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In vitro evaluation of N-(2-hydroxy) propyl-3-trimethyl ammonium chitosan for oral insulin delivery

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ABSTRACT

The present investigation explores an oral insulin delivery system based on the modification of chitosan, N-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan (HTCC). Synthesis of HTCC was carried out by coupling glycidyl trimethylammonium chloride (GTAC) to chitosan in aqueous medium. Quaternization was confirmed by TNBS assay, FTIR, NMR, SEM studies and zeta potential analysis. Cytotoxicity studies of the derivative were carried out by MTT assay and release profile of insulin from HTCC matrix was monitored under in vitro experimental conditions. Further biological activity and conformational stability of released insulin were confirmed using ELISA and circular dichroism studies. Adhesion studies on mucin and freshly excised rat intestinal sections were carried out to evaluate the mucoadhesive nature of the matrix. Confocal microscopy observations showed that these microparticles were capable of opening tight junctions. By exploiting the mucoadhesive and controlled drug releasing capabilities, HTCC particles seems to be a promising candidate for oral insulin delivery.

1. Introduction

Oral insulin delivery is still a formidable challenge to pharmacists and researchers worldwide due to its low bioavailability. An ideal delivery system for the oral administration of insulin should prolong its intestinal residence time and reversibly increase the permeability of the mucosal epithelium to enhance the absorption of drug. In recent years, biopolymeric nano/microparticles are being widely explored for the oral administration of insulin due to its biocompatibility and safe toxicity profile. Chitosan, hydrophilic cationic polysaccharide, with distinctive biological properties such as non-toxicity, biocompatibility, biodegradability and antimicrobial activity (Mourya & Inamdar, 2008) has been widely used in biomaterial applications. Despite its good biocompatibility, the use of chitosan in drug delivery is limited, owing to its poor solubility in physiological media. Quaternized chitosan derivatives, evaluated to overcome this drawback of chitosan at neutral pH, quaternized chitosan derivatives have been exploited for various pharmaceutical aplications such as drug/protein and gene delivery (Mourya & Inamdar, 2009). The presence of positive charge is expected to increase the mucoadhesive nature of chitosan, which leads to an increased residence time and enhanced bioavailability.

Quaternized chitosan was well known for their antimicrobial activity and moisture retention capacity (Sun & Wan, 2007), but very little effort has been made to develop protein delivery systems using this matrix (Xu, Du, Huang, & Gao, 2003). Taking into account of this previous information, we propose to explore the potential of quaternized chitosan N-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride (HTCC) as a mucoadhesive matrix for oral insulin delivery. It is expected that HTCC would improve mucoadhesivity due to the presence of positive charge and hydroxyl functional group on the side chain.

2. Experimental

2.1. Materials

Chitosan (MW 360 kDa, 86% deacetylated) was gifted from CIFT (Cochin, India). Sodium nitrite was from Merck Specialities Private, Ltd., India and Glycidyltrimethylammonium chloride (GTAC) was purchased from Fluka chemicals, USA. Dulbeccos Modified Eagles Medium (DMEM), Hanks Balanced Salt Solution (HBSS), 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT), Deuterium oxide (D_2O), rhodamine phalloidin, Trinitrobenzene sulfonic acid, mucin from porcine stomach (Type III) were obtained from Sigma–Aldrich, Inc., USA. Eudragit L100-55 was a gift from Rohm Pharma, India. L929 cell lines (mouse fibroblast) and Caco-2 cell lines (human intestinal epithelial cells) were purchased from National Center for Cell sciences Pune, India, ELISA kit from Mercodia and Human Insulin from Eli Lilly, Pvt., Ltd., India. Sodiumtripolyphosphate, methanol, and acetone were purchased from S.D. Fine-Chem, India, Ltd.

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2.2. Synthesis of quaternized chitosan

The low molecular weight chitosan was prepared by oxidative degradation with $NaNO_2$ at room temperature as reported elsewhere (Suzuki, Oda, Shinonu, Saimoto, & Shigemasa, 2000). Molecular weight of depolymerised chitosan was calculated by intrinsic viscosity method using Ubbhelode capillary viscometer (Model Schott AVS, Germany).

Quaternized chitosan (HTCC) was prepared by adding varying amounts of GTAC (0.5 ml and 1 ml) to 1% chitosan (1 g depolymerised chitosan dissolved in 1% acetic acid) solution at neutral condition. The reaction was carried out for 6 h at 70 °C. The reaction mixture was dialysed for 2 days against double distilled water to remove unreacted GTAC. The mixture was then precipitated in 50:50 ethanol/acetone mixture. The precipitate was finally dried at 37 °C and stored under vacuum conditions.

