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Development and characterization of self-aggregated nanoparticles from anacardoylated chitosan as a carrier for insulin

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ABSTRACT

Nanoparticulate carriers made from biodegradable polymers especially from chitosan seems to be an excellent approach to increase the uptake and transport of orally administered insulin. Various approaches have been studied to develop nanoparticles from chitosan including self-aggregated nanoparticles from hydrophobically modified chitosan. Anacardic acid is a naturally occurring fatty acid having bulky aromatic group as well as long aliphatic chain and an amphiphilic monomer of great potential. An attempt has been made to develop and characterize self-aggregated nanoparticles from chitosan modified with anacardic acid. Anacardoylated chitosan spontaneously formed nanoparticles in aqueous insulin solution with a particles size of 214 nm diameter at neutral pH. The hydrophobic nature of the nanoparticles helped in the sustained release of insulin in the intestinal environment and the released insulin was stable and retained its conformation. However, it released insulin in acidic conditions and need to be encapsulated in alginate to render pH sensitiveness.

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1. Introduction

Injections had been the only available route for the delivery of insulin since its discovery by Banting and Best in 1921. Oral delivery of insulin can replace daily injections to diabetic patients, however, poses unique problem of stability and susceptibility to proteolysis, which reduce their bioavailability. Administration of therapeutic peptide drug such as insulin via oral route represents one of the greatest challenges in modern pharmaceutical technology (Morishita & Peppas, 2006). The protection of insulin against self-aggregation as well as enzymatic degradation is an important issue for an oral insulin delivery system (Hega et al., 1990). Successful systemic uptake after oral delivery is difficult to achieve because insulin is a large molecule and hydrophilic with possible enzymatic degradation before reaching the site of absorption (Lee & Yamamoto, 1990). Nanoparticulate carriers made from biodegradable polymers seems to be an excellent approach to increase the uptake and transport of orally administered insulin. Like sub-micron emulsions and liposomes, biodegradable polymeric nanoparticles offer a higher stability when they are in contact with biological fluids (Hans & Lowman, 2002). The polymeric nature of nanoparticles protects the drug from adverse external conditions and controls its release (Calvo, Alonso, Vila-Jato, & Obinson, 1996; Romero-Cano & Vincent, 2002; Sakuma et al., 1997; Takeuchi, Yamomoto, & Kawashima, 2001). Moreover, it has been shown that M-cells located on the surface of Peyer's patches are a possible pathway for transporting the nanoparticles through the epithelium of the gut (Clean et al., 1998; Jung et al., 2000). There is a challenge in encapsulating water soluble drugs in water insoluble polymers efficiently (Niwa, Takeuchi, Huno, Kunuo, & Kawashima, 1994; Park, Lu, & Crotts, 1995). Various reports on investigations to improve the oral bioavailability of insulin suggests delivering insulin utilizing nanoparticles made from chitosan or chitosan derivatives and other bioadhesive polymers (Pan et al., 2002).

Interest in chitosan for pharmaceutical formulation continues to grow. Chitosan is a unique polymer that has demonstrated utility in a number of applications for oral drug delivery. Chitosan's film forming ability makes it a coating agent for conventional solid dosage forms such as tablets. Moreover, its gel and matrix forming abilities makes it useful for solid dosage forms such as granules, microparticles, etc. Chitosan also serve as a controlled-release matrix, in addition to properties such as mucoadhesion and permeation enhancement due to its cationic nature which is capable of opening tight junctions in a cell membrane to improve oral bioavailability of a drug. Nanoparticles are normally utilized for drug delivery applications because of its high stability, prolonged residence time, high drug encapsulation, better storage life and the ability to translocate through the intestinal barrier; by Para cellular pathway or via M-cells in Peyer's patches (Hussain, Jaitley, & Florence, 2001).

Anacardic acid is a natural fatty acid having bulky aromatic group as well as long aliphatic chain, found in the exudates of

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plants which belong to Anacardeacea family. Moreover, it is a naturally occurring biocompatible amphiphilic monomer of great potential. It is a natural salicylic acid derivative with an unsaturated non-isoprenoid long aliphatic side chain. It is also a natural aspirin derivative which has shown bioactivity (Acevedo et al., 2006; Balasubramanyam, Swaminathan, Ranganathan, & Kundu, 2003; Bhattacharya, Mukhopadhyay, Rao, Bagchi, & Ray, 1987; Pillai, 1997). Various reports on chitosan have shown that control of drug release was improved by hydrophobic stabilization of matrices and substitution degree (Kurita, 2001; Martin et al., 2002; Noble, Gray, Sadiq, & Uchegbu, 1999). In the present study an attempt has been done to modify chitosan region-specifically by *N*-acylation with anacardic acid, a natural salicylic acid derivative which is biocompatible; to develop self-aggregates of hydrophobically modified chitosan through acid chloride route as a matrix for oral delivery of insulin. Nanoparticles with an average size of about 214 nm diameter were developed without any loss of conformational variation and aggregation of encapsulated insulin. The nanoparticles were further encapsulated in sodium alginate for obtaining pH sensitiveness to the final formulation.

