, with low toxicity and has been applied to avoid the possible toxicity of other crosslinkers agents, it can interact with the cationic chitosan by electrostatic forces.

Numerous studies describe the use of nanoparticles as a way to encapsulating insulin in order to enhance or prolong the hypoglycemic effect and improve the treatment of diabetes patients [6–8]. Chitosan/TPP and chitosan/alginate nanoparticles were studied to encapsulate insulin for other authors [9–12]. The interaction of insulin with chitosan in chitosan/alginate nanoparticles, by differential scanning calorimetry (DSC) and Fourier-transform infrared (FTIR) spectroscopy, was reported by Sarmento et al. [13]. Shift in endothermic and exothermic peaks and changes in the spectra of individual polyelectrolytes and nanoparticles have been reported as ionic interactions that led to a new chemical entities formation with different thermal properties. In addition, the encapsulated insulin also led to minor

modifications of nanoparticles peaks, demonstrating the influence of protein in the formulation. Therefore, the aim of this study was to prepare and characterize by thermal analysis and FTIR chitosan nanoparticles as delivery system for insulin.

Experimental

Materials

Low viscosity chitosan and insulin (standard substance powdered) were purchased from Sigma-Chemical. The sodium tripolyphosphate (TPP) was obtained from SYNTH®. Insulin in solution (Novolin R®, 100 IU/mL), used as raw material, was generous donated by the Secretary of State for Health in Aracaju city. All other chemicals were of reagent grade.

Nanoparticles preparation

Nanoparticles (NP) were prepared according to the ionotropic gelation method first reported by Calvo et al. [2]. Chitosan (0.4% w/v) was dissolved in aqueous acetic acid solutions (1% v/v) (pH 6.1), while TPP (0.1% w/v) was dissolved in deionized water. Insulin solution was premixed with 2 mL of the TPP solution before the addition of the TPP solution dropwise into the chitosan solution under magnetic stirring (600 rpm) at ambient temperature [2, 14]. The inert nanoparticles (without insulin) were obtained similarly and used as control.

Particle size and zeta potential

The nanoparticles size was analyzed by Photon Correlation Spectroscopy (Malvern Instruments, Malvern, UK). Samples were diluted in purified water and the measurements were performed at a scattering angle 173° at ambient temperature. The particle charge was quantified measuring the zeta potential by Laser Doppler Anemometry using a Zetasizer® ZS. For the measurements samples were diluted with purified water and placed in the electrophoretic cell. Each batch was analyzed in triplicate.

Association efficiency

The association efficiency (AE%) of the process was determined upon nanoparticles separation by centrifugation at 14,000 rpm, 4 °C for 3 h from the aqueous medium containing non-associated insulin. The amount of free insulin in the supernatant was measured using high-performance liquid chromatographic (HPLC). Twenty microliters were injected into a chromatograph (Perkin Elmer® series 200) equipped with a UV detector, and C8 column (Zorbax®, 5 µm, 4.6 × 250 mm). The mobile phase was a mixture of 27% acetonitrile and 73% buffer 0.1 M KH_2PO_4 containing 1% triethylamine adjusted to pH 3.0 with phosphoric acid. The flow rate was 1.5 mL/min, the wavelength was set at 214 nm [15]. Insulin association efficiency of nanoparticles was calculated according to Eq. 1, established by Fernandez-Fernández-Urrusuno et al. [9].

$$\text{Association efficiency} = \frac{\text{Total amount of insulin} - \text{free insulin}}{\text{Total amount of insulin}} \times 100. \quad (1)$$

FTIR analysis

The infrared absorption spectra of the freeze-dried nanoparticles with and without drug, chitosan, TPP, and insulin were obtained at room temperature in the range 4,000–400 cm^{-1} in KBr pellets using a Perkin Elmer® spectrophotometer.

Thermal analysis

DSC curves were obtained in a DSC-50 cell (Shimadzu) using aluminum crucibles with about 2 mg of samples, under dynamic nitrogen atmosphere (100 mL min^{-1}) and heating rate of 10 °C min^{-1} in the temperature range from 25 to 400 °C. The DSC cell was calibrated with indium (m.p. 156.6 °C; $\Delta H_{fus.} = 28.54$ J g^{-1}) and zinc (m.p. 419.6 °C).

