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Formulation of pH-Responsive Carboxymethyl Chitosan and Alginate Beads for the Oral Delivery of Insulin

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ABSTRACT: pH-sensitive beads were prepared from the interpenetrating polymer networks (IPNs) of carboxymethyl chitosan (CMCTS) and alginate to study their application in oral insulin administration. CMCTS was synthesized from chitosan and was characterized by Fourier transform infrared spectroscopy and wide-angle X-ray diffraction. Calcium chloride, a relatively less toxic material, was used to crosslink both the alginate and CMCTS to prepare the IPN beads. The IPN beads were used to encapsulate insulin at different weight ratios. The swelling behavior and insulin release profile in response to simulated gastric and intestinal media of the beads were investigated. The beads observed under scanning electron microscopy were almost spherical, with an average diameter of 780 μm. The bead with about a 54% insulin loading efficiency was observed to contain about 98% encapsulated insulin. The maxima of about 20% insulin retention at acidic pH (1.2) and about 94% insulin release at intestinal pH (7.4) justified the good pH-responsive nature of these IPN beads. Moreover, the insulin released from the IPN beads was stable and biologically active.
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INTRODUCTION

To overcome the barriers of oral insulin delivery and improve insulin absorption in the intestine, polymers have gained special attention from scientists.[1] Polymeric hydrogels have proven to be competent in several peptide deliveries, including insulin, in a controlled manner. Different polymeric hydrogels composed of natural[2] and synthetic[3] systems have been evaluated in oral insulin delivery. Among them, naturally occurring chitosan is the most widely used. Chitosan, obtained from the alkaline hydrolysis of chitin, is a naturally occurring linear binary cationic polysaccharide consisting of D-glucosamine and *N*-acetyl-D-glucosamine repeating units linked by a β-(1→4)-glycosidic bond. It possesses the good biological properties necessary for drug delivery, including biocompatibility, biodegradability, nonimmunogenicity, nontoxicity, and bioadhesiveness.[4,5] However, the poor water solubility of chitosan limits its biological application. It is only soluble in acidic aqueous solutions (e.g., formic, acetic, pyruvic, 10% citric, and lactic acids) where the pH is less than 6.5 because of the protonation of its amino groups. Therefore, chitosan in aqueous media at a pH less than or equal to 6.5 may not be desirable for applications such as cosmetics, foods, and biomedicines because, at this pH, proteins will be degraded. So, modifications of chitosan become necessary to improve its water solubility and pH sensitivity. Several modifications have already been implemented to make water-soluble chitosan derivatives; these modifications include acylation, quaternization of the amino groups of chitosan, and the introduction of carboxymethyl group and PEG in chitosan. Among these various modifications, carboxymethyl chitosan (CMCTS) has improved the water solubility and pH sensitivity. CMCTS would be a good candidate for oral insulin delivery. It is a water-soluble derivative of chitosan, where the substitution of some of the amino and primary hydroxyl groups of the native glucosamine units of chitosan is carried out with carboxymethyl groups.[6]

CMCTS has been reported to be a very good biocompatible polymer with excellent pH sensitivity due to the presence of $-NH_2$ and $-COOH$ groups in its structure. The application of CMCTS can be effective in subcutaneous, oral, or intraperitoneal routes of drug delivery.[7] Moreover, it is a nontoxic polymer that can preserve the bioavailability of entrapped drug molecules.[7,8] Thus, CMCTS has drawn attention in biomedical applications, including artificial skin, bone formation, wound healing, blood anticoagulants, and bacteriostatic agents.[9–11] As the oral delivery of insulin demands a more efficient and pH-sensitive polymeric system, another biopolymer alginate is used to increase oral delivery success. Alginate is a water-soluble linear polysaccharide containing various amounts of 1,4-linked

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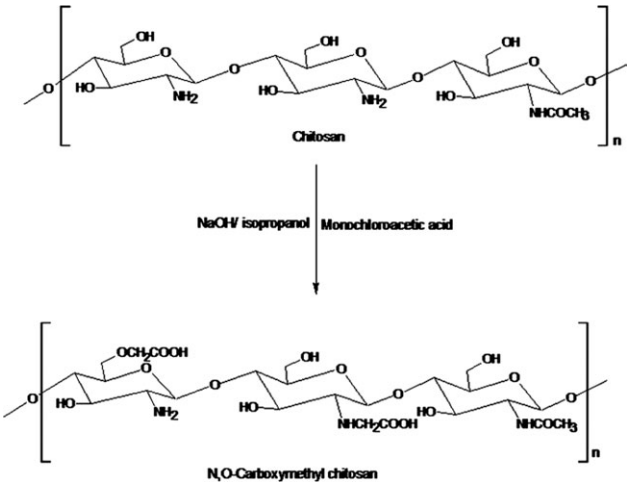
β-D-mannuronic acid and α-L-guluronic acid residues extracted from brown seaweed. It is very popular as a pH-responsive polymer because of its shrinkage at lower pH,[12] which enables encapsulated drug retention in the stomach and leads to its protection against enzymatic deactivation. Alginate is also popular for its low toxicity, low immunogenicity, and good mucoadhesion properties.[13,14] However, its rapid dissolution at higher pH (the intestinal pH range is 6.8–7.4) limits its wide application in oral drug delivery. Thus, although it possesses good qualities, the physical instability of alginate at alkaline or neutral pH hinders its applications in pharmaceutical research.

Alginate can also be crosslinked, either by physical or chemical means, for sustained drug release. With regard to toxicity, physical crosslinking is usually preferred over chemical crosslinking.[15] Calcium, a divalent cation used for the crosslinking of alginate is reported to maintain the biological efficiency of the drug molecules.[16,17] Although calcium ions serve as good physical crosslinkers of alginate, sometimes the strong interaction between the carboxylic groups of both alginate and calcium hinders the progressive swelling of the hydrogels at neutral pH and limits the release of encapsulated drugs in the intestine.[18]

To combat this flaw, CMCTS is used as an efficient polymer for oral insulin delivery as a pH-sensitive chitosan derivative. Being a protein, insulin is highly susceptible to enzymatic degradation in the gastrointestinal (GI) tract. Both the stomach and intestine produce proteolytic enzymes, and the harsh acidic pH of the stomach may degrade the orally administered proteins and peptide drugs such as insulin. Therefore, the primary protection of encapsulated insulin against enzymatic degradation in the stomach is required.[19] Thus, polymeric hydrogels must possess pH-responsive swelling behavior. In the harsh acidic stomach region, hydrogels should provide minimal swelling to protect the drug from degradation. In contrast, considerably greater swelling is required during passage through the neutral or slightly alkaline intestinal region for the complete release of the encapsulated drug. Chitosan can be crosslinked with glutaraldehyde to form chitosan hydrogels. Alginate can also be crosslinked by calcium ions. Although it has reported that chitosan–alginate hydrogels can be used as polymeric carrier systems for protein and peptide drug delivery, a few drawbacks hinder its wide application in biomedical research, especially in oral drug delivery. Again, the unsatisfactory release behavior of chitosan–alginate hydrogels at stomach and intestinal pHs create major barriers to its application in oral insulin delivery. In contrast, CMCTS and alginate can overcome these barriers.