2. Materials and methods

Chitosan (degree of deacetylation of 86% with a molecular weight of 196 kDa) was obtained as a gift from India Sea Foods Pvt. Ltd., Cochin, India. Anacardic acid (AA) was obtained as a gift from Adarsh Industries, Karnataka, India. Acetic anhydride was from Merck KGaA, Darmstadt, Germany. Thionyl chloride and pyridine were from SD Fine Chem India Ltd., Mumbai, India. Insulin (human, 400 IU/ml) was a gift from USV Ltd., Mumbai, India. The chemicals and other solvents used were of analytical reagent grade.

Anacardoylation of chitosan was done in two steps. In the first step anacardic acid was acetylated by heating a mixture of 12 g anacardic acid and 36 g acetic anhydride under stirring for 3 h in an oil bath at 80 °C. After the reaction the mixture was cooled to room temperature. This was precipitated in cool distilled water. The precipitate obtained was then extracted in ether. The ethereal layer was separated using a separating funnel and dried to obtain acetylated anacardic acid. In the second step the acetylated anacardic acid was stirred with 10 ml of thionyl chloride in the presence of a drop of pyridine for 2 h at 60 °C in an oil bath. Excess thionyl chloride was allowed to evaporate by raising the temperature to 80 °C. To this 10 ml of dimethyl formamide and 2 g of chitosan was added and stirred for 16 h at room temperature. This was precipitated in distilled water, washed three times with distilled water and dried to obtain anacardoylated chitosan. Anacardoylated chitosan was characterized by FTIR spectroscopy utilizing a Nicolet Impact 410 FTIR spectrometer. For NMR measurements, the sample was dissolved in D_2O acidified with acetic acid, freeze dried to displace adsorbed moisture, and then dissolved in the same solvent. The sample concentration was 10 mg/ml in D_2O (99.9%). ^1H NMR was carried out in a Bruker spectrometer with 500 MHz.

Specific amounts of anacardoylated chitosan were dissolved in DMF to obtain three different concentrations of anacardoylated chitosan. This was added to the insulin solution (400 IU/ml) to obtain insulin-loaded chitosan self-aggregated nanoparticles. This was centrifuged at 10000 RPM for 10 min and the pellet obtained was dried in a refrigerator at 4 °C. Chitosan nanoparticles as a control were prepared by mild ionotropic gelation with sodium tripolyphosphate as per standard reported procedure (Fernández-Urrusuno, Calvo, Remuñán-López, Vila-Jato, & Alonso, 1999). The particle size and zeta potential and the pH titrations of these self-aggregated nanoparticles were analyzed using a zetasizer, Nano ZS and MPT-2 autotitrator (Malvern Instruments Limited, UK).

The in vitro cytotoxicity of the nanoparticles was evaluated by MTT assay (Mosmann, 1983) done on mouse fibroblast (L929) cell lines as per the directions of ISO standard (ISO, 1999).

Encapsulation efficiency and insulin loading of the nanoparticles were evaluated. A known quantity of insulin loaded nanoparticles was incubated at 30 °C in a known quantity of phosphate buffer (pH 7.4) for 24 h. This was filtered using a 0.4 μm syringe filter and the insulin content estimated. The insulin content was estimated by evaluating the protein content by Lowry's method at 750 nm using UV spectrophotometer (UV l60A, Shimadzu). The insulin content was also estimated using RIA which also indicates its immunoreactivity. RIA was performed following the Coat-A-Count Protocol. The radioactivity was measured by counting the tubes in a gamma counter (1470 Automatic Gamma Counter, Perkin Elmer Wizard).

The release of insulin from the nanoparticles was carried out in simulated gastric (SGF, pH 1.2) and intestinal (SIF, pH 6.8) fluids. SIF and SGF were prepared fresh in the laboratory with the composition as per USP without the addition of enzymes (The United States Pharmacopeia, USP 28, 2005, United States Pharmacopeial Convention Inc., Rockville, MD, USA, pp. 2855–2858). The nanoparticles (10 mg) were introduced into 5 ml of SGF and SIF, respectively, in a screw-capped bottle under sterile conditions. Aliquots of 0.5 ml were withdrawn at various time intervals and insulin content was estimated as described earlier. An equal amount of respective buffers was added in order to maintain a constant volume. To bypass the hostile environment of the gastric region these nanoparticles were encapsulated in calcium alginate. The drug loaded nanoparticles (100 mg) were mixed with 2 ml of 2% sodium alginate solution and added drop wise into 2% calcium chloride gelling bath using a syringe. The capsules obtained were rinsed with water and dried at 4 °C in a refrigerator. The dry particles obtained were in the range of 100 μm with a final weight of 140 mg.

The supernatant of the released insulin solution was concentrated to 50 IU/ml (2 mg/ml approx) using a stirred cell, attached with an ultrafiltration filter of 5 kDa cutoff (Millipore, Model 8050) which is generally used for protein purification. CD spectra at 25 °C were acquired using a Jasco J-810 spectropolarimeter as reported (Ramachandran, Paul, & Sharma, 2009).

