Glutamine-Chitosan Microparticles as Oral Insulin Delivery Matrix: *In Vitro* Characterization

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Received 14 June 2010; accepted 6 February 2011
DOI 10.1002/app.34315
Published online 17 June 2011 in Wiley Online Library (wileyonlinelibrary.com).

ABSTRACT: Chitosan at physiological pH lacks positive charge which reduces the mucoadhesivity and permeation enhancing capacity. Therefore glutamine conjugated chitosan (GC) was developed to enhance the protonation of chitosan at intestinal pH. Particles were prepared by sodium tripolyphosphate ionic crosslinking and were evaluated *in vitro* for its application toward oral insulin delivery. The particles had high positive charge of 35.6 ± 7.3 mV at physiological pH and a size of 4.434 μm. The mucoadhesive capacity was established *in vitro* using rat intestinal tissue. Transepithelial electrical resistance (TEER) and confocal microscopy studies proved the ability of the particles in opening the tight junctions in Caco 2 monolayers. The permeation of fluorescent dextran (M_W 4000; FD4) across intestinal tissue was evaluated using Franz diffusion apparatus. It was observed that the GC particles enhanced the permeation by 1.52 fold in comparison with native chitosan (NC) particles. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 122: 2374–2382, 2011

Key words: glutamine-chitosan; insulin; microparticle; Caco-2

INTRODUCTION

Tremendous advancements in biotechnology have led to the mass production of therapeutic peptides and now it has become a necessity for a noninvasive delivery system with appreciable bioavailability. A Type I diabetic patient is dependent on daily injections of insulin, which is of low patient compliance. Also injection does not follow the normal physiological pathway but delivers insulin into systemic circulation. Hence only a small percentage of insulin reaches liver which is the primary site of action according to normal physiology.[1] Oral insulin has advantages over other forms of insulin delivery as it follows the normal physiological pathway.

Chitosan is a cationic copolymer of D-glucosamine and *N*-acetyl D-glucosamine units and is obtained by partial deacetylation of the natural polysaccharide chitin which is widely exploited for various biomedical applications like protein/peptide, gene delivery, and tissue engineering. The hydroxyl groups and its reactive amino group can be easily modified under mild reaction conditions to prepare modified chitosan of desired properties. Chitosan is biocompatible, bioresorbable, biodegradable, and has mucoadhesive properties. Because of these biological properties in the recent years chitosan and its numerous derivatives are extensively investigated towards the drug delivery applications. Many attempts have been done to develop chitosan-based oral insulin delivery system.[2–5] To improve the bioavailability of orally administered therapeutic peptides, derivatives of chitosan are also tried.[6–9]

It is reported that chitosan can enhance the paracellular permeation by mediating a structural reorganization of the tight junction proteins ZO-1 and occluding.[10,11] However, chitosan is soluble only in acidic pH and pKa of chitosan is 6.5, hence is not as effective as an absorption enhancer in the small intestine, the main absorption area in the gastrointestinal tract. Quarternised chitosan which has improved solubility and mucoadhesive properties is developed and reported by various groups and evaluated for insulin delivery applications.[12] Though evaluation of chitosan glutamate salt as permeation enhancer is reported glutamine conjugated chitosan (GC) or particles prepared from GC is not evaluated towards this application. Chitosan glutamate salt is prepared by dissolving chitosan base in glutamic acid solutions. It is well established and reported that the chitosan salts are insoluble at pH 7.4 and these salts will be un-protonated and therefore will be ineffective or less effective as permeation enhancer or as a mucoadhesive. But when the

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Contract grant sponsor: Department of Science and Technology, Government of India; contract grant number: 8013.

glutamine is covalently linked to chitosan, it is soluble at pH 7.4 and is protonated as the conjugated molecule is an integral part of chitosan. Hence by this method the high pka value of the glutamine can be used to the fullest as a permeation enhancer and mucoadhesive. Therefore the aim of the present study was to develop a mucoadhesive chitosan with net positive charge at intestinal pH (6–7.5). Glutamine conjugated chitosan was developed in view of this objective for oral peptide delivery. The advantage of glutamine is that the pKa value of the α-amino group of glutamine is 9.13 which will contribute to the net positive charge of the derivative at pH above 6.5. The effect of glutamine-chitosan on tight junctions was evaluated *in vitro* using Caco 2 cell lines.

MATERIALS

Chitosan (M_W 270,000 Da; 85% deacetylated), *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC), Trinitro benzene sulfonic acid (TNBS), fluorescein isothiocyanate (FITC), from Aldrich, USA, Fluorescent dextran (FD4; M_W 4000), phalloidin-TRITC, Modified Eagles Medium, penicillin, streptomycin, Millipore *trans*-membrane inserts (Sigma, USA), L-Glutamine (SD Fine, India), human insulin solution (400 IU mL^{-1}), human insulin ELISA kit (Mercodia, Sweden), Eudragit® L100-55 rabbit anti ZO1 antibody (Zymed), FITC antirabbit IgG (BD Biosciences), Fetal bovine serum (Gibco, Invitrogen, US).