Despite remarkable advancements in oral insulin delivery for combating diabetes mellitus, no such efficient oral delivery device is available up to this point. In this study, the efficiency of alginate and CMCTS hydrogel beads for oral insulin delivery was examined. The swelling properties of the beads and the release of insulin from these beads were examined at gastric and intestinal pH. The aim of our study was to develop pH-sensitive polymeric vehicles for the controlled oral delivery of insulin. The polymeric system must be biodegradable and biocompatible, producing no toxic or immunogenic effects within animal system. The bioavailability of insulin after oral administration should also be maintained to produce the desired functions.



Scheme 1. Synthesis of the CMCTS.

Beads of a water-soluble chitosan derivative, CMCTS, and alginate interpenetrating polymer network (IPN) were prepared by crosslinking with calcium chloride for the administration of insulin. IPN beads should provide effective protection to insulin during passage through the acidic environment of the GI tract, but insulin absorption through the intestinal tissue must be properly maintained.

EXPERIMENTAL

Materials

Chitosan (molecular weight = 222 kDa, degree of deacetylation = 87%) was obtained from Acros Organics, Kolkata, West Bengal, India. White crystalline KBr was purchased from (Merck, Mumbai, India). The molecular weight of chitosan was determined by gel permeation chromatography (Waters, Milford, Massachusetts, Ireland), and the degree of deacetylation of chitosan was obtained with a potentiometric titration method. Sodium alginate was purchased from Loba Chemie, Mumbai, India and insulin (bovine insulin, 27 USP units/mg) was obtained from Sigma-Aldrich (Bangalore, India.). Coomassie brilliant blue was purchased from (SRL, Mumbai, India.). Other chemicals were analytical grade and were used as such.

Synthesis of CMCTS

CMCTS was prepared according to a previous report[20] with slight modification. Chitosan (2 g) was swollen in 20 mL of solvent (1 : 4 v/v water/isopropyl alcohol) containing 2.7 g of sodium hydroxide at 50°C for 1 h. Then, 3 g of monochloroacetic acid in 4 mL of isopropyl alcohol was added dropwise to the previous mixture for 30 min, and the reaction was carried out for another 4 h at the same temperature. After that, 70% ethyl alcohol (40 mL) was added to stop the reaction. The solid part was then filtered and rinsed with 70–90% ethyl alcohol and dried *in vacuo* at 55°C for 24 h. The resulting products were sodium salt of CMCTS. Scheme 1 describes a full schematic diagram for the synthesis of CMCTS.

Determination of the Degree of Substitution (DS)

The DS of CMCTS was measured by a potentiometric titration method.[21] Initially, 0.2 g of CMCTS was dissolved in 40 mL of distilled water, and the pH of the solution was adjusted to pH 2

by the addition of hydrochloric acid. Then, the CMCTS solution was titrated with a 0.1*M* aqueous NaOH solution, and the corresponding pH value of the solution was noted with a laboratory pH meter (make CD, model APX175 E/C, Kolkata, India). The volume of aqueous NaOH was determined by the second-order differential method. The DS was calculated as follows:[21]

$$\mathrm{DS} = \frac{161A}{m_{\mathrm{CMCTs}} - 58A}$$

$$A = V_{\mathrm{NaOH}}\, c_{\mathrm{NaOH}}$$

where A is the product of Volume of NaOH and molarity of aqueous NaOH used in the reaction. V_{NaOH} and c_{NaOH} are the volume and molarity of aqueous NaOH, respectively; m_{CMCTS} is the mass of CMCTS; and 161 and 58 are the respective molecular weights of the glucosamine (the repeating unit of chitosan) and carboxymethyl groups, respectively.

Fourier transform infrared (FTIR) Studies

The FTIR characterization of chitosan, synthesized CMCTS, and alginate was done with a Bruker Alpha attenuated total reflectance FTIR spectrometer (model Alpha E, Bruker Alpha, Ettlingen, Germany). The sample was uniformly mixed with potassium bromide at a 1 : 10 weight ratio, and KBr pellets were prepared with 10 tons of hydraulic pressure for 10 min at room temperature. Then, the FTIR spectra of those pellets were recorded within a frequency range of 500–4000 cm^{-1} for 42 consecutive scans.

X-Ray Diffraction (XRD) Studies

The powder XRD patterns of the native chitosan and synthesized CMCTS were examined with a wide-angle X-ray diffractometer with Cu Kα filtered radiation ($\lambda = 1.54060$). The XRD scan rate was fixed at 1°/min, and the step size was 0.04°. The accelerating voltage and current used were 40 kV and 30 mA, respectively.

Preparation of the Blank IPN Hydrogel Beads

Both CMCTS and alginate were dissolved in distilled water at different weight ratios (0 : 1, 0.3 : 1, 0.5 : 1, 1 : 1, 2 : 1, and 3 : 1) and mixed with constant stirring to make a homogeneous mixture. After that, the uniform CMCTS–alginate solutions were dropped through a 21-gauge syringe needle into 100 mL of a 2% (w/v) calcium chloride solution with gentle stirring to form smooth and spherical beads. The beads were kept in the calcium chloride solution for 30 min at room temperature to get the IPN beads. The prepared beads were then washed well with distilled water several times to remove unreacted calcium chloride and air-dried at room temperature until a constant weight was obtained.

Preparation of the Insulin-Loaded IPN Hydrogel Beads

To prepare the insulin-loaded CMCTS–alginate IPN hydrogel beads, insulin with a final concentration of 0.1% (w/v) was added to the dissolved CMCTS–alginate solution with continuous stirring to form the homogeneous CMCTS–alginate/insulin blend solution. The subsequent processes were the same as the preparation of the CMCTS–alginate IPN hydrogel blank beads.

Determination of the Insulin Loading Efficiency (LE) and Insulin Encapsulation Efficiency

A specific amount of insulin-loaded CMCTS–alginate beads were allowed to swell completely in 10 mL of phosphate buffer at pH 7.4 for 24 h at 37°C. The swollen beads were crushed with a glass mortar and pestle, and the mixture was centrifuged to get polymeric debris free of drug solution. The clear supernatant was analyzed for insulin content with a UV–vis spectrophotometer (LAMBDA-25, PerkinElmer, Wiesbaden, Germany) at 280 nm. All of the experiments were done in triplicate to calculate LE and the encapsulation efficiency by the following formulas:[22]

$$\mathrm{LE}(\%) = \frac{\text{Total amount of insulin in beads}}{\text{Total amount of beads}} \times 100 \qquad (1)$$

$$\text{Encapsulation efficiency}\ (\%) = \frac{\text{Actual loading of insulin in beads}}{\text{Theoretical loading of insulin in beads}} \times 100 \qquad (2)$$

Scanning Electron Microscopy (SEM)

The surface morphology and the particle size of the CMCTS–alginate and insulin-loaded CMCTS–alginate beads (before and after insulin release, Model S3400N) were observed under a scanning electron microscope (Hitachi, model 3400N, Japan). The samples were sputter-coated with a thin layer of gold *in vacuo* to neutralize the charging effects before scanning by SEM. An acceleration voltage of 15 kV was used during scanning. For particle size analysis, the image analysis software equipped with the SEM instrument was used.

