International Journal of Peptide Research and Therapeutics, Vol. 12, No. 2, June 2006 (© 2006), pp. 131–138
DOI: 10.1007/s10989-005-9010-3

Development and Comparison of Different Nanoparticulate Polyelectrolyte Complexes as Insulin Carriers

Bruno Sarmento,[1,5] Susana Martins,[1] António Ribeiro,[2] Francisco Veiga,[3] Ronald Neufeld[4] and Domingos Ferreira[1]

(Accepted December 27, 2005; online publication March 25, 2006)

The overall objective of our research is to produce polyanion/chitosan nanoparticulate oral delivery systems for insulin. Specific objectives of the present study were to study dextran sulfate or alginate complexation with chitosan on mean particle size, insulin association efficiency, loading capacity and release profile. Nanoparticles were formed by ionotropic complexation and coacervation between polyanions (dextran sulfate and alginate) and chitosan. Diameter was evaluated with photon correlation spectroscopy, polymer interaction was confirmed by DSC and FTIR and particle morphology was assessed by SEM and TEM. Mean nanoparticle diameter ranged from 423 to 850 nm, insulin association efficiency from 63 to 94% and loading capacity from 5 to 13%. Dextran sulfate provided highest insulin association efficiency and retention of insulin in gastric simulated conditions. These nanoparticle systems show promise as insulin and potentially other therapeutic polypeptides carriers.

KEY WORDS: alginate; chitosan; dextran sulfate; DSC; FTIR; insulin; nanoparticles.

INTRODUCTION

Polyelectrolytes are polymers containing ionizable groups. As charged macromolecules, they can form polyelectrolyte complexes (PEC) with oppositely-charged molecules or polymers through intermolecular interactions, such as hydrogen bonding, Coulomb forces, van der Waals forces, and transfer forces. These properties have been applied to the encapsulation of therapeutic proteins, cells and enzymes (Dumitriu and Chornet, 1998; Simsek-Ege et al., 2003).

A significant number of natural polyelectrolyte-based colloidal systems are being described as promising carriers for bioactive molecules applying simple and mild encapsulation processes free of heating and organic solvents. Polymers like chitosan (Chit), alginate (Alg) and dextran sulfate (DS) have been described as biocompatible, biodegradable and mucoadhesive, enabling numerous pharmaceutical and biomedical applications (Gombotz and Wee, 1998; Ilium, 1998; Tiyaboonchai et al., 2003). Chitosan polycation is the deacetylated form of chitin (poly-β-(1-4)-*N*-acetyl-D-glucosamine), obtained from exoskeletons of marine arthropods. As a

[1] Faculty of Pharmacy of University of Porto, Porto, Portugal.
[2] Superior Institute of Health Sciences – North, Gandra, Portugal.
[3] Faculty of Pharmacy, University of Coimbra, Coimbra, Portugal.
[4] Chemical Engineering Department, Queen's University, K7L3N6, Kingston, Canada.
[5] Correspondence should be addressed to: Bruno Sarmento, Department of Pharmaceutical Technology, Faculty of Pharmacy of University of Porto, Rua Aníbal Cunha 164, 4050-047, Porto, Portugal. Tel: +351222078949; Fax: +351222003977; e-mail: bruno.sarmento@ff.up.pt.

bioadhesive polymer, absorption enhancer and having antibacterial activity, chitosan is a good candidate for drug delivery (Illum et al., 1994). A variety of chitosan-based colloidal delivery vehicles have been described for the association and delivery of drugs (Prabaharan and Mano, 2005). Alginate is an anionic polysaccharide of (1–4)-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) widely used in bioencapsulation of drugs, proteins and cells. The gelling properties of its guluronic residues with divalent ions such calcium permit the formation of alginate matrices for gels, films, beads, pellets, microparticles and nanoparticles. Dextran sulfate is a branched chain carbohydrate polymer of anhydroglucose units which contains 2.3 sulfate groups per glucosyl residue. In addition to its application for polyelectrolyte complexation, it has been applied in biomedical field for lipoprotein separation and DNA release from DNA-histone complexes (Chen et al., 2004). Polyelectrolyte complexes composed of oppositely charged natural polymers have been previously formulated under mild conditions to carry proteins (Dumitriu and Chornet, 1998; Lacik et al., 2001; Tiyaboonchai et al., 2003), and colloidal carriers made by polymer complexation represents a very promising vehicle to entrap proteins and provide protection and sustained release. Insulin is a 5.8 kDa protein used exogenously to treat insulin-dependent diabetes mellitus (IDDM) when normal pancreatic production is insufficient. Bioavailability of insulin after oral administration is normally low, due to acidic gastric pH, the enzymatic barrier of the intestinal tract and the physical barrier made up of the intestinal epithelium. The use of nano and microparticulate systems potentially provides gastric protection, controlled release and enhanced absorption of perorally poorly absorbed drugs like insulin by mucosal adhesion and nanoparticle direct uptake (Fasano, 1998; Pan et al., 2002; Tiyaboonchai et al., 2003). Particles smaller than 10 μm can be taken by the M-cells and transported into the Peyer's patches. Most microparticles larger than 5 μm remain in the Peyer's patches but those smaller than 5 μm are transported through the efferent lymphatics (Fasano, 1998; Vauthier et al., 2003).