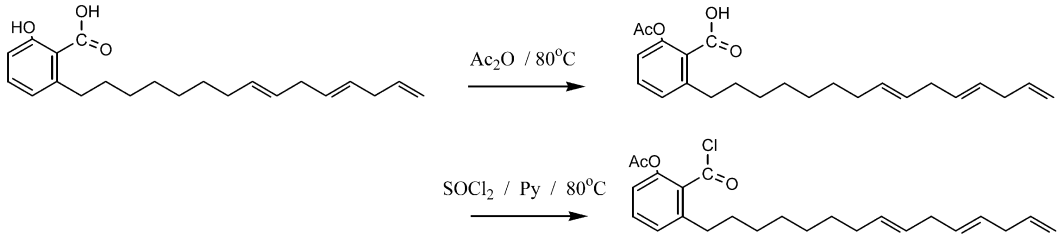

Fig. 1. Scheme of acid chloride reaction with anacardic acid.

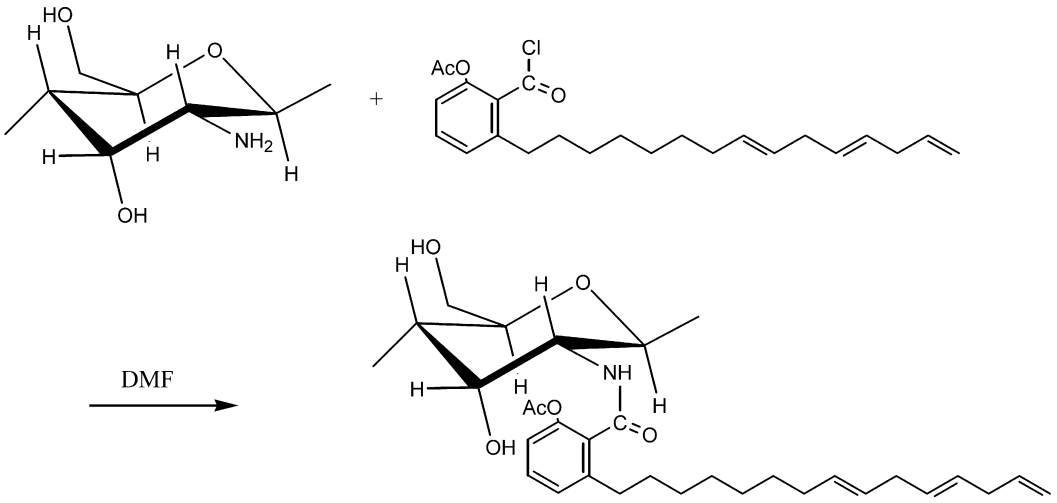

Fig. 2. Scheme of the reaction of chitosan with chlorinated anacardic acid to obtain anacardoylated chitosan.

3. Results and discussion

Chitosan was modified with anacardic acid which is a hydroxy acid having both bulky aromatic group as well as long aliphatic chain. Moreover, it is a naturally occurring biocompatible amphiphilic monomer of great potential. Hydroxyl functionality of anacardic acid is first protected with acetylation using acetic anhydride (Fig. 1) and the acetoxy derivative of anacardic acid which is a long alkyl modified aspirin was reacted with amino group of chitosan using acid chloride chemistry to get region-specific *N*-amidation (Fig. 2). Modified chitosan with anacardic acid showed better solubility in organic solvents like dimethyl formam-