Swelling Study of the CMCTS–Alginate Hydrogel Beads

The swelling characteristics of the prepared CMCTS–alginate IPN hydrogel beads were determined by incubation of the dry beads in a 10-mL buffer solution at pH 1.2 (simulated gastric fluid), pH 6.8 (simulated intestinal fluid), and pH 7.4 (simulated colonic fluid) with gentle agitation. The simulated gastric fluid and simulated intestinal fluids were prepared as described in the pharmacopeia.[23] The beads were allowed to swell in the solutions at 37°C for 6 h.[10] At specific time intervals, the swollen beads were taken out of the swelling media, blotted completely with a paper towel to remove excess water on the surface, and weighed in a microbalance. Each run of the experiment was replicated at least three times.

The swelling ratio (Q_s) was calculated with the following formula:[24]

$$Q_s = \frac{w_s - w_d}{w_d} \qquad (3)$$

where W_s and W_d are the weights of the swollen and dry beads, respectively.

Study of the Swelling–Deswelling Behavior of the CMCTS/Sodium Alginate Hydrogel Beads

To get an idea of the pH sensitivity, the swelling and deswelling behavior of the CMCTS/sodium alginate hydrogel beads was investigated in two buffer solutions (at pHs of 1.2 and 7.4) with pH values corresponding to those of the stomach and intestine.[23] Initially, bead samples of a known weight were allowed

to swell at intestinal pH (7.4) until equilibrium was reached. Then, the swollen beads were again placed in the acidic buffer, with a pH corresponding to the pH of the stomach (1.2) until those deswelled to a constant weight. The cycle of swelling–deswelling was repeated at least three times. The swelling capacity of the hydrogel beads was determined by eq. (3) at a consecutive time interval of 10 min.[25]

Release Profiles of Insulin from the CMCTS–Alginate Hydrogel Beads

To evaluate the insulin release profiles of the insulin-loaded CMCTS–alginate hydrogel beads, insulin with a final concentration of 0.1% (w/v) was added to the dissolved CMCTS–alginate solution with continuous stirring to make a homogeneous mixture. After that, the uniform CMCTS–alginate solutions were dropped through a 21-gauge syringe needle into 100 mL of a 2% (w/v) calcium chloride solution with gentle stirring to prepare the smooth and spherical beads. The beads were kept in the calcium chloride solution for 30 min at room temperature to complete the crosslinking process for the formation of the IPN beads. The prepared beads were then washed well with distilled water several times to remove unreacted calcium chloride and air-dried at room temperature until a constant weight was obtained.

Then, the dried test samples were immersed in buffer solutions[23] at pHs corresponding to those of the GI tract (i.e., 1.2, 6.8, and 7.4). At specific time intervals, an aliquot of 100 μL of the sample was taken out, and the corresponding concentration of the released insulin was determined with a UV spectrophotometer at 280 nm.[10] The samples were collected until all of the insulin (~100%) was released. The percentage cumulative release profile of insulin from the hydrogel beads was calculated from the obtained results.

Assessment of the Structural and Functional Stability of Insulin after Release from the CMCTS–Alginate Hydrogel Beads

The stability of insulin after encapsulation within polymeric beads is one of the major criteria in the production of desired biological function within the animal body. To assess the structural stability of the encapsulated insulin after its release from the CMCTS–alginate hydrogel beads, insulin was run in native polyacrylamide gel electrophoresis (PAGE) at 60–70 V. Insulin released from the CMCTS–alginate hydrogel bead samples was analyzed by native PAGE with a 5% stacking and 15% resolving gel. After electrophoresis, the gel slab was stained with 0.25% Coomassie brilliant blue solution and destained with destaining solution. Fresh insulin with 0.5 and 0.25 mg/mL concentrations were run as controls.

RESULTS AND DISCUSSION

DS of CMCTS

The DS values of CMCTS were measured by a potentiometric titration method, and the obtained DS values were 23.5% (N position) and 15.3% (O position) in native chitosan.

FTIR and XRD Study of CMCTS

To study the interaction of carboxymethyl groups with the native chitosan structure, FTIR analysis was carried out. The FTIR spectra of chitosan and CMCTS are shown in Figure 1. The basic characteristic peaks of chitosan at 3427.73 cm^{-1} (O—H stretching and N—H stretching, overlapped), 2922.49 and 2859.82 cm^{-1} (C—H stretching), 1652.90 cm^{-1} (NH_2 deformation), 1154.09 cm^{-1} (bridge —O— stretching), and 1092.74 cm^{-1} (C—O stretching) are shown in Figure 1(A). Figure 1(B) shows the IR spectrum of the sodium salt of CMCTS, where the strong peaks at 1596.95 and 1416.73 cm^{-1} corresponded to the respective asymmetric and symmetric stretching vibrations of COONa,[20] and the acidic form of CMCTS showed characteristic peaks at 1711.57 cm^{-1} (for carboxylic acids, —COOH) and 1604.98 cm^{-1} (for carboxylate ions, —COO^-) in Figure 1(C). From the IR spectra, we concluded that the carboxymethyl group was successfully attached to the chitosan backbone. This was also confirmed by the DS value. The DSs of the carboxymethyl groups on the amino (N position) and primary hydroxyl (O position) sites were approximately 23.5 and 15.3%, respectively. The addition of carboxymethyl groups can improve the water solubility of native chitosan. The mucoadhesion properties of the chitosan will also be elevated after the modification of chitosan.

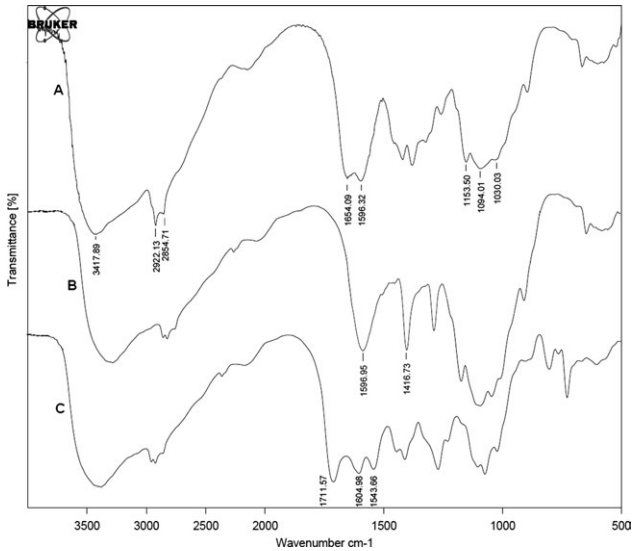


Figure 1. FTIR spectra of (A) chitosan, (B) sodium salt of CMCTS, and (C) H form of CMCTS.

