BIOENCAPSULATION INTO NANOPLEX CARRIER FOR ORAL INSULIN DELIVERY

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1. Introduction

Oral delivery of insulin via nanoplex carrier has presented important potential on improving uptake and translocation of the peptide-based drug through the gastrointestinal tract, mainly via M cells of the gut-associated lymphoid tissues (Morishita et al. 2006). Nanoplexes of natural polyelectrolytes formulated through a bioencapsulation process avoiding exposure to elevated heating and organic solvents are devised to maintain the biological activity of insulin and to overcome barriers for gastrointestinal absorption (Sarmento et al. 2007).

The purpose of the study is to prepare nanoplex carriers by ionotropic gelation using alginate, dextran sulfate, chitosan, polyethyleneglycol and albumin to explore the acid- and enzyme-protective, mucoadhesive and absorptive-enhancer properties on enabling uptake and translocation of insulin. Polyethyleneglycol is considering for the modification of chitosan to improve biocompatibility and to improve insulin stability. The insulin association efficiency (AE) is analyzed and, the polyelectrolyte complexes are characterized in terms of size distribution, chemical and thermal interactions.

Formulations following systematic determination of the aims and critical parameters will be used for *in vitro* studies based on analysis of cytocompatibility and insulin transport using Caco-2 cell monolayers (Prego et al. 2006) and *in vivo* based on analysis of glucose reduction and physiological response after oral administration to diabetic rats.

2. Materials and Methods

Low viscosity sodium alginate, chitosan (50 kDa) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Chemie (L'Isle d'Abeau Chesnes, France). Dextran sulfate (5 kDa) was purchased from Fluka Biochemika (Buchs, Switzerland). Polyetheneglycol 4000 (PEG 4000) was purchased from Fluka Chemie GmbH (Buchs, Switzerland). Calcium chloride was purchased from Panreac Quimica (Barcelona, Spain). Actrapid, INN- insulin human (rDNA) was kindly donated by *Hospitais da Universidade de Coimbra*.

Polyelectrolyte complex is obtained by mixing aqueous solutions of polymers carrying opposite charges under limited pH and stoichiometric conditions. Nanoplexes are prepared dropping 7.5 ml of 18 mM calcium choride solution for 60 minutes under 800 rpm at room temperature into a beacker containing 117.5 ml of 0.06% (w/w) alginate solution, 0.025% (w/w) dextran sulfate solution and insulin equivalent to 200 UI. Into the pre-gel, 25 ml of 0.07% chitosan solution dissolved in 1% acetic acid solution and 0.35% PEG 4000 solution is added dropwise for 90 minutes and, 25 ml of 1% albumin solution is added for further 30 minutes to stabilize the pre-gel nucleus into nanoplexes. The pH of alginate solution is initially set to 4.9, chitosan-PEG solution set to 4.6 and albumin solution set to 5.1. Nanoplexes are recovered from colloidal suspension by centrifugation at 20,000xg for 30 minutes at 4ºC. Insulin-free nanoplexes are also prepared, lyophilized and stored at 4ºC.

Polyelectrolyte complexes and physical mixtures are characterized by laser diffraction spectroscopy (LDS) for size determination and, differential scanning calorimetry (DSC) and Fourier-transform infrared spectroscopy (FTIR) to verify chemical and thermal interactions among polyelectrolytes and between insulin and polymers.

3. Discussion

3.1. Insulin association efficiency (AE)

Insulin is quantified in triplicate using LC-2010 HT HPLC system (Shimadzu Co., Japan) equipped with a reversed-phase X-Terra RP 18 column, 5µm, 4.6 mm x 250 mm (Waters Co., USA), a Purospher® STAR RP-18 precolumn, 5µm, 4 mm x 4 mm (Merck KGaA, Germany) and a UV detector set at 214 nm. The mobile phase consists of acetonitrile and 0.04% trifluoroacetic acid (TFA) aqueous solution initially set in the ratio 30/70 (v/v) and linearly changed to 40/60 over 5 to 10 minutes. The analysis is carried out at room temperature, run time of 12 minutes, flow rate of 1.2 ml/min and injection volume of 20 µl.

The AE is determined indirectly after centrifugation. The amount of insulin associated with nanoplexes is calculated by the difference between the total amount of insulin used to prepare the nanoplexes and the amount of insulin in the supernatant after centrifugation.

$$AE = \frac{\text{Total amount of insulin} - \text{Free insulin in supernatant}}{\text{Total amount of insulin}} \times 100$$

Previously studies reported the AE is probably more dependant on opposing charges between alginate and insulin than between chitosan and insulin (Sarmento et al. 2007). The mean value of insulin AE is directly related to the mechanism of protein association to polyelectrolyte complexes mediated by ionic interactions. The presence of higher concentrations of polyelectrolytes may shift the ionic equilibrium between insulin and polyelectrolytes towards the associated complexes.