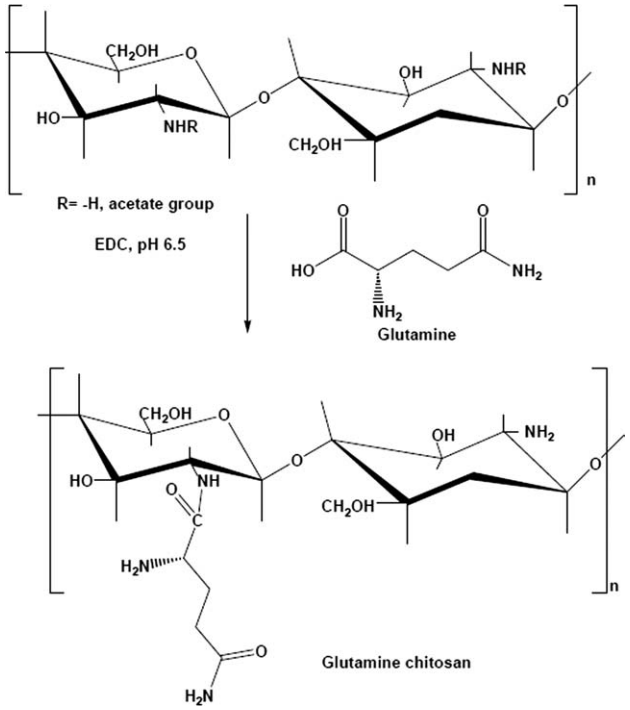
METHODS

Synthesis of chitosan-glutamine

Glutamine–chitosan (GC) was synthesized using EDC chemistry. A 1% (w/v) chitosan solution was prepared in 0.2*M* HCl and the pH was adjusted to 6.5 using 2*M* NaOH under vigorous stirring. Glutamine was added at a concentration of 5% (w/v) to chitosan solution under stirring. To this solution EDC was added drop wise at a final concentration of 0.1*M* (Scheme 1). The reaction was continued for 12 h and the resultant solution was dialyzed against distilled water with four changes, lyophilized and stored at 4–8°C.

Characterization of the derivative

The derivatisation was analyzed using FTIR Impact 410 spectrometer. The chitosan derivatives were used in powder form and the spectra was analyzed with KBr pellet method. The extent of derivatisation of the amino groups was determined chemically using trinitro benzene sulfonic acid (TNBS) method.[13] Same concentration of chitosan was used as control and glucosamine was used as the standard.



Scheme 1 Synthesis of glutamine-chitosan.

Preparation and characterization of microparticles

The glutamine–chitosan was dissolved in 0.2*M* HCl to prepare a 1 g % solution. To 25 mL of this solution, 2 mL of 1 g % sodium tripolyphosphate was added using syringe and needle under stirring (1000 rpm) which was continued for twenty minutes. The particles formed were collected by centrifugation at 7500 rpm for 15 min. The particle pellet was washed by re-suspending in distilled water to make the product free from excess crosslinker and centrifuged. This process was repeated thrice; pellet was collected and dried in refrigerator. Particles from native chitosan (NC) were also developed similarly.

Particle size was determined based on dynamic light scattering using particle sizer. The measurements were performed in distilled water. The zeta potential was measured in folded capillary cells using Nanosizer (Malvern instruments). The particle size and zeta potential of the GC and NC particles were done at pH 1.2 and 7.4. Each batch was analyzed in triplicate.

Insulin loading and release studies

The particles were insulin-loaded by remote loading process. A known quantity of particles (400 mg) was suspended in 1.0 mL of insulin solution (400 IU mL^{-1}, pH 7.0), kept in refrigerator overnight. After 16 h excess insulin if any was removed using filter paper and the particles were dried at 2–4°C. The dried particles were rinsed with 0.01*N* HCl to

remove the surface bound insulin if any, immediately centrifuged and dried under refrigerated conditions (2–4°C). The dried particles were collected and stored for further studies. FD4 loaded particles for the *in vitro* permeation studies were prepared similarly.

To determine the loading, a known weight of NC and GC particles was suspended in phosphate buffer pH 7.4 (USP) at 50 mg/10 mL and incubated overnight (20 h) in refrigerator with occasional stirring. The particle suspension was centrifuged at 8000 rpm for 20 min and the supernatant was taken for the insulin assay by Lowry method and enzyme linked immunosorbent assay (ELISA). Aliquots of 200 μL were taken for insulin assay by Lowry method and an insulin standard was maintained. Insulin analysis by ELISA was done as per manufacturer's instructions and the absorbance was taken in plate reader (Finstruments Microplate Reader).

Insulin loaded particles were coated with Eudragit L100-55 dissolved in acetone and air dried to remove the solvent. The release studies were done on Eudragit® L100-55 coated particles at pH 1.2 (HCl-KCl buffer; USP) and pH 7.4 (phosphate buffer; USP). The particles were suspended in the respective buffers (50 mg/10 mL). Aliquots of 200 μL were taken at one-hour intervals for 8 h and the released insulin was estimated by means of Lowry method. Equivalent volume of the fresh buffer was replaced each time after the sampling. The experiments were done in triplicates. The amount of insulin in the test solution was calculated from the insulin standard maintained during the assay. A comparison was done with uncoated native chitosan particles.