The carboxymethylation of chitosan was also confirmed by an XRD study. The XRD profiles of the polymers are shown in Figure 2. Chitosan showed two distinct crystalline peaks around 10 and 20° due to 020 and 110 reflections, respectively [Figure 2(A)]. This was explained by the regularity in the chain structure due to strong intermolecular hydrogen bonds formed between the hydroxyl and amino groups present in the chitosan. However, in the case of CMCTS, the peak at 10° disappeared, and the peak at 20° weakened [Figure 2(B)]. The reason for the disappearance and weakening of the peaks might have been the destruction of the intermolecular hydrogen bonds and the crystalline regions of chitosan, an indication of the formation of CMCTS by the process of the carboxymethylation of chitosan.

Preparation of the CMCTS–Alginate IPN Hydrogel Beads

The CMCTS–alginate polymeric hydrogel beads were prepared by the addition of different weight ratios of the polymeric blend

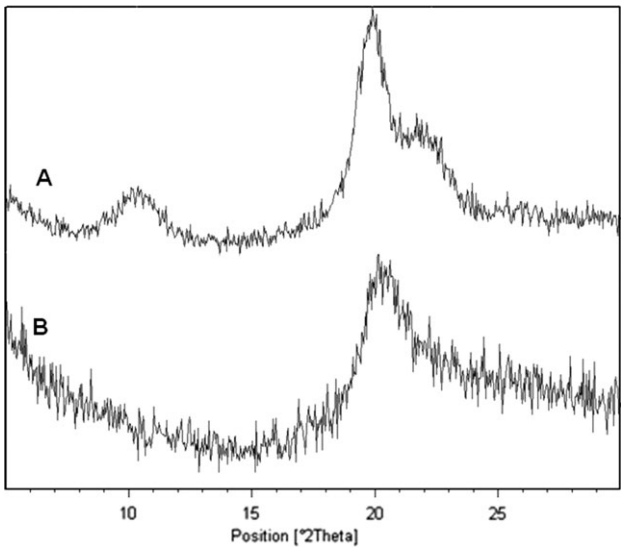
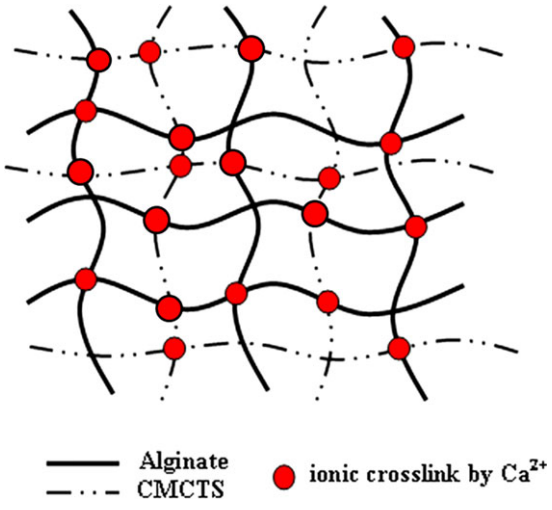


Figure 2. XRD curves of (A) chitosan and (B) CMCTS.

into the crosslinking solution (2% calcium chloride) with the help of a syringe needle. It is known that alginate immediately forms ionic crosslinks in a Ca^{2+} solution and thus forms micro-encapsulated beads.[16] On the other hand, aqueous CMCTS also formed a gel-like structure in calcium chloride solution as the substitution of the amino (N position) and primary hydroxyl (O position) groups by carboxymethyl groups were 23.5 and 15.3%, respectively, as determined by DS. This indicated that ionic crosslinking between the carboxylate ions (—COO^-) on CMCTS could also be established by Ca^{2+}. Thus, during the formation of the CMCTS–alginate hydrogel beads, alginate entangled through the CMCTS network resulted in the formation of an IPN, as shown in (Scheme 2). These IPN beads were further studied for insulin delivery.



Scheme 2. Alginate entangled through the CMCTS network-forming IPN hydrogel beads. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

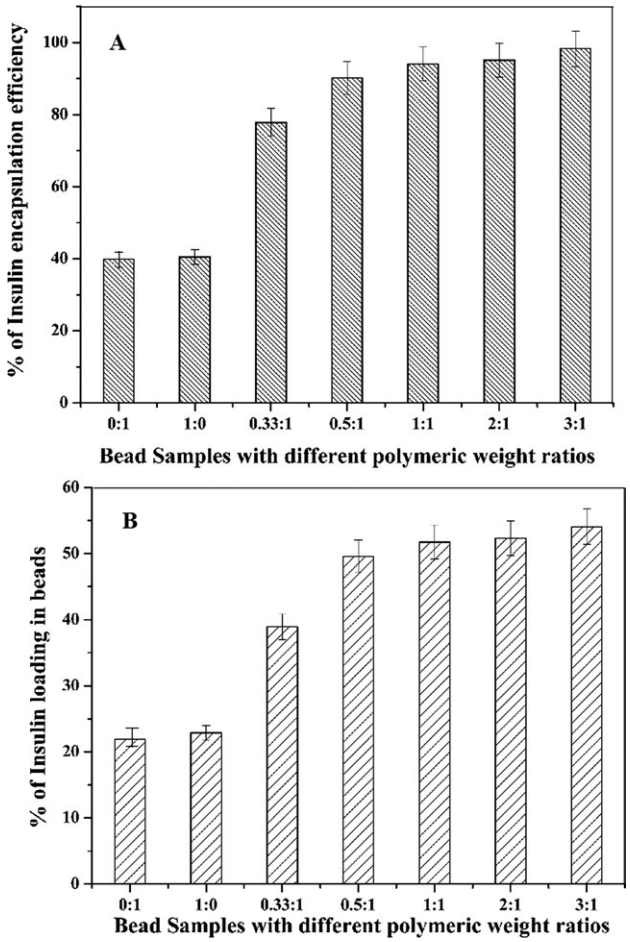


Figure 3. (A) Insulin encapsulation efficiency and (B) LE of the bead samples with different CMCTS–alginate mass ratios.