2.3. Trinitrobenzene sulfonic acid assay

The extent of derivatisation of chitosan was determined using TNBS assay (Trinitrobenzene sulfonic acid assay). Briefly, to 5 mg of the test samples, 200 μl water, 200 μl 4% $NaHCO_3$, and 200 μl 0.1% TNBS were added. The solution was then incubated for 2 h at 37 °C. Following incubation, 200 μl of 2 N HCl was added. The absorbance was measured at 344 nm using UV–visible spectrophotometer (Varian 50Conc spectrophotometer, USA) (Rekha & Sharma, 2009). Chitosan of same concentration was used as control system and glucosamine was used as standard.

2.4. Particle preparation

Following modification, chitosan and its derivative were used for particle formation via ionotropic gelation with sodium tripolyphosphate (TPP). Briefly 6 ml of 1% TPP solution was added dropwise to 30 ml chitosan solution (4 mg/ml) under magnetic stirring for 30 min. The particles were washed several times with water to remove unreacted TPP and dried at 4 °C.

2.5. Instrumental analysis

FTIR spectra of chitosan and its derivative, HTCC were recorded in the 4000–400 cm^{-1} region using NICOLET 5700 FTIR spectrophotometer, USA. ^1H NMR spectra of HTCC were measured in D_2O using a Bruker Avance DPX 300 spectrometer (Bruker, Newark, DE). The hydrodynamic diameter and surface charge density of the particles were measured using Zetasizer Nano ZS (Malvern Instruments, Ltd., UK). These measurements were performed in triplicate. The surface morphology of the particles was studied using scanning electron microscope (HITACHI S-2400, Hitachi, Japan). Samples were mounted on metal stubs using double sided adhesive tape coated with gold under vacuum and then examined.

2.6. Swelling studies

Swelling studies were carried out on phosphate buffer pH 7.4. The dried test samples were placed in the buffer solution and at specific time intervals, the samples were removed from the buffer. After removing excess water from surface, the weight of the swollen samples was noted (Sajeesh, Vauthier, Gueutin, Ponchel, & Sharma, 2010). The degree of hydration of the samples was calculated with the following equation:

$$\text{Degree of hydration} = \frac{W_s - W_d}{W_d} \times 100$$

where W_s is the weight of the swollen particles and W_d is the weight of the dried particles.

2.7. Insulin loading and release studies

In order to evaluate the potential application of the polymer to oral insulin delivery, insulin incorporation and release properties of the microparticles were examined. Drug loading was performed by the diffusion filling method. A known weight of dried microparticles say 100 mg was kept in 200 μl insulin solution (400 IU/ml) for remote loading. After 24 h, microparticles were taken out and excess insulin solution was gently wiped off. Loaded microparticles were kept for drying at low temperature (2–4 °C). Due to the hydrophilic nature of the matrix, insulin loaded particles were coated with Eudragit L100-55 (0.5% Eudragit L100-55 was dissolved in 2-propanol with the help of 400 μl triethylcitrate which is a plasticizer). 0.5% Eudragit L100-55 was used for the present study. About 200 μl of 0.5% Eudragit L100-55 was added to100 mg of insulin loaded microparticles and the obtained microparticles were dried at 2–4 °C.

About 25 mg of drug-loaded microparticles was suspended in 10 mL of buffer solution (pH 1.2 and 7.4) in a glass beaker. At specified time intervals of 1 h, an aliquot of sample (200 μl) was withdrawn and protein content was estimated by Lowry protein assay (Lowry, Rosebrough, Farr, & Randall, 1951). The release study was conducted for 6 h. The dissolution medium was replaced with fresh buffer to maintain the volume after each withdrawal. The amount of insulin in the test solution was calculated from the insulin standard measurements.

The percentage of drug loading was calculated as follows:

$$\text{Percentage of drug loading} = \frac{C_i - C_f}{C_i} \times 100$$

where C_i and C_f are the initial amount of insulin loaded and insulin content in the supernatant solution, respectively.

Biological activity of insulin loaded microparticles was investigated using ELISA technique as per standard protocol. CD spectra of free insulin and insulin loaded quaternized chitosan particles were recorded on JASCO-J-810 spectropolarimeter (Jasco, UK) equipped with a JASCO PTC-423S Peltier type temperature control system using a 1 cm cell, speed of 100 nm/min, a response time of 0.25 s, and bandwidth of 1 nm.