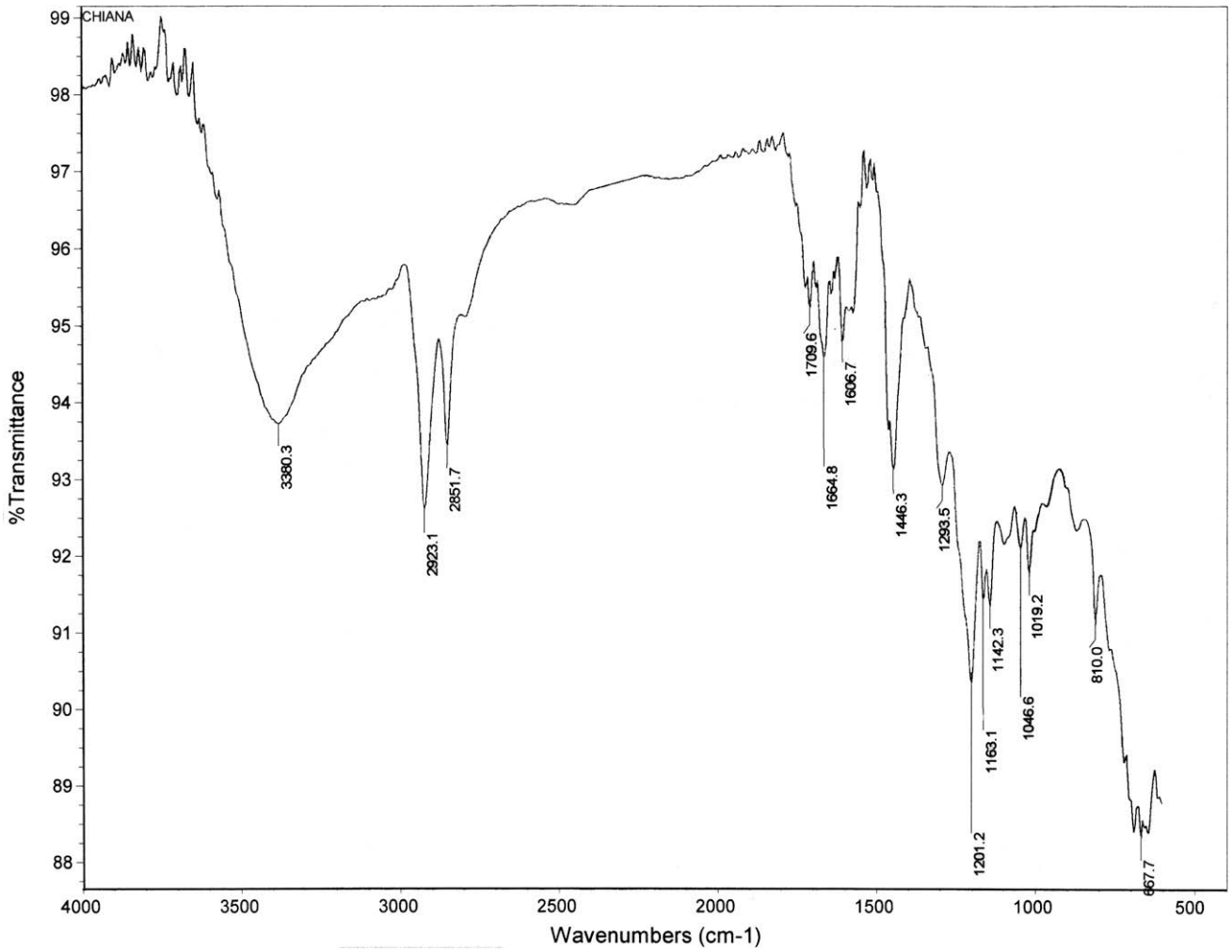

Fig. 3. FTIR spectra of anacardoylated chitosan.

ide compared to unmodified chitosan. This may be due to the reduced crystallinity of chitosan with the incorporation of bulky groups and the disruption of the H-bonding due to the acylation at C-2 position. It has been reported that reduction in crystallinity due to branching leads to increased susceptibility for biodegradation in the case of branched chitosan (Kurita, 2001).

Anacardoylated chitosan was characterized by FTIR spectroscopy and the spectrum is shown in Fig. 3. Absorptions observed at 1709 and 1664 cm^{-1} corresponds to $\nu_{-C=O}$, ν_{-N-H} bending, respectively which confirms amidation. IR absorption at 1606 cm^{-1} corresponds to ν_{Ar} indicated the aromatic ring of anacardic acid. IR absorption at 810 cm^{-1} corresponds to two neighboring aromatic C–H which confirms the aromatic ring of anacardic acid. A broad absorption band observed at 3380 cm^{-1} in the FTIR spectrum corresponds to ν_{OH} further confirmed the absence of C-3 and C-6 ester groups. Normally the secondary alcohol esterification in chitosan is rather difficult as reported by Kurita (2001). Absorption peaks corresponding to hydroxyl stretching frequency in anacardoylated chitosan does not show any noticeable reduction compared to that of standard chitosan spectrum which confirms the absence of acylation at C-3 and at C-6. The ^{1}H NMR spectra of anacardoylated chitosan, is given in Fig. 4. A peak at 1.928 ppm ascribed to $-CH_2$-(CO) is of anacardic acid residue. Peak at 4.6 ppm is assigned to the proton of GlcN residues. Peak at 7.8 ppm is attributed to the protons of the benzene ring. This confirms the derivatisation of chitosan with anacardic acid. The nanoparticles were also found to be non cytotoxic in nature.

It has been reported that the colloids are induced by the self-aggregated polymer (Jane, Calvo, & Alonso, 2001) to form micro- or nano-particles under specific conditions, such as the hydrophobicity/hydrophilicity (Kim, Shin, & Lee, 1998), or ionic strength, hydrogen-bonding network, etc. Recently, self-aggregated nanoparticles received much attention as a material for drug delivery systems since the drug incorporation can be achieved without degradation under harsh reactions in conjugation steps, and at the same time the self-aggregate is eventually formed without a cross-linker. Polymeric self-assemblies are usually formed from block copolymers or hydrophobically modified water-soluble polymers. A study by Ouchi, Nishizawa, and Ohya (1998) has been considered as the first approach on self-aggregation of chitosan. Self-aggregated nanoparticles from several chitosan derivatives like deoxycholate-chitosan, chitosan phthalate, linoleic acid modified chitosan, cholesterol modified chitosan, etc. have since been reported (Chen, Lee, & Park, 2003; Wanga, Liua, Jianga, & Zhanga, 2007; Yoksan, Matsusaki, Akashi, & Chirachanchai, 2004).