The aim of this work was to entrap insulin in different polyanion/chitosan nanoparticulate systems with high efficiency, to study nanoparticulate complexes morphologic and physical properties and to investigate insulin release behaviour under gastrointestinal conditions.

MATERIALS AND METHODS

Materials

Low-G (F_G = 0.39) low viscosity sodium alginate, chitosan low molecular weight (MW) (≈50 KDa), low MW dextran sulfate (5 kDa) and calcium chloride were purchased from Sigma (Oakville, Canada). Polyanion stock solutions were prepared in deionised water (Milli-Q®) overnight under magnetic stirring and chitosan samples were dissolved in 1% acetic acid solution in deionised water followed by filtration using a Millipore #2 paper filter and stored at 4°C. Human zinc-insulin crystal was a gift from Lilly Farma, Portugal.

Nanoparticulate Systems Production

Three types of nanoparticles were prepared. Alg/Chit nanoparticles were formulated by ionotropic pre-gelation or by coacervation, and DS/Chit nanoparticles were produced by coacervation. Alg/Chit nanoparticulate systems were prepared by ionotropic gelation of polyanion with calcium chloride followed by polycationic crosslinking (Sarmento et al., 2005). Briefly, 18 mM calcium chloride solution was dropped for 60 min into 117.5 mL of a 0.063% alginate solution mixed at 800 rpm, to provide an alginate pre-gel. Then, 0.05% chitosan solution was added dropwise into the pre-gel over 90 min giving a final alginate and chitosan concentration of 0.05 and 0.016%, respectively. The pH of alginate and chitosan solutions was initially set to 4.9 and 4.6. A colloidal dispersion at pH 4.7 formed upon polycationic chitosan addition. Nanoparticle complexation between DS and Chit were performed employing aqueous solutions of oppositely charged polymers in a final volume of 20 ml. Complexes were obtained after dropwise addition of chitosan solution at pH 5.0 to DS solution at pH 3.4 followed mixing for 15 min at 500 rpm to final concentrations of 0.1% chitosan and 0.15% polyanion (Chit/DS mass ratio 2:3). For Alg/Chit nanoparticles produced by coacervation, chitosan solution at pH 4.6 was dropped onto alginate solution at pH 4.9 under magnetic stirring for 15 min resulting final concentrations of 0.05 and 0.0116% to alginate and chitosan, respectively (Alg/Chit mass ratio 4.3:1) These pH values were selected in order to provide ideal ionic interactions. Insulin was incorporated by prior mixing with polyanion solution before nanoparticulate complexes formation. Nanoparticulate complexes were isolated by centrifugation (20 000×g/30 min) and stored at 4°C.

Size Analysis

The particle size analysis was assessed by photon correlation spectroscopy with a Malvern Zetasizer and Particle Analyzer 5000 (Malvern Instruments, UK). Collective ten readings were performed three times on a sample of particles at 25°C with a detection angle of 90°.

Scanning (SEM) and Transmission (TEM) Electronic Microscope

Nanoparticulate systems morphology was studied using both scanning (SEM) and transmission electron microscope (TEM). For SEM, samples of nanoparticulate complexes were mounted on metal stubs, gold coated under vacuum and then examined in a

JEOL JSM-840 SEM (10 kV, Japan). For TEM, samples were placed in a grid, treated with uranil acetate and observed in a Zeiss EM 902A TEM.

Differential Scanning Calorimetry (DSC)

Thermograms were obtained using a Shimadzu DSC-50 system (Shimadzu, Kyoto, Japan). Samples were lyophilized, crimped in a standard aluminium pan and heated from 20 to 350°C at a heating rate of 10°C/min under constant purging of nitrogen at 20 ml/min.