Swelling studies

The swelling characteristics of the matrices were evaluated at pH 7.4 for 5 h. At specific time intervals the samples were centrifuged, removed the buffer and the weights were determined. The swelling ratios of the matrices were calculated from the following equation:

Swelling ratio $= (W_s - W_d)/W_{d;}$ where W_d is the weight of the dry particles and W_s is the weight of swollen particles.

In vitro studies using Caco-2 cell lines

Caco-2 cells (Passages 30–33) were grown at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity. Cells were maintained in T-75 flasks using Modified Eagle's Medium (MEM) supplemented with 20% fetal bovine serum, 1% nonessential amino acids, 10,000 U mL^{-1} penicillin and 10,000 μg mL^{-1} streptomycin. Growth medium was changed every alternate day. Cells were passaged at 80–90% confluency using 0.25% trypsin/ethylenediamine tetra acetic acid (EDTA) solution.

The cells were seeded in 24-well plates at a density of 2.5×10^5 cells/well and cultured for 24 h in incubator at 37°C under 5% CO_2. The medium was replaced with particles at a concentration of 5 mg mL^{-1} and incubated for 20 h. To assess the toxicity, the particles were removed and MTT assay was conducted.

Transepithelial electrical resistance and permeability studies were done as reported elsewhere.[14] Caco-2 cell cultures were grown in transmembrane inserts (Millipore) of 0.4-μm pore size with MEM containing 20% fetal bovine serum supplemented with streptomycin/penicillin for 21 days until they achieved a constant transepithelial electrical resistance. The cell seeding density was 2.5×10^5 cells $well^{-1}$. Before the start of experiment cell membranes were allowed to equilibrate with the permeability study medium, HBSS containing Ca^{2+} ions. The transepithelial electrical resistance (TEER) was monitored using a voltmeter with a chopstick electrode (Milli ERS system). After the equilibration period, the apical chamber medium was removed, replaced with medium containing native chitosan or GC particles and TEER of the cell monolayer was monitored at different periods of time at 37°C. The reduction in the transepithelial electrical resistance was determined by calculating the change in TEER from the initial value. TEER%: $[(R_t - R_b)/(R_0 - R_b)] \times 100\%$, where R_t is the resistance value at time t, R_0 is the initial resistance value, and R_b is the resistance value in the absence of the cell monolayer.

The cells were grown, maintained and prepared for experiments as mentioned above. The donor chamber medium was removed and replaced with fresh HBSS containing the insulin-loaded NC or GC microparticles. Aliquots were withdrawn from the receiver chamber at different time intervals for 3 h and the samples were analyzed using human insulin ELISA kit (Mercodia, Sweden). In all cases triplicates were used. The apparent permeability coefficient of insulin was calculated using the following equation,

$$P_{\text{app}} = (dQ/dt)/A \cdot C_0$$

where dQ/dt is the permeability rate, A is the surface area of the membrane filter, and C_0 is the initial concentration in the apical chamber.

Caco-2 cells were seeded at 2.5×10^5cells/well onto four well cell culture plates (Nunc). The cells were maintained under incubation conditions as mentioned above and used for transport experiments 6 days postseeding.[14] Growth medium was replaced with HBSS transport medium, and cells were equilibrated with HBSS at least for 2 h before uptake experiments. Cells were treated with microparticles at a concentration of 5 mg mL^{-1} for 1 h. The particles

were removed by washing the cell layers three times with phosphate buffered saline (PBS). The cells were fixed with 250 μL of 4% (v/v) paraformaldehyde for 20 min at room temperature. Then cells were permeabilized using 0.2% (v/v) Triton X-100 in blocking solution, made of 1 g % 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 g % BSA for 30 min. The blocking solution was removed and cells were incubated with 200 μL rhodamine phalloidin solution (0.2 μg mL^{-1}) for 20 min at room temperature. After removal of rhodamine phalloidin, the cells were treated with 1 g % 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.

The cells were permeabilized as mentioned above. The permeabilized cells were then washed twice with PBS and incubated with 250 μL of 1 g % BSA for 30 min. The blocking solution was removed and cells were incubated with 200 μL of ZO 1 antibody (0.1 μg mL^{-1}) overnight at 4°C. After removal of ZO 1 antibody the cells were treated with 1 g % BSA as before. The blocking solution was removed and the cells were incubated with 250 μL FITC antirabbit IgG for 1 h at room temperature. 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 Argon2 laser. The visualization of FITC was done using excitation and emission wavelengths of 488 and 505–530 nm, respectively.