The *Z*-average particle size of the self-aggregated nanoparticles of anacardoylated chitosan was 214 [d nm] at neutral pH. Nanoparticles were formed with different concentrations of anacardoylated chitosan. The particle size variation of these nanoparticles was studied with respect to the pH of the medium and it was found that the particles formed with the lower concentration (3.125 mg/ml) aggregated only above the pH of 7.5 whereas other particles, i.e., particles formed from 6.25 and 12.5 mg/ml aggregated at pH 6.2 and 5.5, respectively (Fig. 5). Whereas chitosan nanoparticles prepared by mild ionotropic gelation with sodium tripolyphosphate (Fernández-Urrusuno et al., 1999) were having a size of approximately 400 nm and it aggregated above pH 8. The nanoparticles formed from low concentration solution were having a net negative charge with a mean zeta potential of −20.3 mV at neutral pH as shown in Fig. 6. It was found that compared to chitosan nanoparticles whose isoelectric point is 6.5, anacardoylated chitosan nanoparticles (3.125 mg/ml) showed an isoelectric point of 5.91. Nanoparticles prepared with other concentrations of anacardoylated chitosan exhibited similar isoelectric point; however, the zeta potential in the alkaline region was near zero (data not shown). As shown in Fig. 5 the aggregation of nanoparticles near

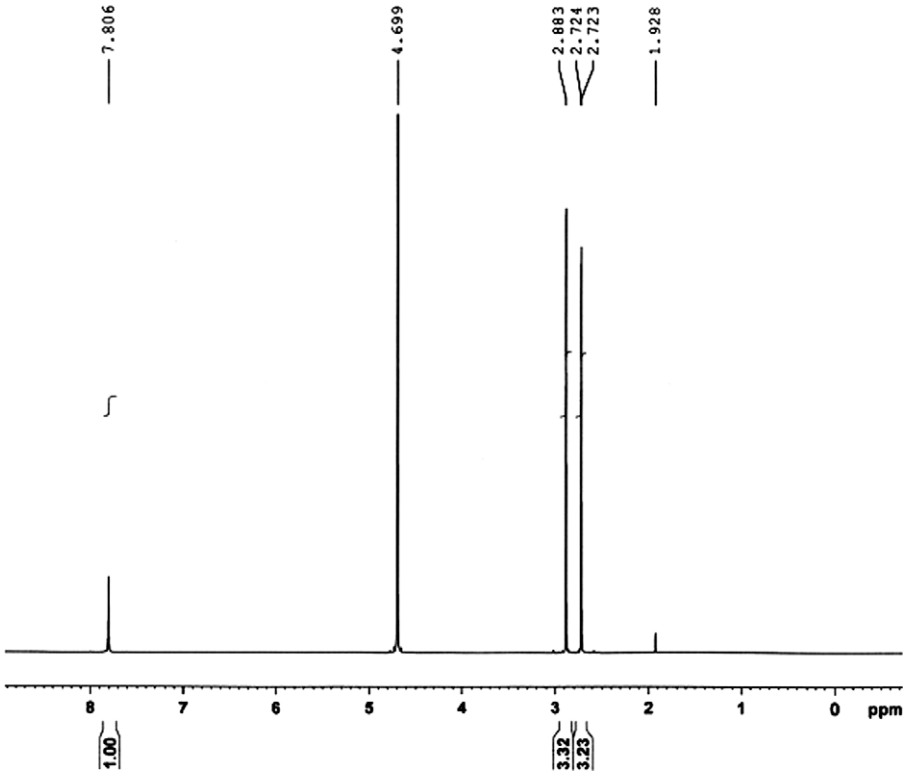

Fig. 4. ^{1}H NMR spectra of anacardoylated chitosan derivative.

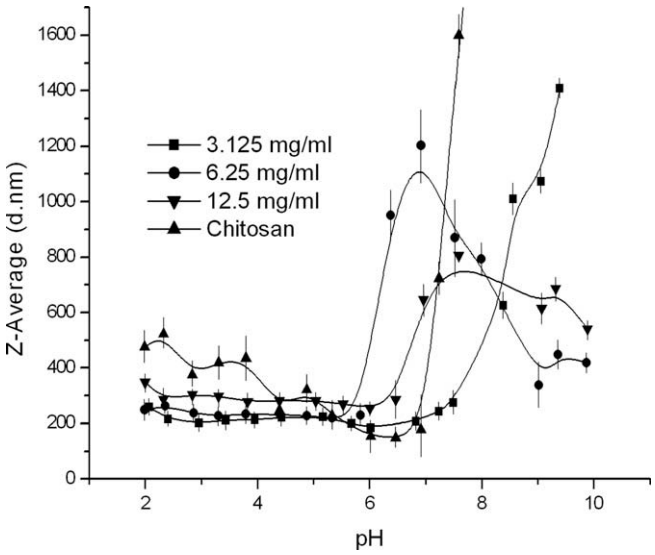

Fig. 5. pH titration versus particle size of anacardoylated chitosan nanoparticles prepared from three different concentrations and chitosan.

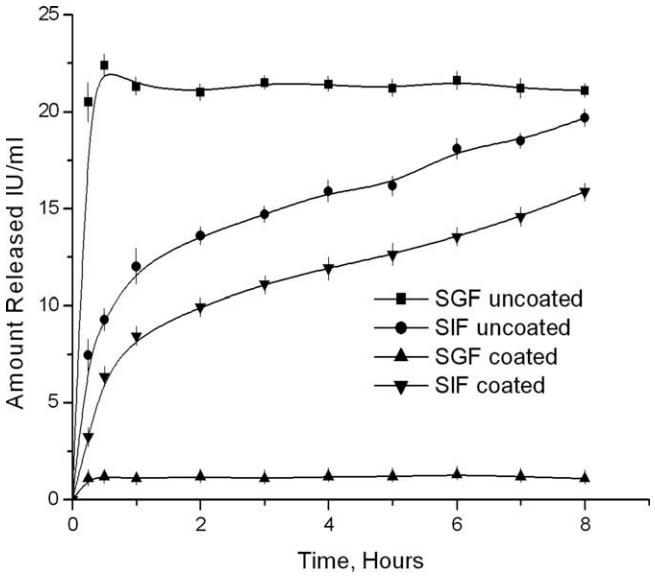

Fig. 7. In vitro release profile of insulin from chitosan–anacardic acid nanoparticles (uncoated) and chitosan–anacardic acid nanoparticles encapsulated in alginate (coated).