3.2. Particle size analysis

Size distribution is determined by laser diffraction spectroscopy (Fraunhofer model) using Coulter LS130 particle analyzer (Beckman Coulter Inc., USA). Particle size is expressed as volume mean diameter and standard deviation (SD) values of the mean. Size distribution depends on the preparation method and conditions, and the concentrations of polyelectrolytes. Previously studies reported that physical properties are strongly influenced by the polyelectrolytes complexes mass ratio (Sarmento et al. 2006). Particle size is an important parameter to determine the absorption, distribution and fate of nanoplexes. In addition, particle size influences the stability, and the insulin loading and release due to larger surface area. Decrease in diameter leads to increased absorption below 1000 nm and, particles above 3000 nm are taken up by the Peyer's patches but are not absorbed (Florence 2005).

3.3. FTIR analysis

Fourier transform infrared (FTIR) analysis is proposed to monitor the complexation of oppositively charged polyelectrolytes as insulin nanoplex carriers for oral delivery. IR spectra of lyophilized complexes are collected in triplicate using ATR-FTIR Magna IR, spectrometer 750, Nicolet (Thermo Fisher Scientific Inc., USA) setting up 64-scan interferogram with 4 cm^{-1} resolution in the mid-IR region at room temperature. FTIR spectrum of isolated polyelectrolyte and comparative studies between nanoplexes and physical mixtures are carried out in order to confirm the polyelectrolyte interactions.

FTIR spectra indicate the peak shifts mainly in absorption bands of carboxyl acid groups of alginate near 1410 and 1600 cm^{-1}, amide and amine groups of chitosan near 1640 and 1570 cm^{-1} and, amide groups of BSA near 1645 and 1540 cm^{-1}. For alginate-chitosan complexes, the asymmetric $-CO^-$ stretching disappear and a new broader band with peak of amine coupled with amide II appear,

indicating that NH_3^+ present in chitosan interacts with $-COO^-$ of alginate. The formation of alginate-chitosan-BSA complexes is mainly driven by an electrostatic mechanism due to large number of functional groups that either are charged or can become charged at specific conditions (Ribeiro et al. 2005). An advantage of FTIR spectroscopy is the possibility of attaining information about the secondary structure of proteins in complex systems without any interference of the polyelectrolytes which are subtracted (Jorgensen et al. 2006). The spectra of insulin present α-helix band at 1654 cm^{-1} and β-sheet and β-turn band at 1684 and 1632 cm^{-1}, respectively (Ribeiro et al. 2005). If the main peak corresponding to β-sheet and α-helix remains almost uncharged, it indicates that insulin is slightly affected after encapsulated into nanoplex carrier.

3.4. DSC analysis

Differential scanning calorimetry (DSC) is used to characterize the thermal behaviour of polyelectrolytes in terms of their structure, hydrophilic properties and association state and, to the folding thermodynamics of proteins (Jorgensen et al. 2006). The technique is based on the heat capacity as a function of the temperature. It is possible to obtain in one analysis the complete temperature profile of the Gibbs energy change associated with the loss of water in polymers, with the denaturation process in proteins and with depolymerization at high temperatures. Shifts of exothermic and endothermic peaks are usually associated with interactions between drug-loaded and polyelectrolytes.

Thermograms of lyophilized nanoplexes and isolated polyelectrolytes crimped into a standard aluminum pan are collected in duplicate using Shimadzu DSC-50 system (Shimadzu Co., Japan) heated from 20 to 350ºC at constant rate of 10ºC/min under constant purging of nitrogen at 20 ml/min.

Peaks of the complexes are shifted from the peaks of physical mixture that appeared to be a combination of each material in contrast to the complexation of polyelectrolytes. Endothermic peaks are correlated with evaporation of water associated to hydrophilic groups of polymers while exothermic peaks are related to degradation of polyelectrolytes due to dehydration, depolymerization and pyrolitic reactions (Sarmento et al. 2006). Thermogram of alginate and chitosan physical mixture show a broader endothermic peak around 76ºC which may represent coalescence of isolated endothermic polymer peaks (Sarmento et al. 2006) and exothermic peaks around 250 and 310ºC related to alginate and chitosan, respectively. The interaction between chitosan and alginate registers an exothermic peak at around 287ºC. Characterization of polyelectrolyte interactions among alginate, chitosan and BSA shows broad and early endothermic peaks of BSA around 70ºC and 220ºC and a new peak at 150ºC, which can be attributed to interactions between the polyelectrolytes. Thermogram of zinc-insulin presents endothermic peaks around 62 and 83ºC and a tiny and broad exothermic peak around 251ºC. If the two endothermic peaks attributed to denaturation process and water loss continued appearing on insulin-loaded nanoplexes, it indicates that insulin is encapsulated and no damage occurred in insulin structure (Sarmento et al. 2006).

4. Conclusions

Nanoplexes prepared by ionotropic gelation exhibits an alternative to encapsulate insulin and are successfully characterized by HPLC, LDS, DSC and FTIR. HPLC and particle size analyses indicating that oral insulin delivery may occur via nanoplexes. Shifts on maximum infra-red peaks, endothermic and exothermic peaks permit to understand ionic interactions and to confirm the formation of nanoplexes carriers. Optimization of the formulation and preparation method are required for *in vitro* studies using Caco-2 cell monolayers and *in vivo* studies based on analysis of glucose reduction and physiological response after oral administration to diabetic rats.

5. References

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