Fourier Transform Infra-Red (FTIR)

FTIR-spectra were measured using a Bomem IR-spectrometer (Bomem, Canada). The samples were gently triturated with 300 mg of micronized KBr powder and compressed into discs at a force of 10 kN for 2 min sing a manual tablet presser (Perkin Elmer, Norwalk, USA). For each spectrum a 256-scan interferogram was collected in absorption with a 4 cm^{-1} resolution from the 4000–600 cm^{-1} region at room temperature.

Insulin Association Efficiency and Loading Capacity

Association efficiency (AE) and loading capacity (LC) of insulin to nanoparticulate complexes were obtained according to the following equations:

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

$$LC = \frac{\text{Total amount of insulin} - \text{Free insulin supernatant}}{\text{Total weight of nanoparticles}} \times 100$$

Insulin was determined by HPLC running with a Varian 9012 Gradient Solvent Delivery System and a Varian 9050 Variable Wavelength UV–VIS Detector (Varian®, USA) were used to perform all chromatographic runs. The HPLC system was equipped with an XTerra RP 18 column, 5 μm particle size, 4.6 mm internal diameter × 250 mm length (Waters®, USA) and a LiChrospher® 100 RP-18, 5 μm particle size guard column (Merck, Germany) and a mobile phase composed of acetonitrile (ACT) and 0.1% trifluoracetic acid (TFA) aqueous solution in a gradient way were used at a flow rate of 1 ml/min. Protein identification was made by UV detection at 214 nm. The gradient changed from 30:70 (ACT: TFA) to 40:60 in 5 min running following 5 min in isocratic 40:60 ratio. The method was validated and found to be linear in the range of 1–100 μg/ml (R^2 = 0.9996) (Sarmento et al., in press).

Evaluation of Insulin *In Vitro* Release from Nanoparticles

Nanoparticles were placed either into test tubes containing 20 ml of HCl pH 1.2 USP XXVI buffer (120 min/100 rpm) and phosphate pH 6.8 USP XXVI buffer (120 min/100 rpm). Samples were taken and replaced by fresh medium. Released insulin was evaluated by HPLC.

RESULTS AND DISCUSSION

In the present study, nanoparticulate polyelectrolyte complexes composed of cationic chitosan and anionic alginate or dextran sulfate were developed, involving the gentle mixing of two aqueous polyelectrolyte solutions. Nanoparticulate systems were formed from electrostatic interactions between the negative carboxylic groups of alginate or sulfate groups of dextran sulfate and the positive amine groups of chitosan. Table I shows the composition and properties of the nanoparticulate systems developed by three different approaches.

Photon correlation spectroscopy analyses indicated that mean particles size obtained were in the nanoscale range. Alg/Chit nanoparticulate complexes produced by ionotropic pre-gelation of alginate followed chitosan crosslinking presented a mean particle size of 850 nm, while complexes produced by coacervation method presented a mean particle size of 488 nm. DS/Chit nanoparticule complexes produced by coacervation resulted in a narrow size distribution with a mean particle size of 423 nm. In addition, TEM and SEM micrographs (Figs. 1 and 2) revealed that the nanoparticulate complexes were different morphologically. Particles produced by pre-gelation were spheroid and smooth, but coacervation method originated amorphous and porous particles. Micrographs confirmed the range size and exposed spherical and smooth particles.

Values of AE and LC for Alg/Chit nanoparticulate complexes produced by pre-gelation and coacervation methods were found to be similar, but DS/Chit nanoparticulate complexes produced systems with higher insulin association efficiencies. LC obtained was lower, but also the initial amount of insulin used to prepare nanocomplexes was lower in DS/Chit nanoparticle systems. LC appears to increase with the increase of initial amount of insulin used to prepare the nanoparticulate systems, while

Table I. Characterization of Polyelectrolyte Complexes between Polycationic Chitosan and Polyanionic Polymer in Terms of Mean Size, Insulin Association Efficiency and Loading Capacity (n = 3)

Formulation	Polyanion	Method	Mean particle size (nm)	AE (%)	LC (%)
A	Alginate	Pre-gelation	850 ± 88	68.95 ± 1.97	9.76 ± 2.9
B	Dextran sulfate	Coacervation	423 ± 37	94.10 ± 4.80	4.71 ± 1.02
C	Alginate	Coacervation	488 ± 76	66.43 ± 7.40	12.91 ± 0.50