Insulin Loading and Encapsulation Efficiency of the CMCTS–Alginate IPN Beads

For successful oral drug delivery, ideally, the carrier system must provide a high drug loading and encapsulation efficiency.[26] After entrapment within the polymeric carriers, bioactive molecules (e.g., enzymes, peptides, and proteins drugs) need to be maintained as biologically efficient to produce the desired effects within the animal system. So, a lower drug loading or poor encapsulation efficiency might hinder the use of polymeric delivery devices in drug delivery as either would cause the waste of expensive drug molecules. The insulin loading and insulin encapsulation efficiency of the CMCTS–alginate beads were calculated with eqs. (1) and (2), and they were graphically plotted and are presented in Figure 3. The percentage of insulin encapsulation and the loading percentage of insulin in the IPN beads with different weight ratios of CMCTS to alginate are shown in Figure 3(A,B), respectively. We found that the maximum insulin loading and encapsulation efficiency of the IPN beads were about 53 and 95.3%, respectively. Beads prepared with alginate only showed poor insulin loading (21.9%) and encapsulation (39.8%). However, the addition of CMCTS with alginate seemed to significantly improve both the amount of drug loading and the drug entrapment efficiency. In each set, we found that an increase in the concentration of

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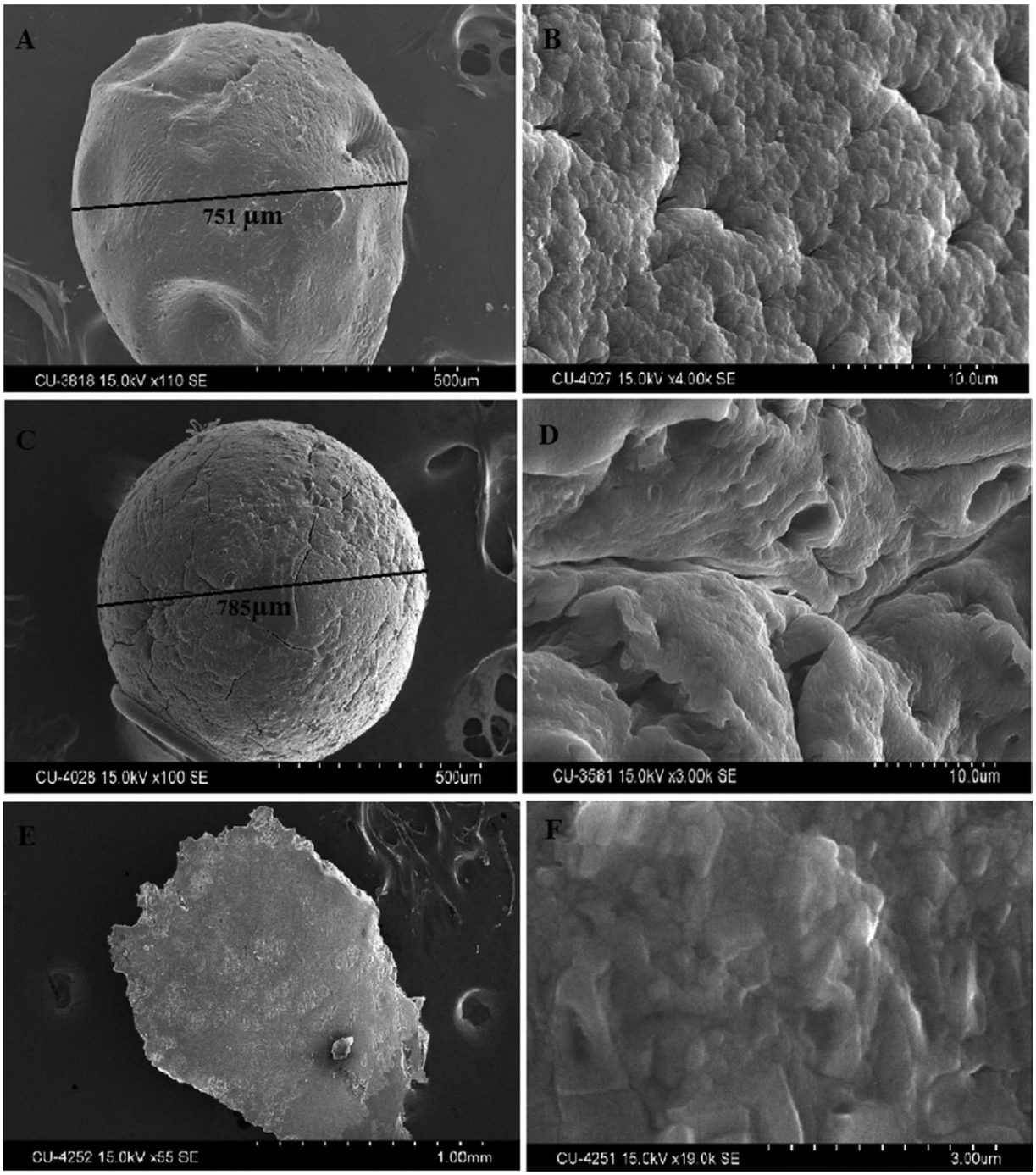


Figure 4. SEM images of the blank and insulin-loaded CMCTS–alginate IPN hydrogel beads.

CMCTS also increased the amount of insulin loading and entrapment. Not only the polymeric amount but also the crosslinking medium were very essential factors influencing the drug encapsulation and LE. This was due to ionic interaction occurring between the polymeric chains and the crosslinking medium. We observed that increasing the concentration of calcium chloride led to better insulin entrapment within the polymeric beads. However, the insulin release was also lower because of the higher crosslinking density. Significant encapsulation of insulin and its complete release through a sustained manner from the beads with a 2% calcium chloride solution as a crosslinking medium were observed. These findings led us to use that particular concentration of calcium chloride (2%) for the crosslinking of all of the formulations of beads in our experiments.

SEM

SEM was used to study the size, shape, and surface morphology of the insulin-loaded native alginate beads and insulin-loaded CMCTS–alginate hydrogel beads. The SEM images of all of the beads are shown in Figure 4. The SEM image of the