Mucoadhesion studies

Mucoadhesion studies of the native and modified chitosan particles were done on rat intestinal mucosa.[15] Intestinal tissue from jejunum of ~ 5 cm length was taken, flushed with normal saline to remove the free mucus and cut open longitudinally. The tissue was mounted on a semicylindrical polypropylene support and washed with saline. Twenty five mg quantity of particle was spread on the tissue and kept in a humidity chamber for 5 min. The humidity of the chamber was maintained at 90% RH for particle-mucosal interaction. The tissue was then washed with phosphate buffered saline, pH 6.8 for 60 min at an angle of 45°. For washing, the flow rate of buffer was maintained at 120 mL h^{-1}. The dislodged particles were collected, dried and the weight was noted. The mucoadhesive capacity was calculated as the percentage of the dry particles retained by the mucosal tissue at the end of the process.

In vitro permeation studies

A fabricated Franz diffusion cell with an internal diameter of 0.8 cm was used. The studies were performed using rat intestine. Jejunum from male Wistar rat was collected and flushed with saline to remove the free mucins and cut open longitudinally. The segment was attached onto the surface of the receptor compartment so that it is between the donor and receptor compartments. The receptor compartment was filled with phosphate buffer pH 7.4 (USP). NC or GC particles applied on to the intestinal patch, FD4 was used to evaluate the permeation. Samples were collected at 1-h intervals for 3 h and the amount of FD4 permeated was determined by fluorescent spectroscopy (Varian Cary Eclipse Fluorescence Spectrophotometer). The excitation wavelength was 488 nm and the emission at 525 nm.

RESULTS

Chitosan-glutamine synthesis and characterization

FTIR spectroscopy revealed the derivatisation of chitosan with glutamine as shown in Figure 1. Strong peak at 1528–1572cm^{-1} is indicative of the amide groups. It is also observed that the vibrational band corresponding to primary amino groups at 1583 cm^{-1} disappeared while a new prominent band appeared. For GC there was a major change in this region, a broad band including 1623–1572 cm^{-1} was observed. This could be due to the fact that glutamine has a primary amino group and another amino group via amide bonding to the carboxyl group.

TNBS assay confirmed the increase in amino groups. TNBS reacts with amine functional group to give an orange colored complex. The extent of derivatisation was evaluated by determining the remaining underivatised primary amino groups in chitosan. The amount of free amino groups was evaluated using glucosamine as the standard. The amino group content was higher in the glutamine derivative as seen from Figure 2.

Characterization of particles

The particle size is 4.434 μm with a PDI of 0.238 (Fig. 3). The zeta potential of GC particle was 35.6 ± 7.3 mV (Fig. 4) at neutral pH, where as it was 22.1 mV for NC particles. The particle size is higher compared to native chitosan particles which are in the nanorange of 450 nm.

Loading and release studies

The loading of insulin in GC particles was 30.2 IU/100 mg with a loading efficiency 75.5 ± 0.3%. Eudragit®-coated particles were evaluated for release

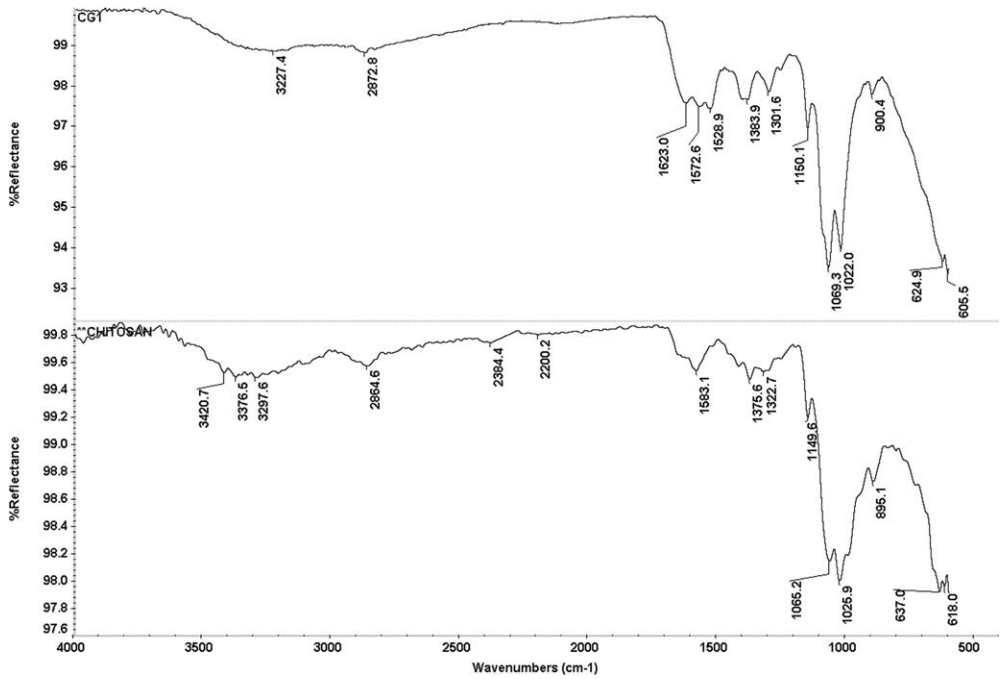


Figure 1 FT-IR spectra of native chitosan and glutamine chitosan.

characteristics. Chitosan is easily soluble in acidic pH and the particles release the loaded drug at this pH. For efficient use of chitosan based oral therapeutic delivery, proper steps need to be taken to prevent unwanted release at gastric pH. Here Eudragit® L 100-55, a well known enteric coating material was used for the purpose. The enteric coating will dissolve at a pH above 5.5, which takes place soon after the intestinal entry into dudodenum. As seen from the data shown (Fig. 5) it is observed that the release at pH 1.2 is restricted as expected and the release at pH 7.4 is controlled. From chitosan nanoparticles there is a burst release and within an hour itself about 70% of the loaded insulin gets released.