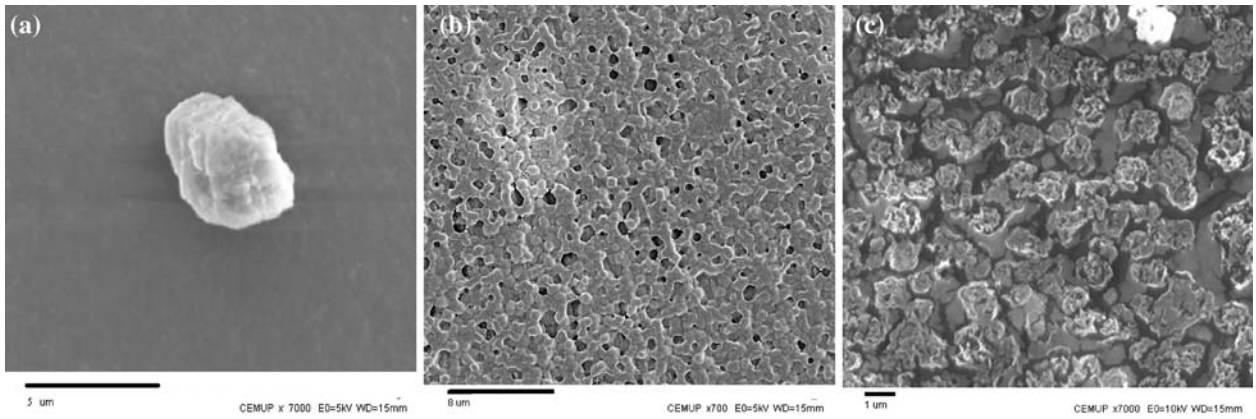

Fig. 1. SEM micrographs of formulation A (a), formulation B (b) and formulation C (c).

AE did not changed significantly with the same initial insulin increase (results not presented). The interaction between insulin and polymers is essentially ionic, but may also take into account hydrogen and van der Waal's forces. Positive amino radicals of insulin are strongly and electrostatically attracted by sulfate/carboxylic groups, and thus pH becomes an important factor controlling this interaction. Since the pI of insulin is around 5.3, positively charged insulin would interact strongly with negatively charged dextran sulfate/alginate. Positively charged chitosan then complexes with free negative residues of polyanion and insulin. The results obtained suggested that the affinity of insulin for DS sulfate groups is higher than for Alg carboxylic groups, as indicated by comparing the association efficiencies of nanocomplexes containing DS and Alg (Table I).

Thermograms plotted in Fig. 3 show differences between individual polymers and complexed suggesting ionic interactions expressed on the rearrangement of endothermic peaks and also on the migration of exothermic decomposition peaks temperature.

Isolated polymers were characterized by the presence of initial endothermic peaks at 86.6, 62.0 and 60.6°C for alginate, chitosan and dextran sulfate, respectively, and higher exothermic peaks at 257.8, 311.0 and 210.5°C, respectively. Endothermic peaks are correlated with loss of water associated to hydrophilic groups of polymers while exothermic peaks resulted from degradation of polyelectrolytes due dehydration, depolymerization and pyrolitic reactions (Zohuriaan and Shokrolahi, 2004).

Association of insulin with nanoparticulate complexes can also be observed by a delay of its endothermic peak at 62–78°C after complexation with formulation A (Fig. 3B). The two endothermic peaks associated with insulin which are attributed to

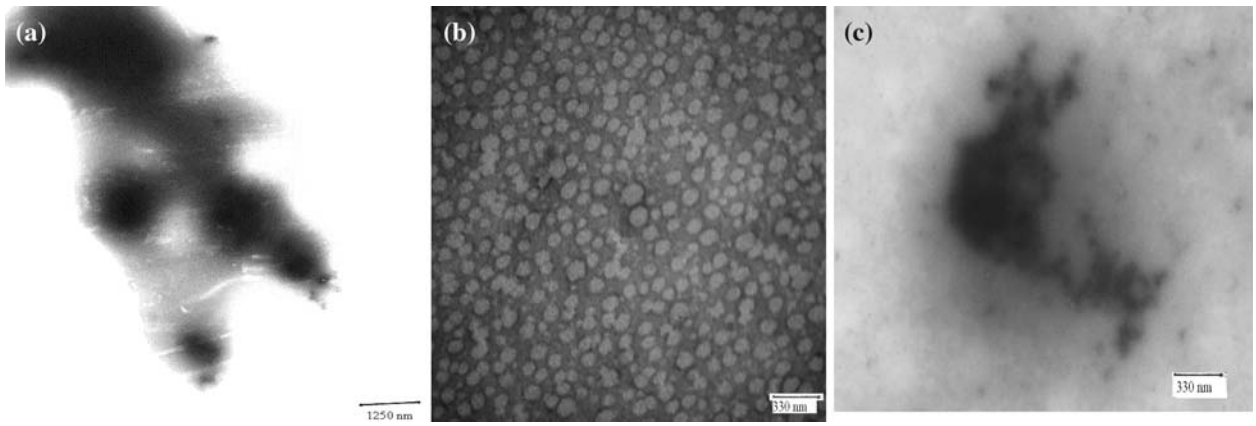

Fig. 2. TEM micrographs of formulation A (a), formulation B (b) and formulation C (c).