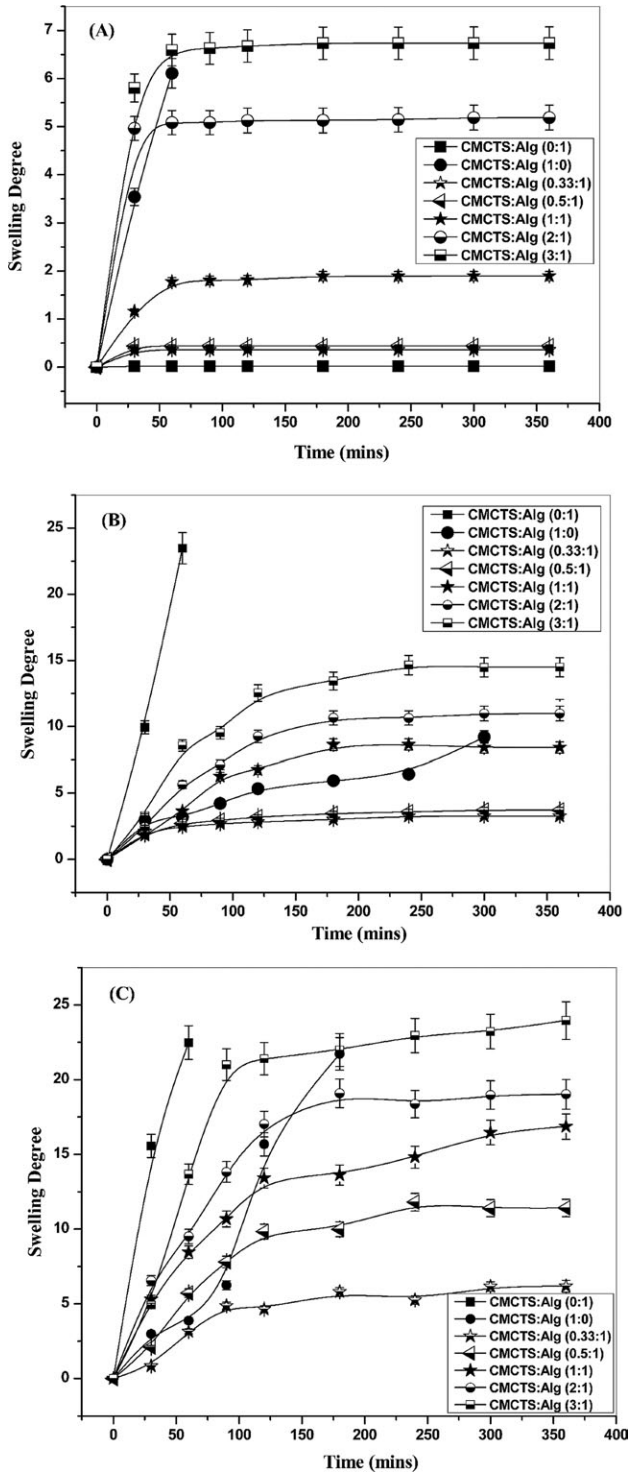


Figure 5. Comparative swelling study of the CMCTS–alginate (Alg) IPN bead samples at different pH values.

insulin-loaded alginate hydrogel beads after drying is shown in Figure 4(A). We found that the beads were spherical in shape. The average particle size of the dried beads was 751 μm. However, the insulin-loaded CMCTS–alginate hydrogel beads had average size of 785 μm, as illustrated in Figure 4(C). A small increase in size may have resulted from introduction of CMCTS to the bead formulation. The CMCTS–alginate hydrogel beads possessed a coarser surface with comparatively larger pores; this differed from the insulin-loaded alginate-only beads, as shown in Figure 4(B,D). After the blending of CMCTS with alginate, there may have been some molecular interaction between those polymers, and this may have caused a better porous surface of the beads. The structural changes in the morphology of the CMCTS–alginate hydrogel beads after insulin release at pH 7.4 is shown in Figure 4(E). The round uniform structure was destroyed after incubation in simulated intestinal fluid (pH 7.4). An enlarged picture of the surface of the bead after insulin release is depicted in Figure 4(F). The clearly distorted surface morphology of the bead supported the release of insulin through the pores. It is evident from the SEM study that porous surface can aid in insulin release.

Swelling Behavior of the Insulin-Loaded CMCTS–Alginate IPN Beads

The swelling behavior of 2% calcium chloride crosslinked insulin-loaded CMCTS–alginate beads was studied in buffers with three pH values (1.2, 6.8, and 7.4) corresponding to the pH values in the GI tract. All of the results are presented later in Figure 6. The swelling characteristics of the IPN beads at pH 1.2 are shown in Figure 5(A). Beads formulated with an increasing amount of CMCTS in the blend with alginate exhibited significant swelling. On the other hand, beads prepared with only alginate did not swell significantly at the same pH. The phenomenon eventuated because of the pH sensitivity of the polymeric carriers. At this pH, Q_s was found to be very low because of hydrogen-bond formation between the —COOH and —OH groups of CMCTS and the —OH groups of alginate. On the contrary, beads having an increasing amount of CMCTS significantly swelled at acidic pH (pH 1.2) within 30 min of incubation. This may have been due to the protonation of the primary amino groups ($—NH^{3+}$) of CMCTS structure; this created repulsive force within the beads and led to their high degree of swelling. So, the beads containing more CMCTS at pH 1.2 may have been ineffective at retaining a sufficient amount of encapsulated insulin for oral delivery application.

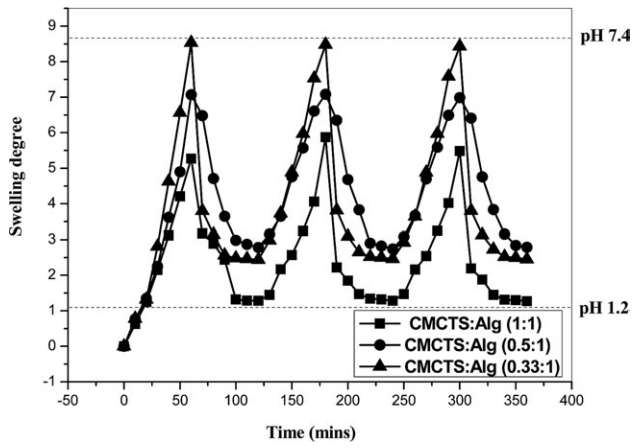


Figure 6. Swelling–deswelling study of the CMCTS–alginate (Alg) IPN bead samples at pH 1.2 and pH 7.4.

With a gradual increase in the pH (from acidic to alkaline), the swelling behavior of the CMCTS–alginate beads was also altered accordingly. At pH 6.8 (that of the duodenum), the beads prepared with different ratios of CMCTS to alginate showed sustained swelling after 90 min of incubation at 37°C, whereas the alginate-only beads at pH 6.8 became completely disintegrated within 30 to 60 min of incubation [Figure 5(B)]. It has been reported that alginate possesses pH-sensitive swelling properties at alkaline pH, and it is easily swollen at alkaline pH; this leads to complete dissolution.[27] The repulsive force between the ionized groups of —COO^- on alginate and the CMCTS structure may influence the swelling properties of the beads. In this study, we crosslinked the alginate CMCTS blend with a 2% calcium chloride solution as a standard because a greater extent of crosslinking limited the free mobility of the polymeric chains and resulted in a poor Q_s of the beads. The crosslinking density may have influenced the swelling kinetics of the polymeric beads in this study, too. Again, bead formulations with comparatively lower polymer concentrations were weakly crosslinked by 2% calcium chloride and did not show sustained swelling, whereas beads prepared with a greater amount of both alginate and CMCTS revealed better crosslinking and showed sustained swelling kinetics over a long period of time.

A remarkable swelling of the beads occurred at pH 7.4 compared to those at both pH 1.2 and 6.8 [Figure 5(C)]. However, the beads composed of only alginate (CMCTS–alginate ratio = 0 : 1 w/w) were easily dissolved at pH 7.4, and their fast swelling behavior will limit their application at this pH. In contrast, the beads prepared with both alginate and CMCTS at different weight ratios resulted in sustained swelling behavior over a long period of time. Initially, the swelling was lower, but with increasing incubation time, the beads swelled significantly. With the introduction of greater amounts of alginate and CMCTS (or an increase in the concentration of —COO^-), the swelling profile of the beads improved significantly. The presence of acidic (carboxyl) groups may have attracted water and led to higher swelling. So, to improve the pH responsiveness of beads, a higher concentration of carboxylic groups needs to be introduced to the polymeric structure.