TG/DTG curves were obtained with a thermobalance model TG-DTA 2960 (TA instruments) in the temperature range 25–800 °C, using platinum crucibles with ~5 mg of samples, under dynamic nitrogen atmosphere (100 mL min^{-1}) and heating rate of 10 °C min^{-1}. TG/DTG was calibrated using a $CaC_2O_4 \cdot H_2O$ standard in conformity to ASTM.

Results and discussion

Particle size and zeta potential

The inert and insulin-loaded nanoparticles showed an average size of 260.56 nm (PDI 0.502) and 312.80 nm (PDI 0.481), respectively. These results indicated that size of the nanoparticles was affected by the presence of insulin. More specifically, the incorporation of insulin led to a slight enlargement of the nanoparticles. Similar results were obtained by Fernández-Urrusuno et al. [9].

The zeta potential results showed that the surface charge of chitosan/TPP nanoparticles without insulin was 29 ± 3 mV, while nanoparticles with insulin exhibited load of 23 ± 2 mV. The zeta potential reduction was probably related by the presence of insulin on the nanoparticles surface [2, 9, 16].

Association efficiency

The AE% of chitosan/TPP nanoparticles obtained was 69.37 ± 4.71%, similar to that was found by Ma et al. [14] at pH 6.1 formulation. According to these authors, at pH 6.1, the some used in this study, the formulation could contain zwitterionic insulin molecules and chitosan molecules in globular state, both of which favored hydrophobic interactions, then the association between insulin and chitosan could be driven by hydrophobic interactions and hydrogen bonding. However, higher values of AE% were also obtained when insulin was incorporated into chitosan/TPP nanoparticles, even in a pH, in which it was positively charged [9, 10]. This shows that not only hydrophobic interactions, hydrogen bonding, and other intermolecular forces, but also the physical mechanism of incorporation caused by the process of chitosan gelation, at particulate form, probably can be involved at nanoparticles drug incorporation.

Other authors showed also that the chitosan molecular weight, the solution viscosity, the weight ratio chitosan/TPP, as well as the drug concentration can be adjusted to modulate the AE% of chitosan/TPP nanoparticles [9, 10, 16].

Drug–polymer interaction

To examine the chitosan–insulin interactions were employed DSC, TG/DTG, and FTIR spectroscopy. As a result, there were changes in the absorption bands in FTIR spectra of the amino, carboxyl, and amide groups. The chitosan spectrum (1) revealed the presence of characteristic bands, among them the amide I (axial deformation C=O) located at 1,658 cm^{-1} [16, 17]. The intense band at 3,424 cm^{-1} is related to the stretching vibration of O–H and/or N–H, as well as the hydrogen bonds of the polysaccharide chains. In 1,380 cm^{-1} appears as a band on the angular deformation CH_3 symmetric [16]. The absorption bands at 1,156 cm^{-1} (anti-symmetric stretch C–O–C) in 1,072 and 1,024 cm^{-1} (vibrations involving the C–O stretch) are characteristic of chitosan structure [18, 19].

The TPP spectrum showed their characteristic bands at the region of 1,094 cm^{-1} related to the phosphate group (P=O). The insulin spectrum showed the amide groups absorption: bands 1,652 cm^{-1} (amide I) and 1,540 cm^{-1} (amide II) characteristic of the protein (1) [13, 20].

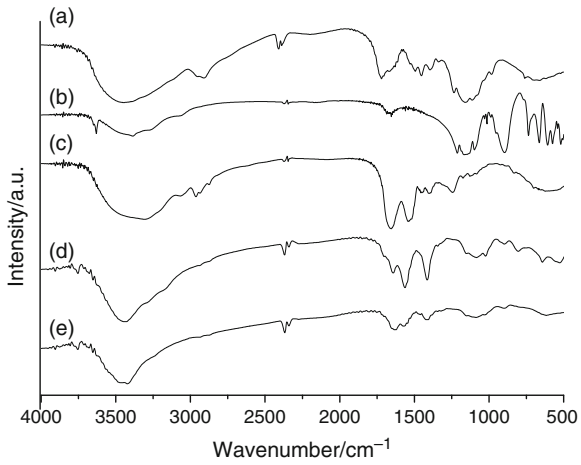

Fig. 1 FTIR spectra of chitosan (*a*), TPP (*b*), insulin (*c*), inert nanoparticles (*d*), and insulin nanoparticles (*e*)