to pH 6 may be because of this near zero zeta potential. A negative zeta potential of the nanoparticles at neutral pH is sufficient to provide good stability and shelf life for a formulation prepared from it.

Encapsulation efficiency of the anacardoylated chitosan nanoparticles was found to be 27 ± 3%. The total insulin load in these nanoparticles was 10 ± 0.5 IU/mg of dried nanoparticles as estimated via Lowry's protein estimation method. It was 10.2 ± 0.4 IU/mg when estimated using RIA procedure. This also shows that the loaded insulin is immunoreactive. In order to obtain some preliminary information about the potential use of anacardoylated chitosan as a drug delivery systems for oral administration, in vitro hydrolysis studies were performed subjecting the hydrolysis in simulated gastric (SGF, pH 1.2), and intestinal fluids (SIF, pH 6.8). A moderately sustained release of insulin was observed for anacardoylated chitosan in intestinal fluid as shown in Fig. 7. This seems to be due to the hydrophobic barrier of anacardoylated chitosan limiting access of water and dissolution of the drug. Diffusion of drug may occur through the anhydrate polymer matrix but will generally be facilitated as the polymer gradually swells in contact with the body fluids. Since these modified chitosan nanoparticles swell in acidic pH, complete insulin was released in SGF in 15 min. Therefore, these insulin loaded nanoparticles were encapsulated in calcium alginate to impart pH sensitiveness. Alginate coated nanoparticles released no insulin in the gastric fluid; however, in intestinal fluid it demonstrated a sustained release of over 8 h and the release pattern was similar to that of uncoated nanoparticles. The rapid disintegration of acid treated calcium alginate capsules has been reported earlier (Bajpai & Sharma, 2004; Kim, Chung, Shin, Yam, & Chung, 2008). Alginate capsules reaching the intestinal fluid after a residence time of 1 h in the gastric fluid disintegrates spontaneously, dispersing the nanoparticles into the medium.

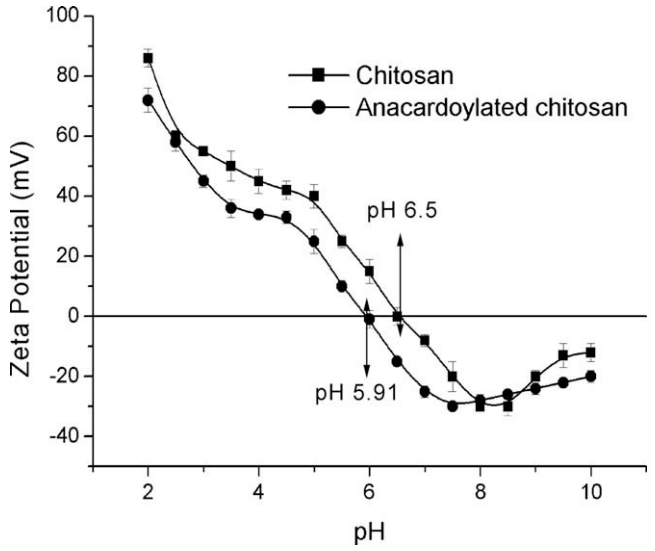

Fig. 6. pH titration versus zeta potential of chitosan and anacardoylated chitosan nanoparticles.

CD spectra provide both qualitative and quantitative information about protein conformations (Prieto, Wilmans, Jimenez, Rico, & Serrano, 1997). At near neutral pH the far-UV CD spectra revealed no significant differences in the secondary structure of the released insulin as compared to the native insulin as shown in Fig. 8.

The degradation of unprotected insulin by the high acid content in the stomach and enzymes of the digestive tract makes the effective bioavailability to less than 1% (Morishita et al., 2006). Several attempts have been made to encapsulate insulin in pH responsive polymers like alginate, chitosan and polymethacrylic acid for safely delivering insulin to the intestine. Insulin is susceptible to conformational changes and related loses of its biological activity in these polymeric formulations (Sah, 1999a, 1999b). In the present study it has been shown that the nanoparticles prepared from hydrophobically modified chitosan can release insulin in sustained manner and is having a negative zeta potential encouraging its compatibility with cells. The alginate encapsulated anacardoylated chitosan nanoparticles seems to be a good candidate for delivering insulin orally.