From the study of the swelling kinetics of beads formulated with various ratio of alginate and CMCTS, we observed that the beads with CMCTS–alginate ratios of 0.5 : 1 and 1 : 1 w/w efficiently maintained sustained swelling. These beads did not swell much at acidic pH but swelled more in alkaline pH solutions. The use of these beads will be more fruitful in the release of insulin in the intestinal environment and will protect it in the harsh acidic conditions of the stomach.

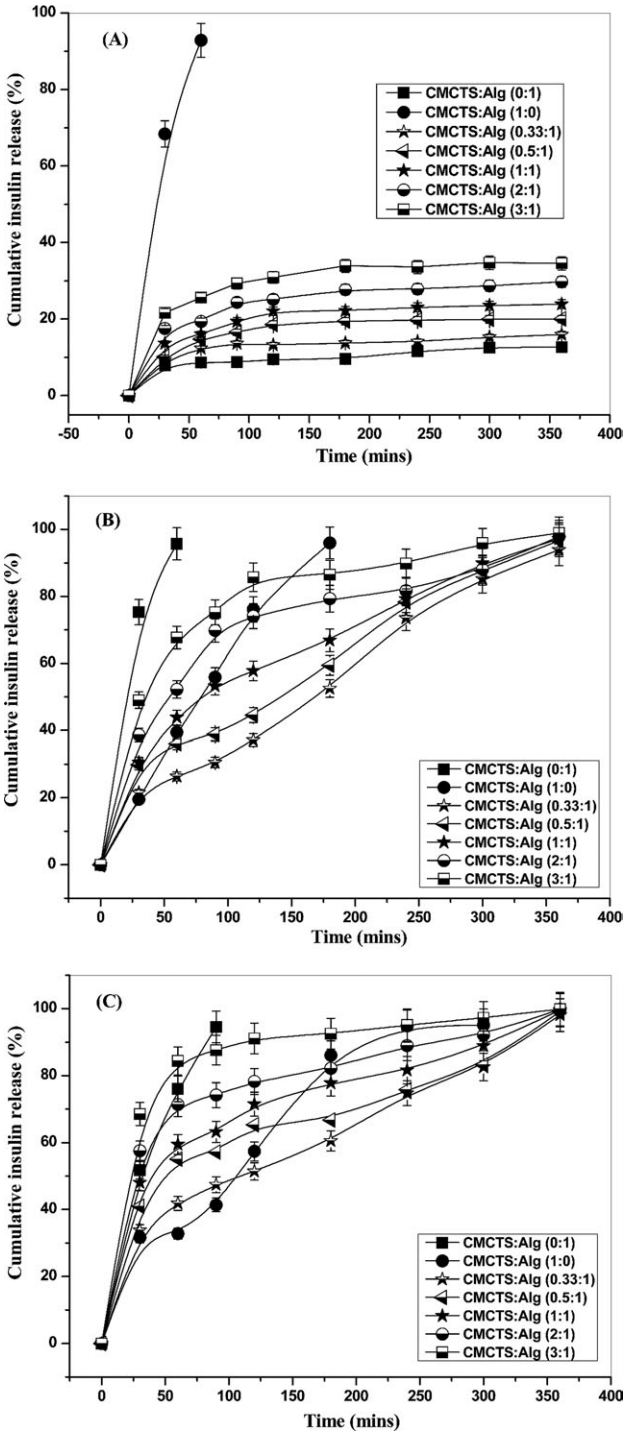
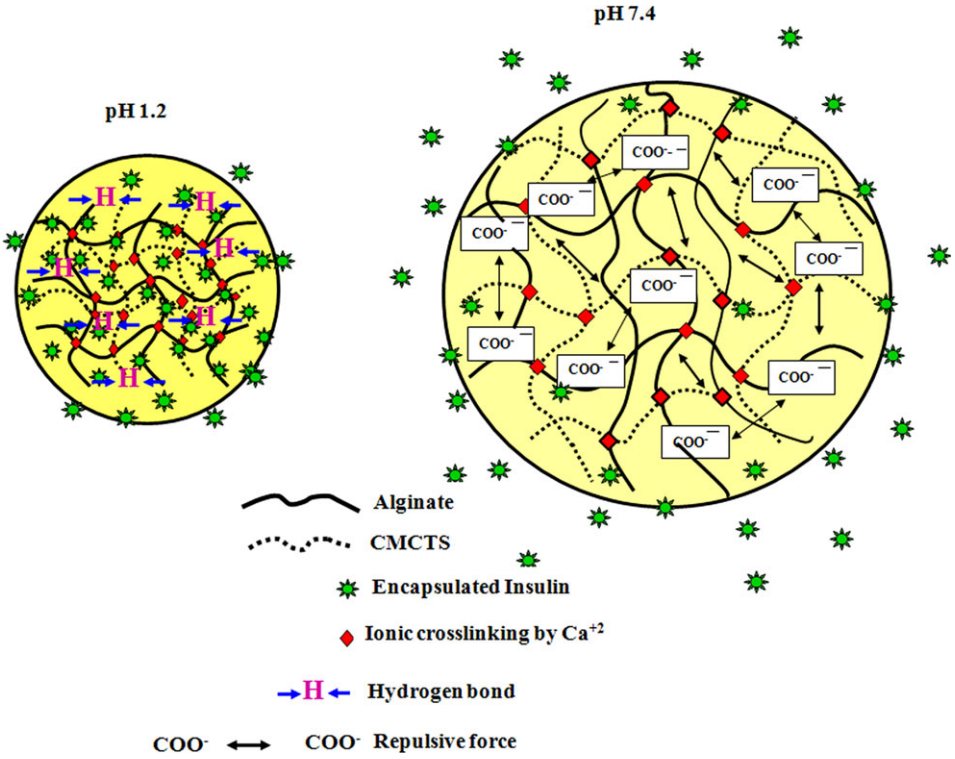


Figure 7. Insulin release profile of the CMCTS–alginate (Alg) IPN bead samples at different pH values.