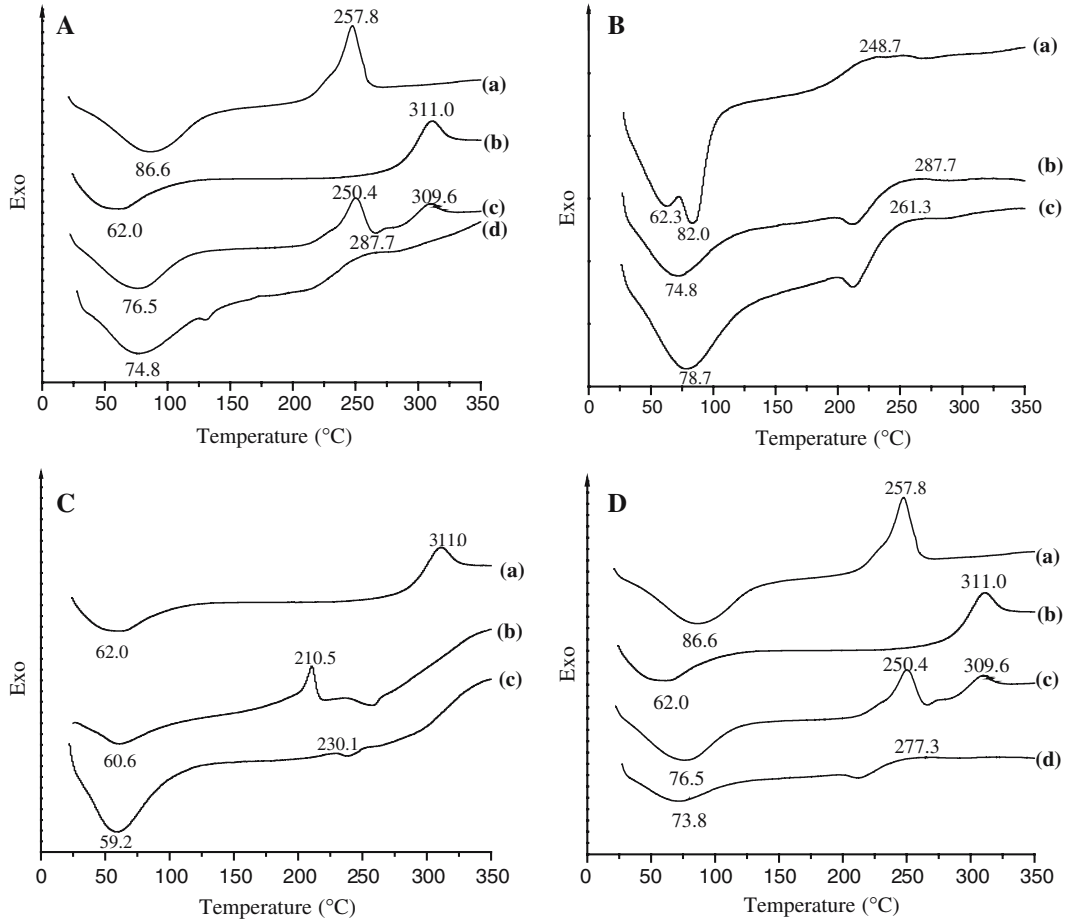

Fig. 3. (**A**). Thermograms of (a) Sodium alginate, (b) Chitosan, (c) Chitosan and alginate physical mixture, (d) Alginate/Chitosan nanoparticles produced by ionotropic pre-gelation (**B**). Thermograms of (a) Insulin, (b) Alginate/Chitosan nanoparticles, (c) Insulin-loaded alginate/chitosan nanoparticles produced by coacervation (**C**). Thermograms of (a) Chitosan, (b) Dextran sulfate, (c) Dextran sulfate/Chitosan nanoparticles (**D**). Thermograms of (a) Sodium alginate, (b) Chitosan, (c) Chitosan and alginate physical mixture, (d) Alginate/Chitosan nanoparticles produced by coacervation.