2.8. Mucoadhesion studies

The animal experiments were done as per the requirements of the Animal Ethics Committee of the Institute (Sree Chitra Tirunal Institute for Medical Science and Technology). Animals were housed in rooms at controlled temperature and relative humidity. Mucoadhesion studies were carried out on freshly excised rat intestinal tissue. The rats were under fasting for 16 h with free access to water before euthanasia by cervical dislocation. The intestinal tissue of about 5 cm was taken out, flushed with normal saline to remove the luminal contents and cut open longitudinally. The tissue was mounted and fixed on a semi cylindrical polypropylene support and washed with saline to remove free mucin. A known amount of particles was applied on the intestinal tissue and kept in humid condition for 5 min. It was then washed with PBS at a rate of 15 ml/min for 20 min. The dislodged particles were collected and dried. The percentage of mucoadhesion was calculated by comparing the weight of particles adhered to weight of particles applied (Sajeesh & Sharma, 2006).

2.9. Adsorption studies on mucin

Mucin stock solution with a concentration of 2 mg/ml was prepared. Chitosan microparticles (10 mg), both modified and unmodified were dispersed in the above mucin solutions, vortexed and kept for incubation in a shaker at 37 °C for 30 min and 60 min

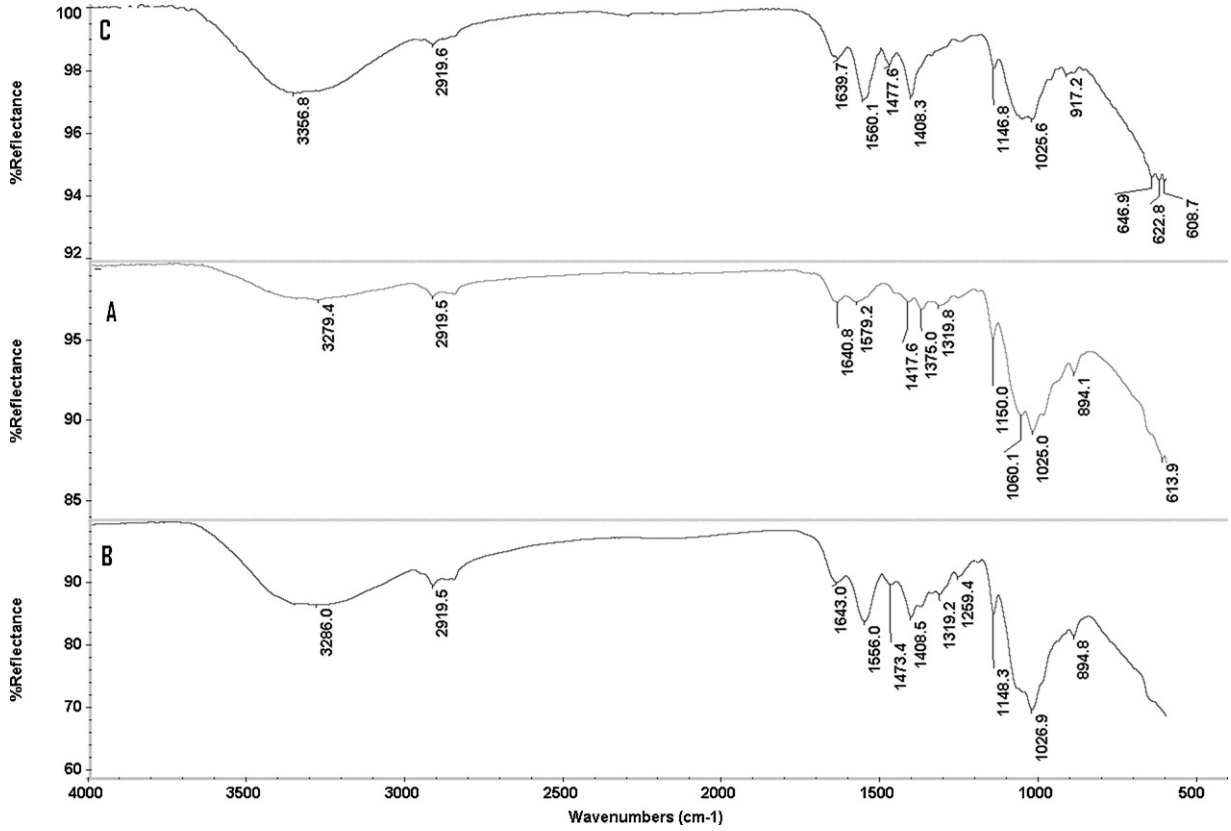

Fig. 1. IR spectra of (A) native chitosan, (B) quaternized chitosan [HTCC (0.5:0.5)], and (C) quaternized chitosan [HTCC (0.5:1)].

separately. The dispersions were then centrifuged at 4000 rpm for 2 min and the supernatant was used for the measurement of free mucin content. An aliquot of 200 μl was taken, and protein estimation was done by Lowry method. The absorbance of mucin was measured by colorimetry at a wavelength of 750 nm. The amount of mucin adsorbed by the microparticles was determined as the difference between its initial concentration and the concentration found in the dispersion after incubation and centrifugation. The calculations were done on the basis of mucin standard curves (Sanju, Anil Kumar, & Vivek Ranjan, 2007).