Swelling–Deswelling Behavior of the CMCTS/Sodium Alginate Hydrogel Beads

From the results of the swelling study of the beads with different weight ratios of CMCTS to alginate, bead samples with CMCTS–alginate mass ratios of.33 : 1, 0.5 : 1, and 1 : 1 showed good swelling in the simulated intestinal pH solution, whereas minimal swelling was observed at the simulated stomach pH. These formulations were again subjected to a reversible swelling study[25] to evaluate their ability to deliver insulin via the oral route. The reversible swelling–deswelling behavior of these bead formulations is shown in Figure 6. We found that the beads swelled significantly in the intestinal pH solution (pH 7.4) and subsequently deswelled in the acidic pH solution. This was attributed to the ionization of functional groups on the polymeric chains. We interpreted from the results that swelling took place at intestinal pH (7.4) because of strong repulsive-force



Scheme 3. Entrapped insulin retention within the IPN beads at pH 1.2 and its sustained release at pH 7.4. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

generation within the ionized acid groups (—COO^-) of the polymeric chains. Simultaneously, when the swollen samples were placed in the acidic medium (pH 1.2), strong hydrogen bond formation started to occur between the —COOH and —OH groups of CMCTS and the —OH groups of alginate at pH 1.2; this led to a gradual deswelling of the beads. Thus, it is evident that a progressive swelling–deswelling behavior in CMCTS–alginate beads may help in the delivery of insulin in a pH-responsive manner and protect the insulin from enzymatic degradation in the GI tract.

Insulin Release Profile from the CMCTS/Sodium Alginate Hydrogel Beads

The insulin release profile from the CMCTS–alginate hydrogel beads was examined in buffers with different pH values to evaluate their efficiency in oral insulin delivery. The release study also followed the same trend as that of the swelling behavior of these hydrogel beads. The insulin release profile at different pH values is presented in Figure 7. All of the bead samples exhibited the release of a very low concentration of insulin; they retained almost their entire loaded amount of the drug at pH 1.2, as shown in Scheme 3. Only about 20% of encapsulated insulin was released from different bead samples prepared with different weight ratios of CMCTS to alginate at this low pH. The formation of hydrogen bonds between the alginate (—COOH and —OH) and CMCTS (—COOH and —OH) probably limited insulin release at pH 1.2. However, the bead sample with the maximum amount of CMCTS released comparatively more insulin (~34%) at pH 1.2 because it swelled because of the protonation of primary amino groups (—NH^{3+}) on the CMCTS structure, as shown in Figure 7(A), at lower gastric pH (1.2).

Although a significant amount of insulin release was observed at pH 6.8 and 7.4, which corresponded to the pH values of the duodenum and ileum, respectively (Scheme 3), a sustained release of encapsulated insulin was found with successive increases in swelling; this led to the disintegration of the beads at alkaline pH. Samples prepared with native sodium alginate showed a burst release of insulin at this alkaline pH. Within 30 min of incubation, almost all of the entrapped insulin (~56.7%) was released because of the high electrostatic repulsion forces between the ionized carboxylic groups on the structural polymers. However, in case of the other polymer beads, insulin was released in a sustained and controlled manner. At pH 6.8, the beads swelled considerably, releasing about 83.9% of their insulin [Figure 7(B)]. On the other hand, at pH 7.4, the beads swelled with a significant release (~94.3%) of insulin within 5 h of incubation (Figure. 7C).

Primarily, the design of successful oral insulin formulations must consider the pH sensitivity as one of the most important factors. It is known that the fasting pH of the stomach is about 2.0–6.0. With the presence of food within the stomach, the pH becomes 1.2–2.0, as HCl secretion is stimulated with the intake of food. The pH of the duodenum ranges between 6.0 and 6.8, and the small intestine shows a pH of 7.4.[28,29] As orally administered drug formulations go through different regions of the GI tract, they are usually absorbed in the small intestine. In the case of healthy subjects, the small intestine transit time varies from 3 to 4 h.[30] In our study, we prepared IPN beads with

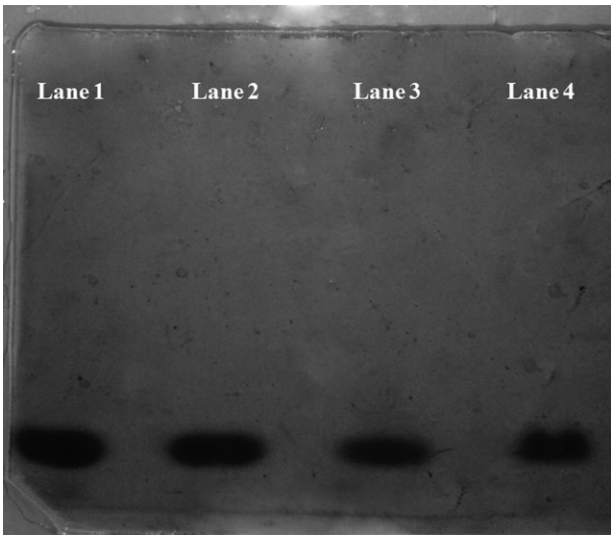


Figure 8. Biological efficiency and stability of insulin after release from the IPN bead samples.

different weight ratios of CMCTS and alginate for use as a potential oral insulin delivery device. The use of higher amount of CMCTS in the formulation of beads also limits the oral administration of insulin because of their fast dissolution at the acidic stomach pH, followed by protonation of amino groups. Therefore, bead formulations with 1 : 1 and 0.5 : 1 CMCTS–alginate weight ratios show the best results in retaining insulin at acidic pH and released significant amounts of insulin at alkaline pH in their passage through the GI tract.

Assessment of the Structural and Functional Stability of Insulin by PAGE

The native PAGE analysis for the released insulin is shown Figure 8. The control insulin at 0.5 and 0.25 mg/mL were run in lanes 1 and 2 in the gel, and insulin released from the bead samples with CMCTS–alginate mass ratios of 0.5 : 1 and 1 : 1 were run through lanes 3 and 4, respectively. We observed that the released insulin (5.8 KDa) had distinct bands in comparison to the insulin band of the control in the gel. So, we concluded that the structure of insulin remained intact after entrapment in the polymeric hydrogel beads. In turn, this will enable these bead formulations to deliver insulin within the animal body to exert their desired biological function.[31]

CONCLUSIONS

Water-soluble chitosan was synthesized by carboxymethylation, and its synthesis was confirmed by FTIR and XRD studies. The DS of CMCTS was determined by potentiometric titration, and the obtained DS values wer e23.5% (N position) and 15.3% (O position). Insulin-loaded CMCTS–alginate IPN beads with different weight ratios were put in 2% calcium chloride solution to get crosslinked IPN in which the insulin was entrapped. The bead samples prepared with alginate and CMCTS at weight ratios of 0.5 : 1 and 1 : 1 showed good insulin encapsulation efficiency with a maximum of 95% insulin encapsulation. The samples also showed good flexibility and tensile strength, which definitely aided in the improvement of their oral insulin delivery efficiency because these bead samples had to cross the different pH environments of the GI tract. These beads also demonstrated excellent pH-responsive behavior in *in vitro* studies. The prepared beads effectively protected the insulin at gastric pH, whereas a significant amount of insulin was released at pH 6.8 (that of the duodenum) and pH 7.4 (that of the ileum). Again, the released insulin was found to be stable and biologically active after encapsulation. The more important aspect of these polymeric formulations was that they were formulated in aqueous media, as both the alginate and CMCTS were water-soluble polymers. So, calcium chloride crosslinked CMCTS–alginate IPN beads could serve as a novel polymeric vehicles for oral insulin administration.

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