4. Conclusion

The self-aggregated nanoparticles from hydrophobically modified chitosan using anacardic acid have been developed and

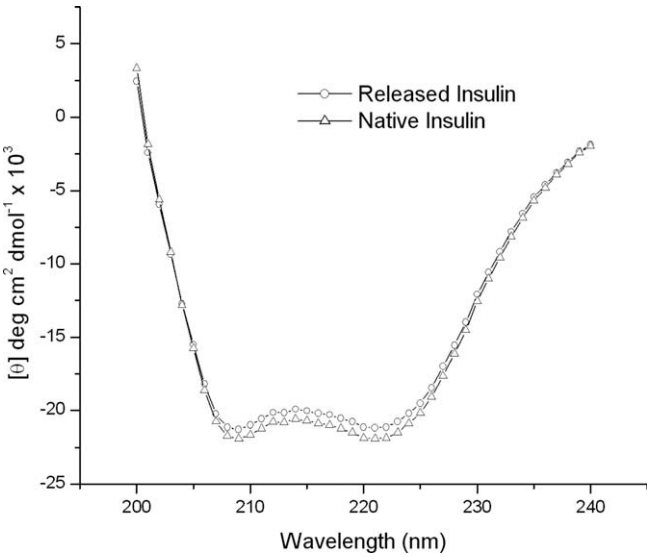

Fig. 8. Far UV CD spectra of native insulin and released insulin.

their possible application towards oral insulin delivery has been discussed. The insulin released from the particles was stable with no conformational changes. It has already been established that fatty acid complexation can improve uptake of particles across epithelium. Since anacardic acid being a natural fatty acid and an aspirin derivative, these nanoparticles may be translocated efficiently across the intestinal epithelium. However, further studies on diabetic animals are required to confirm these findings.

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References

Acevedo, H. R., Rojas, M. D., Arceo, S. D., Hernandez, M. S., Vazquez, M. M., Terrazas, T., et al. (2006). Effect of 6-nonadecyl salicylic acid and its methyl ester on the induction of micronuclei in polychromatic erythrocytes in mouse peripheral blood. *Mutation Research, 609*, 43–46.

Bajpai, S. K., & Sharma, S. (2004). Investigation of swelling/degradation behaviour of alginate beads crosslinked with Ca^{2+} and Ba^{2+} ions. *Reactive & Functional Polymers, 59*, 129–140.

Balasubramanyam, K., Swaminathan, V., Ranganathan, A., & Kundu, T. K. (2003). Small molecule modulators of histone acetyltransferase p300. *Journal of Biological Chemistry, 278*, 19134–19140.

Bhattacharya, S. K., Mukhopadhyay, M., Rao, P. J. R. M., Bagchi, A., & Ray, A. B. (1987). Pharmacological investigation on sodium salt and acetyl derivative of anacardic acid. *Phytotherapy Research, 1*, 127–134.

Calvo, P., Alonso, M., Vila-Jato, J., & Obinson, J. (1996). Improved ocular bioavailability of indomethacin by novel ocular drug carriers. *Journal of Pharmacy and Pharmacology, 48*, 1147–1152.

Chen, X. G., Lee, C. M., & Park, H. J. (2003). O/W emulsification for the self aggregation and nanoparticle formation of linoleic acid-modified chitosan in the aqueous system. *Journal of Agricultural and Food Chemistry, 51*, 3135–3139.

Clean, S. M., Prosser, E., Meehan, E., Malley, D. O., Clarke, N., Ramtoola, Z., et al. (1998). Binding and uptake of biodegradable poly-DL-lactide micro- and nanoparticles in intestinal epithelia. *European Journal of Pharmaceutical Sciences, 6*, 153–163.

Fernández-Urrusuno, R., Calvo, P., Remuñán-López, C., Vila-Jato, J. L., & Alonso, M. J. (1999). Enhancement of nasal absorption of insulin using chitosan nanoparticles. *Pharmaceutical Research, 16*, 1576–1581.

Hans, M. L., & Lowman, A. M. (2002). Biodegradable nanoparticles for drug delivery and targeting. *Current Opinions in Solid State & Materials Science, 6*, 319–327.

Hega, M., Saito, K., Shimaya, T., Maezawa, Y., Kato, Y., & Kim, S. W. (1990). Hypoglycemic effect of intestinally administered monosaccharide-modified insulin derivatives in rat. *Chemical & Pharmaceutical Bulletin, 38*, 1983–1986.

Hussain, N., Jaitley, V., & Florence, A. T. (2001). Recent advances in the understanding of uptake of microparticulates across the gastrointestinal lymphatics. *Advanced Drug Delivery Reviews, 50*, 107–142.

ISO (1999). Biological evaluation of medical devices. Part 5. Tests for in vitro cytotoxicity. *International Organisation for Standards*, ISO 10993-5.

Jane, K. A., Calvo, P., & Alonso, M. J. (2001). Polysaccharide colloidal particles as delivery systems for macromolecules. *Advanced Drug Delivery Reviews, 47*, 83–97.

Jung, T., Kamm, W., Breitenbach, A., Kaiserling, E., Xiao, J. X., & Kissel, T. (2000). Biodegradable nanoparticles for oral delivery of peptides: Is there a role for polymers to affect mucosal uptake? *European Journal of Pharmaceutics and Biopharmaceutics, 50*, 147–160.

Kim, W. T., Chung, H., Shin, I. S., Yam, K. L., & Chung, D. (2008). Characterization of calcium alginate and chitosan-treated calcium alginate gel beads entrapping allyl isothiocyanate. *Carbohydrate Polymers, 71*, 566–573.