Swelling studies

The GC particles have high swelling capacity, as shown in Figure 6, with a degree of swelling (%) up to 225 × 5th hour at pH 7.4. GC as seen from the zeta potential data is positively charged at pH 7.4 and this could be the reason for the high swelling capacity. High swelling capacity exhibited by these particles may be due to two reasons. First, the protonated amino groups tend to repel causing the particle to swell in aqueous medium and the other might be due to the osmotic effect generated by the pH of the solution and the ions present.[16]

Studies using Caco-2 cell lines

The particles were found to be nontoxic to Caco 2 cells. The cell viability following particle exposure for 24 h at a concentration of 5 mg well^{-1} was 83.2% ± 1.2% and 88.7% ± 2.8% respectively, for NC and GC particles.

The effect of native chitosan and glutamine chitosan particles on transepithelial electrical resistance of Caco 2 cell monolayers is as shown in Figure 7. The

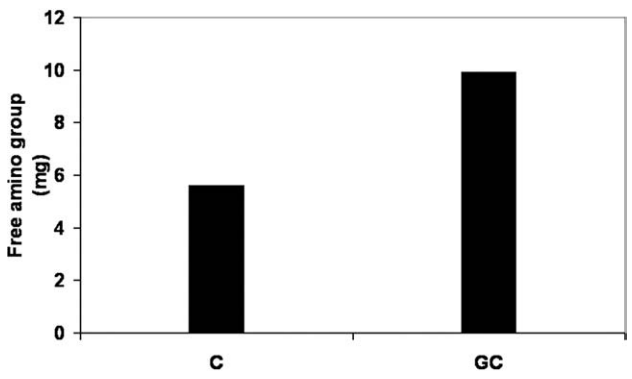


Figure 2 Free amino group content in native chitosan (C) and modified chitosan (GC).

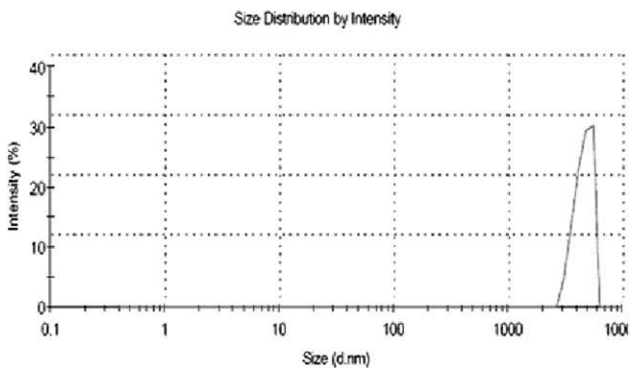


Figure 3 Size distribution of GC particles.

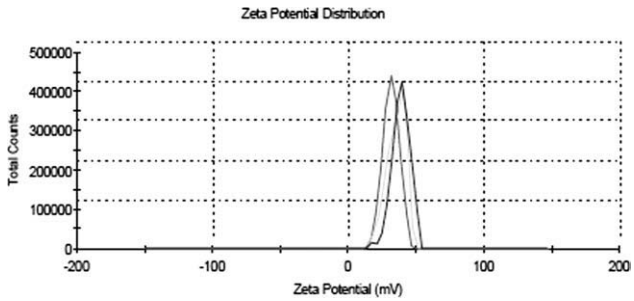


Figure 4 Zeta potential of GC particles.

TEER values are reduced by about 50% in the case of GC where as it was lower for NC particles. The TEER of control cell monolayers remained constant through out the study period. The TEER did not go down further in both the cases after the first 60 min.

The tight junction integrity of the Caco 2 cell layers was lost in both the cases of particle treatment. Compared to NC particles the loss of ZO1immunoflurescence as well as the phalloidin staining was higher in GC treated cell layers which is very obvious from the confocal image (Fig. 8).

In vitro mucoadhesion and permeation studies

About 88.1% ± 3.8% of the applied GC particles remained adhered to the intestinal tissue where as it was 62.4% ± 3.57% in the case of NC particles. Compared to native chitosan particles the positive charge is higher for GC which could be due to the availability of amino groups from glutamine which can remain protonated at physiological pH.

Glutamine chitosan particles were found to be more efficient in increasing the permeability of FD4 across the intestinal tissue. At 3 h 7.47 ± 0.038 μg of FD4 permeated in presence of GC particles and 4.97 ± 0.026 μg with NC particles.