The surface topography of mucin and mucin adsorbed particles was analysed by atomic force microscopy (WITEC Confocal Raman Microscope System with Atomic Force Microscope Extension, Germany). The samples were prepared by mixing 100 μl of aqueous mucin solution with 100 μl chitosan solution (both modified chitosan and unmodified chitosan). After incubation for 1 h, the complexes were placed on a freshly cleaved mica surface and dried at room temperature for 24 h. The image mode was set to tapping mode with a scanning speed of 1–5 Hz.

2.10. Cytotoxicity (MTT assay)

To check the cytotoxicity of the derivative, MTT assay was carried out using the mouse connective tissue fibroblasts, L929, and human colorecteal cancer cells, Caco-2-cells. The cell lines were seeded into a 24-well plate containing DMEM culture medium with 10% FBS. The seeded plate was then incubated for 2 h at 37 °C in a CO_2 incubator with a humidified 5% CO_2/95% air atmosphere to allow the cells to adhere to the well. Samples along with positive and negative control were dispersed in DMEM culture medium (1 mg/ml) and fed into each well. After incubation for an additional 24 h at 37 °C in a humidified 5% CO_2/95% air atmosphere, the samples were removed and MTT reagent (0.5 mg/ml) was added to each well and incubated for 3 h (Sgouras & Duncan, 1990). The reagent was then removed and dimethylsulfoxide (DMSO) was added to dissolve the MTT formazan crystals. Plates were shaken for 5 min. The absorbance of each solution was read at 620 nm using an automated microplate reader (Finstruments Micro plate Reader, USA). DMEM medium without sample was used as negative control.

2.11. Haemolysis

Whole blood was collected from a healthy volunteer and anticoagulated with sodium citrate (ratio of blood to anticoagulant taken was 9:1). Erythrocytes were isolated by centrifuging whole blood at 700 rpm for 10 min. The erythrocytes were washed thrice with saline before use. Microparticles of varying ratios were mixed with RBCs and then incubated for 2 h at 37 °C. The supernatant was spinned off at 1500 rpm for 5 min. Haemoglobin release was monitored spectrophotometrically at 541 nm. Triton X-100 and 0.9% NaCl were taken as the positive and negative control, respectively (Dong, Kewin, & Ronald, 2004).

2.12. Confocal laser scanning microscopy

Caco-2 cells (passages 22–28) were grown at 37 °C under 5% CO_2. Cells were maintained in T-75 flasks using Modified Eagle's Medium (MEM) supplemented with 20% foetal bovine serum, 1% non-essential amino acids, 10,000 U/ml penicillin and 10,000 μg/ml streptomycin. Growth medium was changed every alternate day. Cells were passaged at 80–90% confluency using 0.25% trypsin/EDTA solution. The cells were maintained under incubation conditions as mentioned above and used for transport experiments 6 days post-seeding (Schipper et al., 1997). Medium was replaced with HBSS transport medium, and cells were equilibrated at least for 2 h before the experiments. Cells were treated

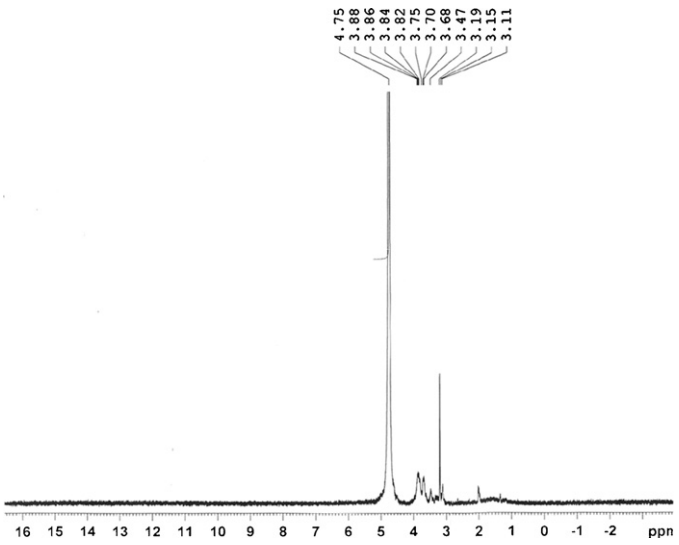

Fig. 2. NMR spectra of quaternized chitosan (HTCC).