denaturation process and water loss (Huus et al., 2005), became indistinct and transformed themselves into a single peak after entrapped to Alg/Chit nanoparticulate complexes. Similar results were obtained for both formulations produced by coacervation process (results not shown). Insulin-loaded systems reached this endothermic condition at lower temperature values compared insulin-empty systems, clearly indicating an interaction between the protein and the polyelectrolytes. Also, comparing the exothermic peak of insulin-loaded and unloaded Alg/Chit nanocomplexes, its onset point started at lower temperature for insulin-loaded particles, possibly indicating that insulin being entrapped, started the decomposition at higher temperature values (261.3°C) than when analyzed separately from particles (248.7°C).

In order to examine relations between components of nanoparticulate systems, preliminary concerns were with the polymers' interaction and insulin entrapment. Figures 4 and 5 represent Fourier transform infrared (FTIR) spectra of pure alginate and chitosan, Alg/Chit nanoparticles, and insulin and insulin-loaded Alg/Chit nanoparticles.

Alginate presented carbonyl peaks near 1615 cm^{-1} (symmetric COO^- stretching vibration) and 1415 cm^{-1} (asymmetric COO^- stretching vibration) and one peak at 1033 cm^{-1} that correspond to vibrations of the carbohydrate ring. The FTIR spectrum of chitosan also shows a peak of amide bond at 1636 cm^{-1} and a strong protonated amino peak at 1569 cm^{-1} because it is obtained from partial N-deacetylation of chitin. Alginate peaks enlarged and slightly shifted from 1613 to 1610 and 1414 to

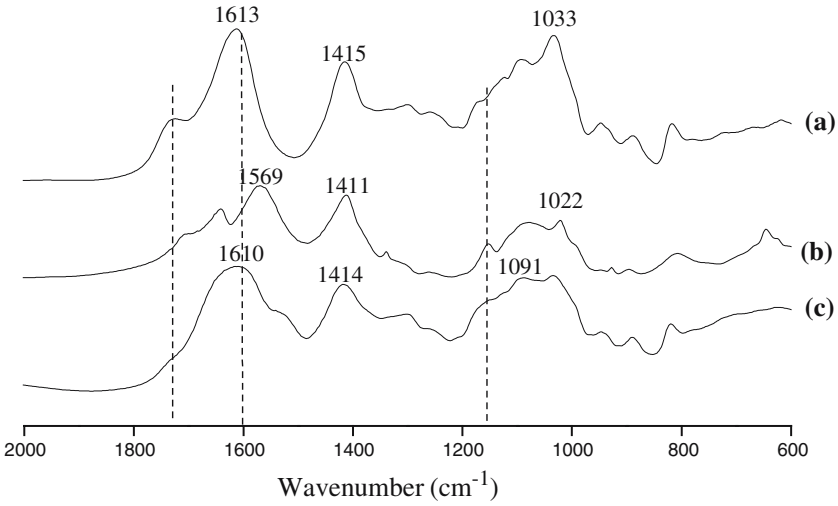

Fig. 4. FTIR spectra of (a) Alginate, (b) Chitosan and (c) Alginate/Chitosan Nanoparticles produced by ionotropic pre-gelation.

1415 after complexation with chitosan. Both chitosan peaks were similarly changed after complexation with alginate, amide peak into singlet band at 1610 cm^{-1} and amino peak to 1534 cm^{-1}. In addition, the peak intensity of amino groups of chitosan at 1153 cm^{-1} was also decreased after complexation. Thus, the results presented here suggest an effective interaction between polymers after complexation and the production of nanoparticles made by opposite charged Alg and Chit, since the peaks of the molecular groups responsible for the ionic interaction had been altered. Similar observations were noted previously (Mitrevej et al., 2001).

The introduction of insulin into nanoparticulate complexes was also investigated by FTIR spectrum of insulin-loaded nanoparticles. The spectrum revealed more pronounced shoulders absorption in the amide I (~1650 cm^{-1}) and amide II (~1540 cm^{-1}) regions as characteristic of protein spectra (Fig. 6).

Dextran sulfate presented sulfyl peaks near 1026 cm^{-1}(symmetric SOO^- stretching vibration) and 1261 cm^{-1} (asymmetric SOO^- stretching vibration) as well as band around 820 cm^{-1} correspondent to S–O–S vibrations (Cakic et al., 2005).