Analyzing the inert nanoparticles (1), the band at 1,658 cm^{-1} of chitosan spectrum was modified to 1,644 cm^{-1} and one new band arises at 1,568 cm^{-1}. The 1,644 cm^{-1} is the acetylated chitosan part which has remained unchanged during the bond formation between the amino groups of polymer chain and the phosphate groups of the anion represented by the band at 1,568 cm^{-1} [21, 22]. At the insulin-loaded nanoparticles spectrum (1) the values of the bands in 1,644 and 1,568 cm^{-1} (1e) were modified for 1,632 and 1,574 cm^{-1}, respectively. Moreover, significant reduction at the bands intensity was observed (1). This behavior could to be related to weak insulin–chitosan interaction, such as reported by Sarmento et al. [12] and Boonsongrit et al. [19].

DSC curve of pure chitosan showed (2) an endothermic event at 55 °C corresponding to its dehydration (water associated to hydrophilic groups of polymer). This event was also shown in the TG curve (3) where a humidity loss of about 11.3% was verified. As seen in 2, chitosan showed a thermal stability region between 120 and 230 °C, and an exothermic event indicated by broad peak at 330 °C, corresponding to the thermal decomposition of this material [13, 23].

DSC curve of TPP (2) showed two endothermic events at 98 and 124 °C characteristic of elimination of water. The endothermic event at 311 °C is possibly related to the merger or change of crystalline phase, because any mass loss is observed in the TG/DTG curves.

DSC curve of insulin (2) showed an endothermic event at 51 °C was a characteristic process that corresponds to denaturation. The exothermic events were characteristics of insulin degradation that may occur by chemical or physical process, influenced by temperature conditions. This thermal profile of insulin is in agreement with other reported in the literature [13, 23]. The chemical instability involves

Table 1 Comparison of IR wavenumbers/cm^{-1} of chitosan, TPP, insulin, inert nanoparticles, and insulin nanoparticles

Chitosan	TPP	Insulin	Inert nanoparticles	Insulin nanoparticles
3424	3632 and 3388	3318	3442	3468
1658	1654	1652	1644	1632
1380	1216	1540	1568	1574
1156	1164	1406	1422	1416
1072	1094	1248	1082	1088

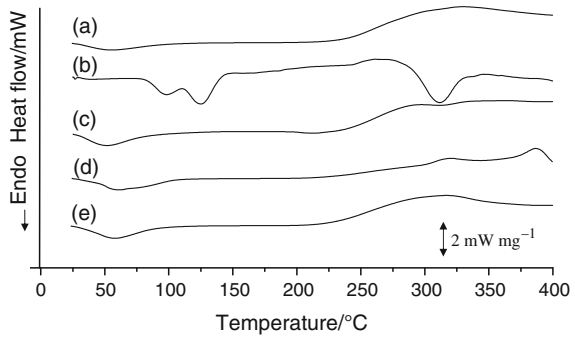

Fig. 2 DSC curves of chitosan (*a*), TPP (*b*), insulin (*c*), inert nanoparticles (*d*), and insulin nanoparticles (*e*) obtained in heating rate of 10 °C min^{-1} under dynamic nitrogen atmosphere (100 mL min^{-1})

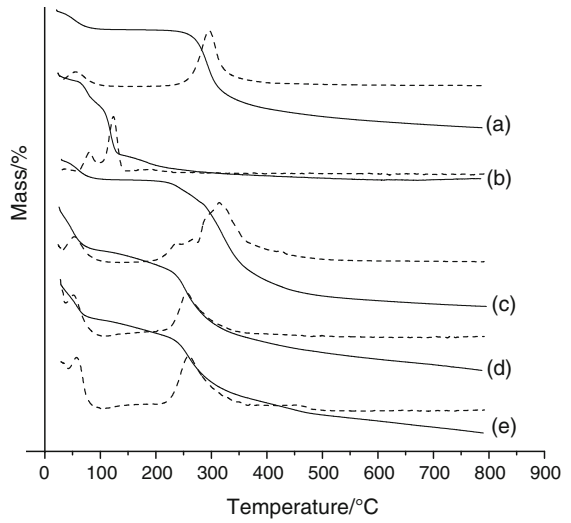

Fig. 3 TG curves of chitosan (*a*), TPP (*b*), insulin (*c*), inert nanoparticles (*d*), and insulin nanoparticles (*e*) obtained in heating rate of 10 °C min^{-1} under dynamic nitrogen atmosphere (100 mL min^{-1})