with 500 μl particles at a concentration of 2 mg/ml for 2 h. The particles were removed by washing the cells three times with PBS. The cells were fixed with 250 μl of 4% paraformaldehyde for 20 min at room temperature. Then cells were permeabilized using 0.2% Triton X-100 in blocking solution made of 1% (w/v) bovine serum albumin (BSA) in PBS for 20 min. The permeabilized cells were then washed twice with PBS and incubated with 250 μl of 1% BSA for 30 min. For actin filament visualization, the blocking solution was removed and cells were incubated with 200 μl rhodamine phalloidin solution (0.2 μg/ml) for 20 min at room temperature. After the removal of rhodamine phalloidin, the cells were treated with 1% BSA as before. The cells were washed with PBS, and dried overnight at 4 °C. Images were obtained using Carl Zeiss LSM Meta 510 inverted confocal laser scanning microscope (Carl Zeiss, Germany), equipped with He/Ne laser 543. The visualization of rhodamine phalloidin was done using excitation and emission wavelengths of 543 and 605 nm, respectively.

2.13. Cellular uptake studies using FITC labeled insulin

Insulin was covalently labeled with Fluorescein isothiocyanate (FITC) and FITC labeled insulin was loaded into microparticles. Cell uptake studies were performed with Caco-2 cell monolayers. FITC insulin loaded microparticles were incubated with Caco-2 cells for 4 h. The cells were then washed with phosphate buffer saline (PBS) and fixed in 1% formaldehyde. The entry of the FITC labeled insulin into the cells was assessed with the help of fluorescence microscope (Leica DMIRB, Germany).

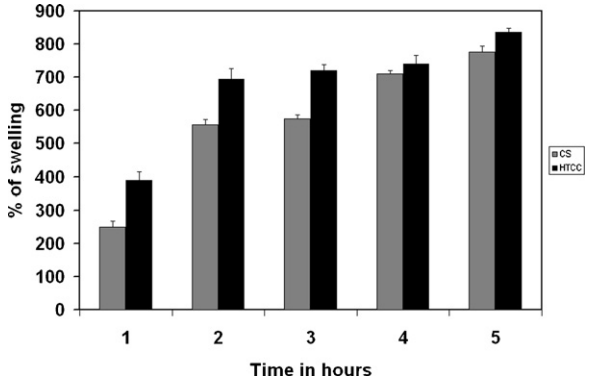

Fig. 4. Swelling studies of chitosan (CS) and quaternized chitosan (HTCC) microparticles at pH 7.4.

3. Results and discussion

The objective of the present study was to explore the potential of quaternized chitosan for oral insulin delivery. Molecular weight of the depolymerised chitosan was around 85 kDa, as determined by viscometric method. Derivatisation of chitosan was confirmed by FTIR, NMR spectra and TNBS assay. The extent of derivatisation was evaluated by determining the remaining underivatised primary amino groups in chitosan (TNBS assay). The percentage of free amino groups was around 60% for HTCC (1:0.5) and 36% for HTCC (1:1) as compared to 78% for native chitosan. HTCC (1:1) was used for further studies due to the high degree of substitution and was abbreviated as HTCC or quaternized chitosan.

Fig. 1 shows the IR spectra of chitosan and quaternized chitosan (HTCC). The peak at 1476 cm^{-1}, was attributed to the presence of methyl groups of quaternary nitrogen atoms. It was observed that the N–H bending (1579 cm^{-1}) of the primary amine disappeared due to the change of the primary amine to the secondary amine (aliphatic). In addition, the spectrum showed a broad peak at around 3356 cm^{-1}, due to the increased number of hydroxyl groups. The characteristic peaks of primary alcohol and secondary alcohol between 1102 and 1082 cm^{-1} did not change in HTCC compared to chitosan molecule, which could be due to the introduction of quaternary amino groups at NH_2 sites on chitosan chains.

Modification of chitosan was further confirmed by NMR spectra (Fig. 2). NMR spectra of HTCC showed strong peaks at 3.1 ppm, which was attributed to the presence of methyl groups in the quaternary nitrogen atoms. All protons of glucopyranosyl ring were located between $\delta = 3.4–3.8$ ppm. SEM micrographs showed the presence of irregularly shaped microparticles and the quaternized derivative appeared to be more compact than native chitosan (Fig. 3). The size of the microparticles was in the range of 2–3 μm.