The cumulative release of insulin at HCl pH 1.2 USP XXVI buffer and phosphate pH 6.8 USP XXVI

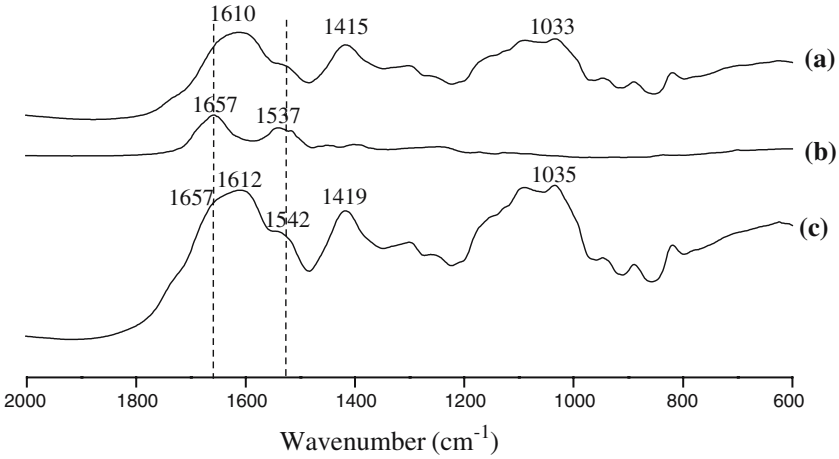

Fig. 5. FTIR spectra of (a) Alginate/Chitosan nanoparticles, (b) Insulin and (c) Insulin-loaded Alginate/Chitosan nanoparticles.

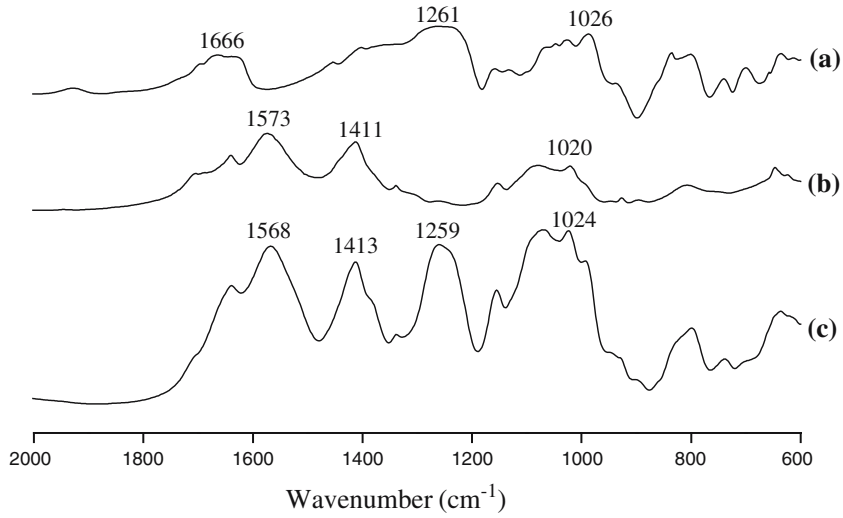

Fig. 6. FTIR spectra of (a) Dextran sulfate, (b) Chitosan and (c) DS/Chitosan Nanoparticles produced by coacervation.

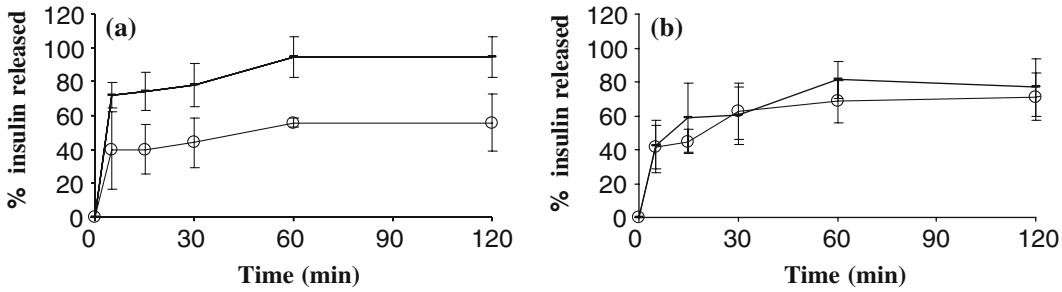

Fig. 7. Insulin release profile at USP XXVI pH 1.2 buffer (a) and USP XXVI pH 6.8 buffer (b) from formulations A (—) and B (o) ($n = 3$).

buffer from insulin-loaded Alg/Chit nanoparticulate complexes prepared by ionotropic pre-gelation and DS/Chit nanoparticulate complexes prepared by coacervation are depicted in Fig. 7a and b, respectively. Nanoparticulate complexes of Alg/Chit prepared by coacervation released all associated insulin after 5 min in contact with both pH buffers (results not shown), indicating that these nanoparticulate complexes lost their integrity very rapidly.