Permeation of FD4 across rat intestinal tissue was evaluated on jejunum. The apparent permeability of FD4 in presence of NC and GC particles is as given in Table I. The enhancement ratio was 8.25 and 12.56 for NC and GC particles, respectively.

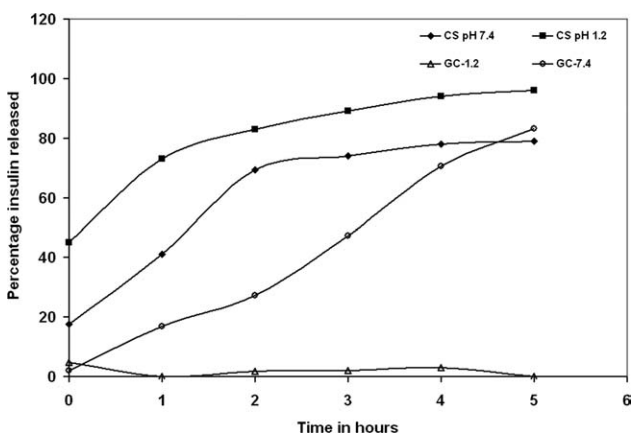


Figure 5 Release profile of insulin from native chitosan particles and Eudragit® L 100-55 coated GC nanoparticles at pH 1.2 and 7.4.

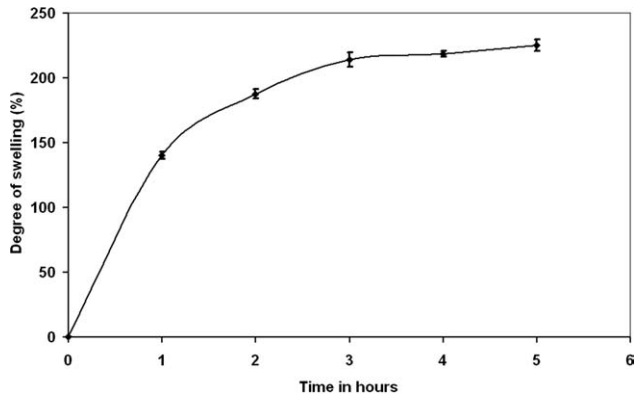


Figure 6 Degree of swelling of GC particles at pH 7.4 with respect to time.

DISCUSSION

Chitosan is a weak base and at intestinal pH chitosan is less protonated which limits its function as a permeation enhancer. However chitosan is an easily modifiable polymer which can be derivatised under mild reaction conditions with any functional group of choice. Here glutamine is covalently linked to chitosan with an aim to improve its solubility and protonation at neutral pH. Higher the positive charge at intestinal pH causes repulsion, leading to swelling and molecular expansion in solution, resulting in a higher chain expansion compared to chitosan. Chitosan at intestinal pH is less protonated and once it is in particulate form the charge will be even further reduced.

The zeta potential of GC particles was higher than native chitosan particles at pH 7.4 indicating the protonated existence of the glutamine conjugated to chitosan. pKa value of chitosan is 6.5 above which

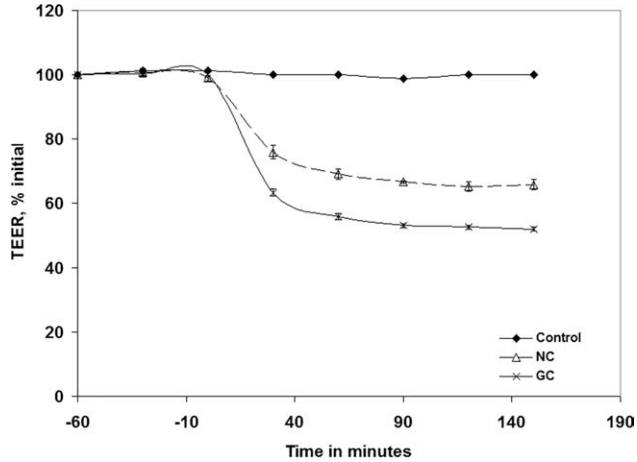


Figure 7 Effect of NC and GC particles on Caco 2 cell TEER.