Zeta potential confirmed the increase in the cationic charge density of the derivative (30.4 mV) as compared to native chitosan (20.6 mV). The microparticles of HTCC exhibited significant

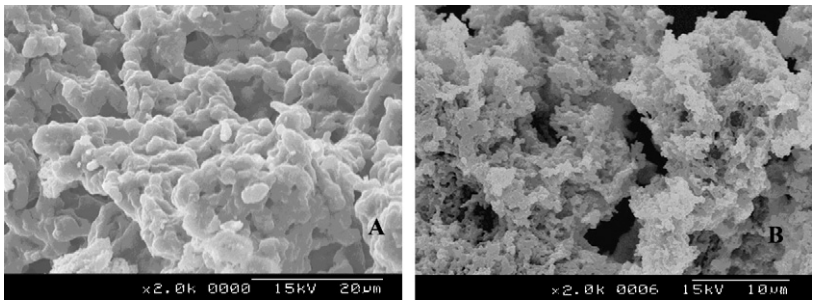

Fig. 3. Scanning electron micrographs of (A) chitosan and (B) quaternized chitosan (HTCC) microparticles.

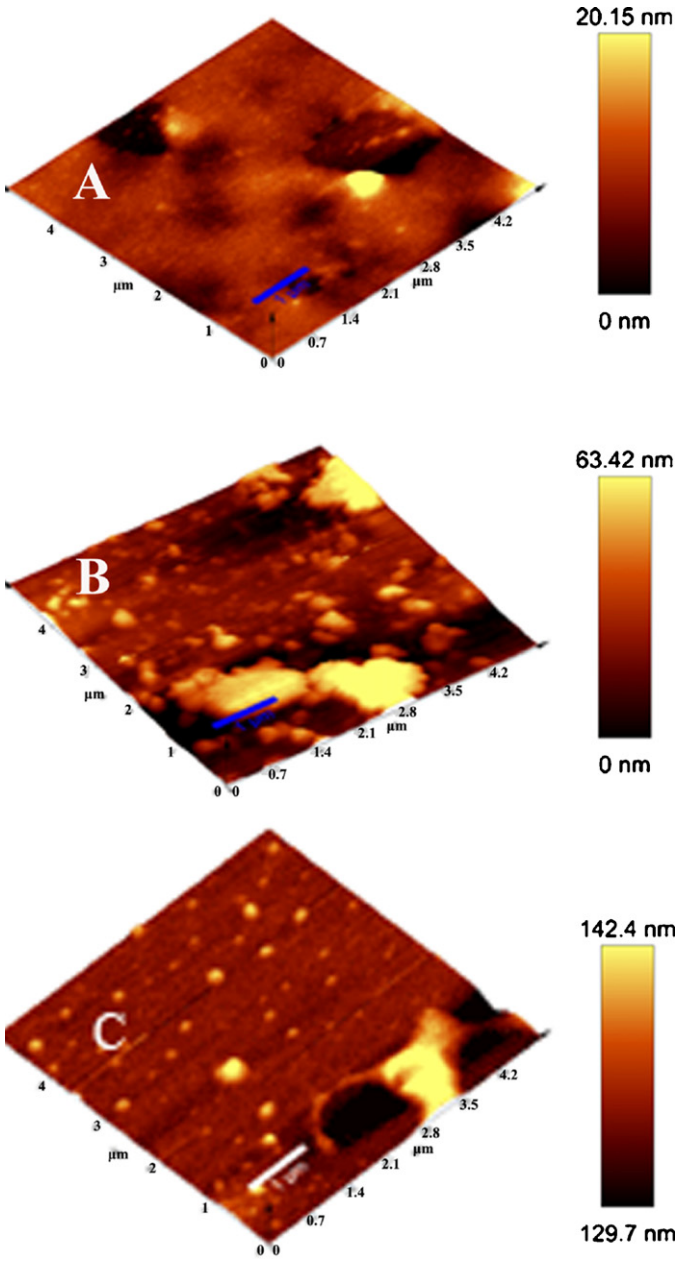

Fig. 5. AFM microscopic images of (A) mucin, (B) mucin mixed with chitosan, and (C) mucin mixed with quaternized chitosan (HTCC) on freshly cleaved mica surfaces.

swelling as compared to that of native chitosan (Fig. 4). Swelling studies indicated that the microparticles are capable of hydrating the matrix without dissolution for a sufficient period of time. This could be due to the increase in the positive charge density of the particles and also due to the presence of hydrogen bonding.