It may be seen that insulin release from Alg/Chit and DS/Chit occurred very rapidly as a burst effect within the first 5 min, followed by a reduction in release rate. It is possible that some insulin was associated with the particle surface and desorbed in contact with the aqueous environment. In simulated gastric conditions a significant increment of insulin retention when using dextran sulfate in the formulation compared with alginate was observed. The sulfate negative groups probably interact strongly with insulin amino groups suggesting higher affinity than alginate carboxylic groups, as observed above in AE values.

In intestinal simulated conditions, the release pattern was similar to that in gastric environment for Alg/Chit nanoparticulate complexes, but slight higher amounts for DS/Chit nanoparticulate complexes. One explanation could be the overall negative charge that insulin adopted at pH 6.8 which contribute to the decrease of ionic interaction with negative DS.

CONCLUSIONS

Insulin was associated with nanoparticulate polyelectrolyte complexes in the nanomeric size range, made with biodegradable, natural polymers. Nanoparticulate complexes interactions themselves

and with insulin were revealed by DSC and FTIR analysis. Its release was evaluated under simulated gastrointestinal conditions and protection improved by complexation with highly negative charged dextran sulfate. These nanoparticulate complexes appear to possess good properties for oral protein delivery, particularly those containing dextran sulfate/chitosan polyelectrolytes, although additional in vitro and in vivo studies must be conducted to confirm such properties.

ACKNOWLEDGEMENTS

This work was supported by *Fundação para a Ciência e Tecnologia*, Portugal and the Natural Sciences and Engineering Research Council of Canada. The authors wish to thank Lilly Farma for insulin supply.

REFERENCES

Cakic, M., Niholic, G., Ilic, L. and Stankovic, S.: 2005, *Chem. Ind. Chem. Eng. Q*. 11, 74–78.

Chen, Y., Mohanraj, V. J. and Parkin, J. E.: 2004, *Lett. Pept. Sci*.. 10, 621–629.

Dumitriu, S. and Chornet, E.: 1998, *Adv. Drug Deliv. Rev*.. 31, 223–246.

Fasano, A.: 1998, *Trend. Biotechnol*.. 16, 152–157.

Gombotz, W. R. and Wee, S. F.: 1998, *Adv. Drug Deliv. Rev*.. 31, 267–285.

Huus, K., Havelund, S., Olsen, H. B., van de Weert, M. and Frokjaer, S.: 2005, *Biochemistry*. 44, 11171–11177.

Ilium, L.: 1998, *Pharm. Res*.. 15, 1326–1331.

Illum, L., Farraj, N. F. and Davis, S. S.: 1994, *Pharm. Res*.. 11, 1186–1189.

Lacik, I., Anilkumar, A. V. and Wang, T. G.: 2001, *J. Microencapsul*.. 18, 479–490.

Mitrevej, A., Sinchaipanid, N., Rungvejhavuttivittaya, Y. and Kositchaiyong, V.: 2001, *Pharm. Develop. Technol*.. 6, 385–392.

Pan, Y., Li, Y. J., Zhao, H. Y., Zheng, J. M., Xu, H., Wei, G., Hao, J. S. and Cui, F. D.: 2002, *Int. J. Pharm*.. 249, 139–147.

Prabaharan, M. and Mano, J. F.: 2005, *Drug Deliv*.. 12, 41–57.

Sarmento, B., Ribeiro, A., Veiga, F. and Ferreira, D. (in press), *Biomed. Chromatogr*. .

Sarmento, B., Ribeiro, A., Veiga, F., Neufeld, R. and Ferreira, D.: 2005, *Rev. Port. Farm*.. LII, 139–140.

Simsek-Ege, F. A., Bond, G. M. and Stringer, J.: 2003, *J. Appl. Polym. Sci*.. 88, 346–351.

Tiyaboonchai, W., Woiszwillo, J., Sims, R. C. and Middaugh, C. R.: 2003, *Int. J. Pharm*.. 255, 139–151.

Vauthier, C., Dubernet, C., Fattal, E., Pinto-Alphandary, H. and Couvreur, P.: 2003, *Adv. Drug Deliv. Rev*.. 55, 519–548.

Zohuriaan, M. J. and Shokrolahi, F.: 2004, *Polym. Test*.. 23, 575–579.