Kim, S. Y., Shin, I. G., & Lee, Y. M. (1998). Preparation and characterization of biodegradable nanospheres composed of methoxy poly(ethylene glycol) and DL-lactide block copolymer as novel drug carriers. *Journal of Controlled Release, 56*, 197–208.

Kurita, K. (2001). Controlled functionalization of the polysaccharide chitin. *Progress in Polymer Science, 26*, 1921–1971.

Lee, V. H. L., & Yamamoto, A. (1990). Penetration and enzymatic barriers to peptide and protein absorption. *Advanced Drug Delivery Reviews, 4*, 171–207.

Martin, L., Wilson, C. G., Koosha, F., Tetley, L., Gray, A. I., Senel, S., et al. (2002). The release of model macromolecules may be controlled by the hydrophobicity of palmitoyl glycol chitosan hydrogels. *Journal of Controlled Release, 80*, 87–100.

Morishita, M., Goto, T., Nakamura, K., Lowman, A. M., Takayama, K., & Peppas, N. A. (2006). Novel oral insulin delivery systems based on complexation polymer hydrogels: Single and multiple administration studies in type 1 and 2 diabetic rats. *Journal of Controlled Release, 110*, 587–594.

Morishita, M., & Peppas, N. A. (2006). Is the oral route possible for peptide and protein drug delivery? *Drug Discovery Today, 11*, 905–910.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods, 65*, 55–63.

Niwa, T., Takeuchi, H., Huno, T., Kunuo, N., & Kawashima, Y. (1994). In vitro drug release behaviour of DL-lactide(glycolide) copolymer nanospheres with nafarelin acetate prepared by a novel spontaneous emulsification solvent diffusion method. *Journal of Pharmaceutical Sciences, 83*, 727–732.

Noble, L., Gray, A. I., Sadiq, L., & Uchegbu, I. F. (1999). A non-covalently cross-linked chitosan based hydrogel. *International Journal of Pharmaceutics, 192*, 173–182.

Ouchi, T., Nishizawa, H., & Ohya, Y. (1998). Aggregation phenomenon of PEG-grafted chitosan in aqueous solution. *Polymer, 39*, 5171–5175.

Pan, Y., Li, Y., Zhao, H., Zheng, J., Xu, H., Wei, G., et al. (2002). Bioadhesive polysaccharide in protein delivery system: Chitosan nanoparticles improve the intestinal absorption of insulin in vivo. *International Journal of Pharmaceutics, 249*, 139–147.

Park, T. G., Lu, W., & Crotts, G. (1995). Importance of in vitro experimental conditions on protein release kinetics, stability, and polymer degradation in protein encapsulate poly(D,L-lactic-*co*-glycolic acid) micro spheres. *Journal of Controlled Release, 33*, 211–222.

Pillai, C. K. S. (1997). High performance polymers from natural monomers and polymers. In N. P. Cheremisinov (Ed.), *Handbook of Adv. Polym. Pater.*. New York: Marcel Dekker Inc..

Prieto, J., Wilmans, M., Jimenez, M. A., Rico, M., & Serrano, L. (1997). Non-native local interactions in protein folding and stability: Introducing a helical tendency in the all β-sheet α-spectrin SH3 domain. *Journal of Molecular Biology, 268*, 760–778.

Ramachandran, R., Paul, W., & Sharma, C. P. (2009). Synthesis and characterization of PEGylated calcium phosphate nanoparticles for oral insulin delivery. *Journal of Biomedical Materials Research: Applied Biomaterials, 88B*, 41–48.

Romero-Cano, M. S., & Vincent, B. (2002). Controlled release of 4-nitroanisole from poly(lactic acid) nanoparticles. *Journal of Controlled Release, 82*, 127–135.

Sah, H. (1999a). Protein behaviour at the water/methylene chloride interface. *Journal of Pharmaceutical Sciences, 88*, 1320–1325.

Sah, H. (1999b). Protein instability toward organic solvent/water emulsification: Implications for protein microencapsulation into microspheres. *PDA Journal of Pharmaceutical Science and Technology, 53*, 3–10.

Sakuma, S., Suzuki, N., Kikuchi, H., Hiwatari, K., Arikawa, K., Kishida, K., et al. (1997). Oral peptide delivery using administered salmon calcitonin by polystyrene nanoparticles having poly(*N*-isopropylacrylamide) branches on their surfaces. *International Journal of Pharmaceutics, 158*, 69–78.

Takeuchi, H., Yamomoto, H., & Kawashima, Y. (2001). Mucoadhesive nanoparticulate systems for peptide drug delivery. *Advanced Drug Delivery Reviews, 47*, 39–54.

Wanga, Y. S., Liua, L. R., Jianga, Q., & Zhanga, Q. Q. (2007). Self-aggregated nanoparticles of cholesterol-modified chitosan conjugate as a novel carrier of epirubicin. *European Polymer Journal, 43*, 43–51.

Yoksan, R., Matsusaki, M., Akashi, M., & Chirachanchai, S. (2004). Controlled hydrophobic/hydrophilic chitosan: Colloidal phenomena and nanosphere formation. *Colloid and Polymer Science, 282*, 337–342.