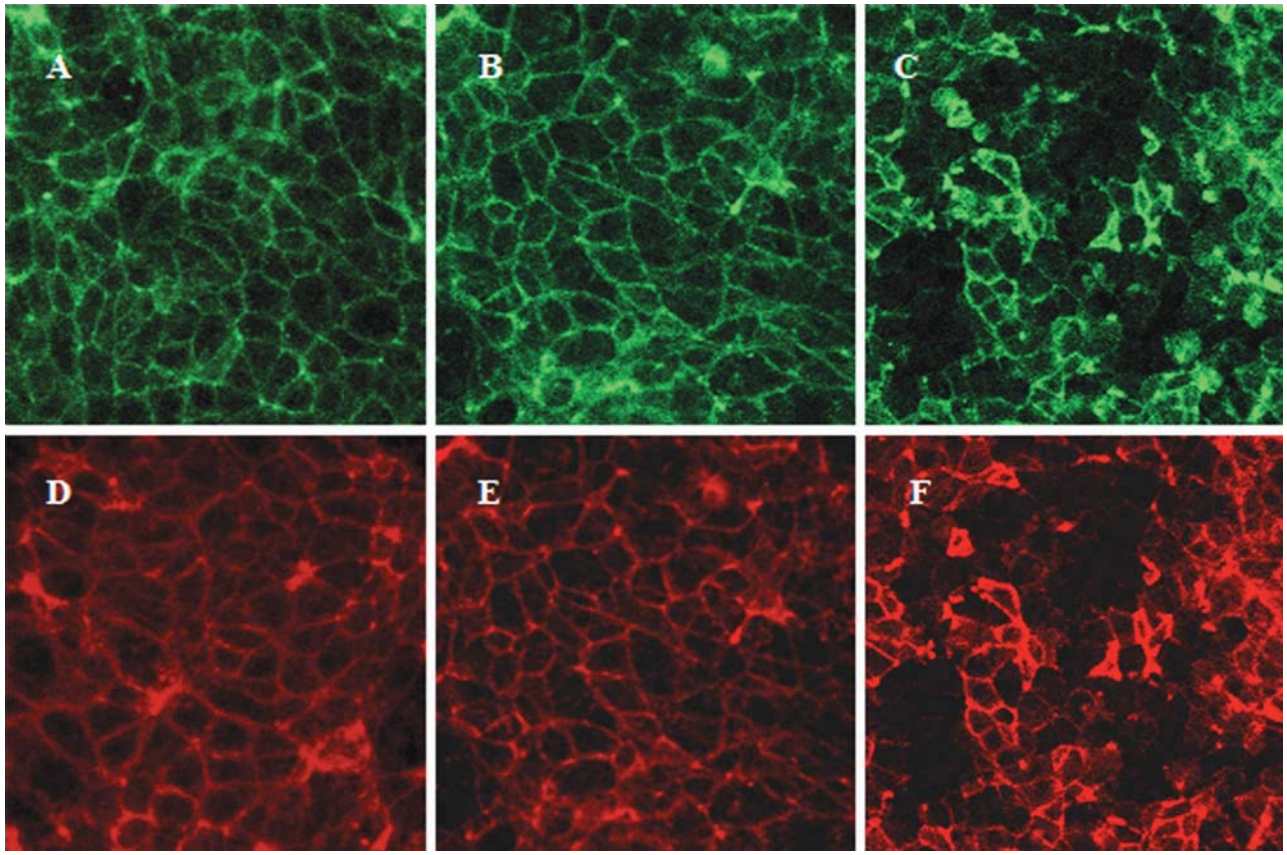


Figure 8 Tight junction visualization; Top panel represents the ZO 1 staining (A) control, (B) Native chitosan (C) Glutamine chitosan particles. Bottom panel represents the actin filament staining. (D) Control, (E) Native chitosan (F) Glutamine chitosan particles. Confocal images at ×20 magnification. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

this polymer is unprotonated which reduces its efficiency as a permeation enhancer. The pKa of the α-amino group of glutamine is 9.13 which could be the reason for the higher zeta potential of the derivative. Moreover, the amount of amino groups was higher as glutamine has two amino groups in its side chain. Sadeghi et al. reported reduction in zeta potential of quarternised chitosan on conversion to nanoparticle. The authors observed a reduction in zeta potential from 43.2 to 22.1 mV for quarternised chitosan when developed into nanoparticles. Here the substitution at the amino group introduces two amino groups and limited use of TPP for particle preparation might be the reason for the higher zeta potential.

Enteric-coated (Eudragit® L 100 55) particles which can prevent the loss of insulin at acidic pH was used for release studies. From the Figure 3 it is clear that this coating is able to prevent the release of insulin from the particle for extended period of time. The coating also helped in controlling the insulin release profile at pH 7.4, the burst release was nullified. It was observed that from unmodified chitosan particles at pH 1.2 and 7.4 the release was significantly higher at initial hours itself.[17] Sadeghi et al., also reported that the release of insulin at acidic pH from chitosan and chitosan derivatives based nanoparticles was about 90% within the first 30 min and 50% at pH 6.8.[18]

The toxicity of the particles was evaluated using Caco2 cells and found to be nontoxic. More than 80% of the cell population was viable. The reduction in TEER values suggests the improvement in permeation properties of derivatised chitosan. Tight junction opening by chitosan is proposed to be via ZO1 binding.[11] Tight junctions appear as a continuous apical belt around the cell periphery. In intact cell tight junctions, these proteins are strongly associated with the plasma membrane.[19] Smith et al.[11] investigated

TABLE I
Permeability Coefficient of FD4 Across Caco-2 Cell Monolayers in Presence of NC and GC Particles in Comparison with FD 4 Alone