Quaternization of chitosan could give rise to a strong electrostatic interaction with mucus, which enhances their mucoadhesive property. The percentage of adhesion was 86% for HTCC (1:0.5) and 95% for HTCC (1:1) as compared to 72% for native chitosan. The higher mucoadhesive properties of quaternized chitosan resulted from the synergistic effect of the interpenetration of polymer chains into the mucus layer and hydrogen bonding/electrostatic interaction between positively charged quaternized chitosan and anionic glycoproteins present in the mucus layer. The extent of mucin adsorption of modified chitosan was found increasing with increasing mucin concentration and time. Fig. 5 shows the AFM images of unmodified mucin, chitosan–mucin complex and HTCC–mucin

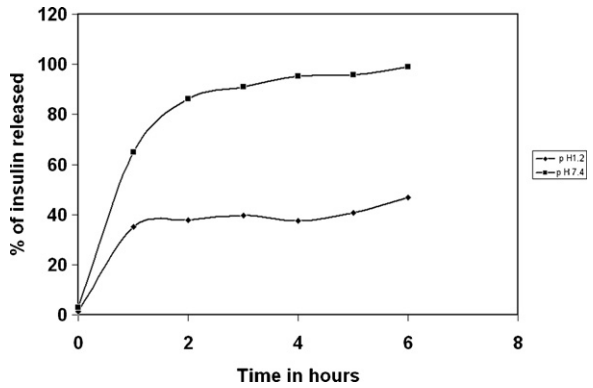

Fig. 6. Release profile of insulin from Eudragit coated quaternized chitosan at (♦) pH 1.2 and (■) pH 7.4.

complex placed on freshly cleaved mica surfaces. There was significant changes in the surface topography of mucin–chitosan and mucin–quaternized chitosan samples. HTCC–mucin complex appeared more spherical in shape and less aggregating as compared to that of chitosan–mucin complex.

Drug loading onto these particles was achieved by diffusion filling method (remote loading), in which the microparticles were exposed to protein solutions. Fig. 6 illustrates the in vitro release profile of insulin from enteric coated HTCC microparticles at acidic (pH 1.2) and alkaline pH (pH 7.4). For oral insulin delivery systems minimal release in the gastric pH is appropriate, as it may save the loaded insulin and increase the bioavailability compared to that of a matrix which does not exhibit pH sensitive release profile. Due to the hydrophilic nature of HTCC, insulin loaded microparticles were enteric coated with Eudragit L100-55. At pH 7.4 the matrix showed a burst release followed by a slow and sustained release. In this investigation, the amount of insulin released within gastric retention period (2 h) was significantly low and sustained release of insulin was observed in the intestinal fluid. Thus it seems that the enteric coated HTCC microparticles could protect insulin from gastric degradation and favour their release at intestinal pH, making them a suitable candidate for oral insulin delivery (Mika, Hiraku, & Yoshiharu, 2005).

The HTCC microparticles exhibited 65% insulin loading efficiency. By diffusion filling method a higher drug loading was achieved inside the particles without affecting its biological activity. ELISA results suggest that quaternized chitosan microparticles are capable of preserving biological activity of encapsulated insulin.

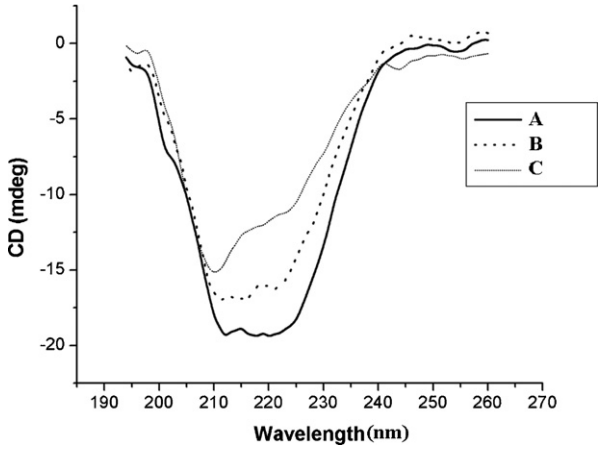

Fig. 7. Far UV circular dichroism spectra of (A) native insulin, (B) insulin loaded chitosan, and (C) insulin loaded quaternized chitosan microparticles.