covalent modification of protein or amino acid residues to produce a new molecule via bond cleavage, bond formation, rearrangement or substitution. Physical instability refers to changes in the three-dimensional conformational integrity and not necessarily involves covalent modification [24].

DSC curve of inert nanoparticles (2) showed different endothermic and exothermic events, when compared to pure chitosan and TPP, confirming a new structure with different thermal characteristics. Comparing to inert nanoparticles with insulin-loaded nanoparticles it observed small modifications at the thermal events (2), which probably were attributed to the insulin presence into the carrier structure [7, 20]. Insulin-loaded systems reached these thermal events at lower temperature values comparing to unloaded nanoparticles. Thus, an interaction between the protein and the components could have occurred. Similar results were obtained for Sarmento et al. [13].

Figure 3 shows the chitosan TG/DTG curves, which confirm the DSC events and show a first mass loss of 11.3% in the temperature range between 30 and 110 °C, relating to the loss of water, and a second mass loss of 30.97%, on the thermal decomposition, with peaks DTG = 292.96 °C. The TPP showed two events of mass loss in the following ranges of temperature and percentages: 25–102 °C (Δm = 1.2%) and 102–144 °C (Δm = 1.84%).

TG/DTG curves of insulin presented four events of mass loss with the following percentages and temperature ranges: 25–123 °C (Δm = 13.2%), 180–280 °C (Δm = 15.61%), 280–440 °C (Δm = 50.96%) and 440–800 °C (Δm = 10.46%). Similarly DSC and TG/DTG curves of insulin-loaded nanoparticles and inert nanoparticles showed only slight changes in the thermal events related to the presence of insulin into the carrier structure. Both had four events of mass loss.

The interaction between insulin and chitosan was also reported by Boonsongrit et al. [19, 25], although the electrostatic reactions occur between the positively charged amino groups of chitosan and carboxyl groups of insulin, this interaction was limited. Boonsongrit et al. [25] studied surface charge of chitosan microparticles contained insulin and observed that surface charge of insulin-chitosan microparticles decreased with increasing the amount of insulin in formulation and accompanied by the increase of entrapment efficiency. However, the surface charge could not be reduced to the zero level. Then, the results reveal that only a small amount of insulin interacts with the positively charged chitosan. Boonsongrit et al. [19] evaluated the interaction between insulin and chitosan by isothermal titration calorimetry technique. According to these authors, the change in enthalpy (ΔH) of the titration between insulin and chitosan

seems to be partly related to the ionic interaction between both molecules. In this study, ΔH modifications were attributed to conformational changes, caused by the presence of electrostatic interactions, hydrophobic interactions and hydrogen bonding, the ionization of polar groups and, especially, due to adsorption phenomena of insulin on the carrier surface. As a result, they concluded that small amount of insulin interacts with the chitosan positive charges and the main reason for this association was the adsorption and/or incorporation phenomena, through the physical mechanism, during the nanoparticles preparation. Thus, the discrete changes found by the nanoparticles FTIR, DSC and TG/DTG analysis could be justified by the results presented by Boonsongrit et al. [19].

Considering the AE% results, we suggest that the insulin encapsulation in chitosan/TPP nanoparticles occurred by electrostatic interactions, as evidenced by analysis of FTIR, DSC, and TG/DTG, as well as physical process and/or by adsorption phenomena during nanoparticles formation.

Conclusions

The chitosan and insulin interaction were successfully evaluated using FTIR and thermoanalytical techniques. Modifications in the thermal events of the inert nanoparticles in relationship with individual spectra were understood like a new structure formation with different characteristics. The peaks of inert nanoparticles after insulin addition and the AE% data suggest the presence of insulin on the carrier structure. However, insulin encapsulation seems to be related not only to electrostatic interactions, but also to physical process and/or adsorption phenomena of drug incorporation.

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