Particulate system	Permeability ($cm\ s^{-1}$)	Enhancement ratio (R)
FD4	$0.16 \pm 0.002 \times 10^{-5}$	1
FD4-loaded NC	$1.32 \pm 0.028 \times 10^{-5}$	8.25
FD4-loaded GC	$2.01 \pm 0.012 \times 10^{-5}$	12.56

and reported first about the mechanism of chitosan mediated tight junction opening. They observed that the chitosan mediated tight junction disruption is caused by a translocation of tight junction proteins ZO1 from the membrane to the cytoskeleton. In our study particle mediated reduction in TEER was observed with in thirty minutes of exposure to the cell monolayer. The loss of tight junction integrity causes the loss of barrier properties resulting in the flux of ions across the monolayer. It is reported that the chitosan and quarternary chitosan derivatives when converted into particles is less efficient as a permeation enhancer.[20,18]

Actin filaments in the case of GC and NC particle treated were observed to be discontinuous and disrupted as evidenced from the staining pattern and the clumping. To further investigate the effect on the tight junction proteins immunofluorescence studies using anti ZO1 was done. In the untreated cells ZO-1 is observed as smooth lines at cell-cell junctions. The immunofluorescent staining intensity of NC or GC particle treated cells were observed to be weaker compared to the control which indicated the loss of ZO-1 from sites of cell–cell contact. The staining intensity was found to be least in the GC particle treated cells.

Mucoadhesive polymer increases the intimacy and duration of contact between the matrix and the mucosal surface[21,22] and various chitosan derivatives have been reported for enhanced mucoadhesivity from our group.[23,24] It is now understood that mucoadhesive nature of the polymer can increase the residence time of the matrix which enhance bioavailability of the drug with a minimal dose and less frequent administration. Chitosan at acidic pH is protonated which forms strong electrostatic interactions with sialic acid component of mucosal glycoproteins and contributes to its mucoadhesivity. As observed from the swelling studies GC particles exhibited high degree of swelling of 225 $\times$ 5th hour at pH 7.4. The swelling of chitosan microparticles was low. According to the various reports in literature it is understood that the chitosan particles have very low degree of swelling at pH 7.4 due to its hydrophobicity at neutral and alkaline pH which prevents swelling.[25,26] Since the pKa of amino groups from the substituted glutamine is above pH 9.0 they will be positively charged at pH 7.4 leading to repulsion and swelling. Swelling of the matrix is important in addition to other parameters. This step determines the extent of entanglement and adhesivity of the matrix. Since the degree of swelling is higher it might have helped in enhancing the mucoadhesivity in addition to the positive charge.

The transport of FD4 across the intestinal tissue was monitored as a function of time and the permeability of FD4 was higher for the GC particles by 1.52 fold (Fig. 9).

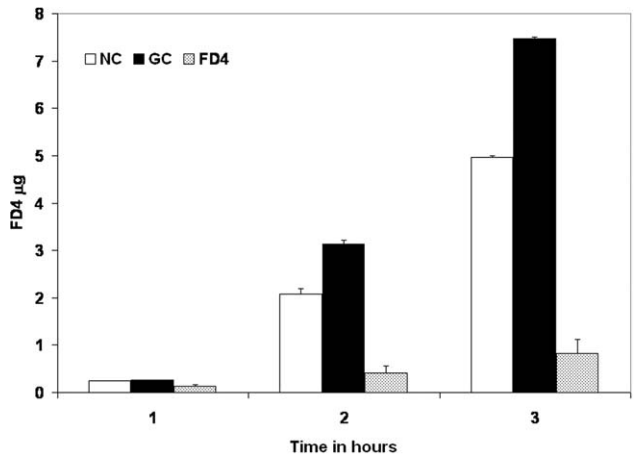


Figure 9 Permeation of FD4 (microgram) across the rat intestinal (jejunum) tissue. The amount of FD4 permeated was determined by fluorescent spectroscopy. The excitation wavelength was 488 nm and the emission at 525 nm.

CONCLUSIONS

The glutamine-chitosan conjugate showed good aqueous solubility at neutral pH and particles were formed by TPP crosslinking. The particles have a size of 4.4 µm and a zeta potential of 35.6 mV and also high degree of swelling at pH 7.4. The NC and GC particles were found to be nontoxic to Caco 2 cells. Owing to protonated amino group content, GC particles showed higher TEER reduction and tight junction opening compared to NC particles. The transport efficiency of FD4 from GC particles, across the rat jejunum was shown to be higher than from NC. This study clearly shows that the GC particles have higher positive charge at physiological pH and hence may be a good permeation enhancer for hydrophilic drugs through the paracellular route. FD4 transport was improved by 1.52 fold compared to unmodified chitosan particles. It can be concluded that for oral drug delivery of hydrophilic therapeutic macromolecules like insulin, positively charged chitosan derivatives are more suitable as drug enhancers for developing particulate drug delivery system. However, further studies are required to prove the efficacy of this formulation under *in vivo* conditions.

The authors express their sincere thanks to The Director, SCTIMST and The Head, BMT Wing for the facilities provided. The authors are thankful to Dr. T.V. Anil Kumar for providing confocal laser scanning microscopy facility and Dr. P.R.H.K. Varma for FTIR facility.

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