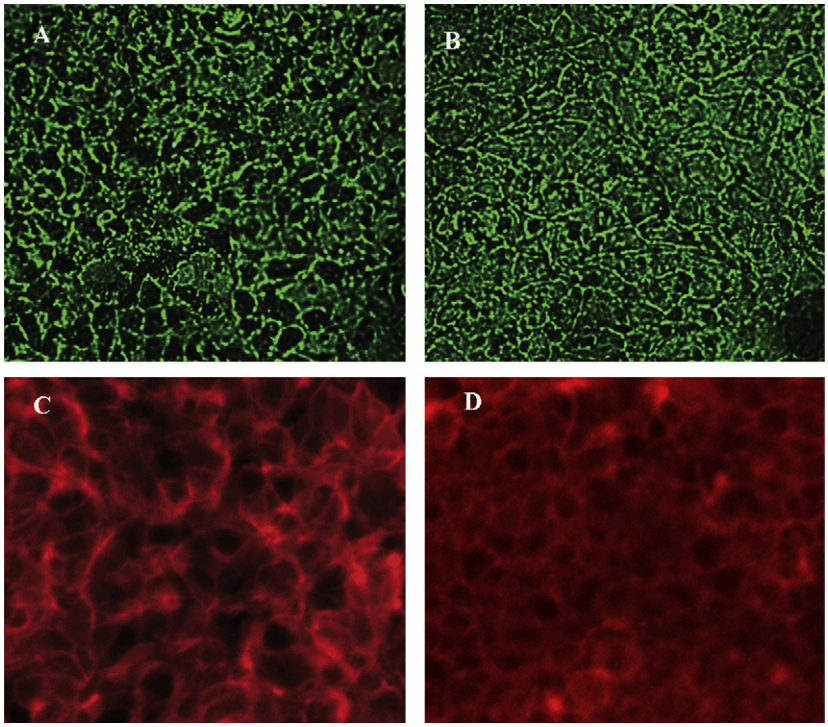

Fig. 8. Fluorescent microscopic images (A and B) and confocal micrographs (C and D) of chitosan and quaternized chitosan microparticles. (A and B) Fluorescent microscopic images of FITC-insulin labeled chitosan and quaternized chitosan (HTCC) microparticles on incubation with Caco-2-cells. (C and D) The tight junction visualization of chitosan and quaternized chitosan (HTCC) microparticles.

Further, the CD spectra revealed that the released insulin from HTC-Cmicroparticles was capable of maintaining the conformation of insulin. There was no significant difference in the conformation of insulin released from the microparticles compared to that of native insulin (Fig. 7).

The non-toxic nature of quaternized chitosan microparticles was established through MTT assay. In contrast to the report by Ji et al. (2009) which revealed a concentration-dependent relative cytotoxicity for HTCC at a relatively higher concentration, all the samples were found non toxic even upto a concentration of 2.5 mg/ml. The percentage of cell viability was around 85% for HTCC (1:0.5) and 89% for HTCC (1:1) as compared to 85% for native chitosan on L929 cells. Similar results were obtained from Caco-2-cell model. At physiological pH, haemolytic activity of HTCC microparticles was 1.39% and that of native chitosan microparticles was 5%.

In vitro uptake studies showed that the HTCC microparticles are capable of enhancing insulin permeation across the intestinal epithelium (Fig. 8C and D). It is reported that calcium ions are essential for the tight junction integrity. Cationic polymers such as chitosan are able to reversibly open the tight junctions between enterocytes allowing the transport of macromolecular drugs (Smith, Wood, & Dornish, 2004). Calcium chelators such as chitosan can disturb the cell–cell adhesion phenomena by depleting the concentration of extracellular calcium ions, and this may in turn lead to the uptake of the particle. Its protonated form can interact with the epithelial tight junctions, inducing a redistribution of actin filaments and of the tight junction protein ZO-1 (Staddon, Herrenknecht, Smales, & Rubin, 1995). This ultimately leads to the opening of tight junctions across the intestinal epithelium as visualized from confocal micrographs (Fig. 8A and B). The control cells showed continuous peri-junctional F-actin rings, which are required for maintenance of tight junction integrity. HTCC matrix induced a total disruption of F-actin distribution. Previous reports showed that the absorption enhancers that can induce structural separation of the tight junctions in Caco-2 cells, induce parallel changes in F-actin distribution (Kotze et al., 1997). Thus, quaternized chitosan can modulate tight junction opening by binding to the Zona-Occludens proteins and by the dislocation of actin filaments.

4. Conclusion

An oral insulin delivery system based on natural polymer chitosan was successfully developed. Quaternized chitosan with high positive charge could establish specific bioadhesive interactions with mucosal tissues because of their ability to inter-diffuse across the mucus network. Moreover this matrix was found to be non-toxic. HTCC microparticles, which were capable of retaining their cationic charge at intestinal pH, enhanced the permeability of intestinal cells by loosening the epitheial tight junctions. Thus, cationic modification of natural polymers could be a promising strategy in designing particles for efficient oral delivery of insulin and similar bioactive peptides across mucosal barriers.

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