Studies on Effect of pH on Cross-linking of Chitosan With Sodium Tripolyphosphate: A Technical Note

Submitted: February 15, 2006; Accepted: April 9, 2006; Published: June 2, 2006

Devika R. Bhumkar[1] and Varsha B. Pokharkar[1]

[1]Department of Pharmaceutics, Poona College of Pharmacy, Bharati Vidyapeeth Deemed University, Erandwane, Pune-411038, Maharashtra State, India

KEYWORDS: chitosan, deprotonation, ionic cross-linking, mechanistic study, tripolyphosphate.

INTRODUCTION

Chitosan, a natural, biodegradable, biocompatible, bioadhesive polymer, is gaining attention in the pharmaceutical field for a wide range of drug delivery. Chitosan is a copolymer of glucosamine and N-acetyl glucosamine linked by β 1–4 glucosidic bonds obtained by N-deacetylation of chitin. The molecular weight and degree of deacetylation can be modified during its preparation to obtain tailor-made properties. Also, chitosan has free amine as well as hydroxyl groups, which can be modified to obtain different chitosan derivatives.[1,2]

Chitosan acts as a penetration enhancer by opening the tight epithelial junctions and hence is particularly exploited in protein and vaccine delivery.[3,4] Chitosan nanoparticles for delivery of polypeptides such as insulin, tetanus toxoid, and diphtheria toxoid are widely explored.[5-9] Van der Lubben et al[5] observed significant rise in systemic and local response after oral administration of diphtheria toxoid–loaded chitosan microparticles, but insignificant systemic response after nasal administration. The basic molecular parameters of chitosan, like molecular weight and degree of deacetylation, influence the protein loading and delivery. Xu et al[6] observed that increase in the degree of deacetylation from 75.5% to 92% promoted the encapsulation efficiency but deaccelerated the release rate. Chitosan being cationic in nature offers great advantages for ionic interactions. The surface modification of chitosan nanoparticles by the diblock copolymer polyethylene oxide polypropylene oxide (PEO-PPO) was performed by Calvo et al[7,8] such that they can be effective carrier systems for parenteral administration. PEO-PPO decreased the protein loading as it interacted electrostatically with amino groups of chitosan, thus competing with protein for association with chitosan. Janes et al[9] studied the efficacy of depolymerized low molecular weight chitosan for encapsulation of insulin and tetanus toxoid stating that the amount of tripolyphosphate (TPP) affected the entrapment efficiency.

Chitosan microparticles and nanoparticles have been made by chemical cross-linking with glutaraldehyde, glyoxal, and ethylene glycol diglycidyl ether.[10,11] Although these are very good cross-linkers, they are not preferred owing to their physiological toxicity. Chitosan is polycationic in acidic media (pK_a 6.5) and can interact with negatively charged species such as TPP and sodium sulfate. This characteristic can be employed to prepare cross-linked chitosan nanoparticles. The interaction of chitosan with TPP leads to formation of biocompatible cross-linked chitosan nanoparticles, which can be efficiently employed in protein and vaccine delivery. The cross-linking density, crystallinity, and hydrophilicity of cross-linked chitosan can allow modulation of drug release and extend its range of potential applications in drug delivery.

The aim of the present work was to carry out a systematic study to gain insight on the mechanistic basis of the ionotropic gelation of chitosan with TPP. With this view, the effect of pH of TPP on chitosan-tripolyphosphate cross-linking was investigated.

MATERIALS AND METHODS

Materials

Chitosan [α (1–4) 2-amino 2-deoxy β-D glucan] was kindly supplied by Marine Chemicals, Cochin, India. The degree of deacetylation of chitosan was 85%. Chitosan is soluble in weak acids like acetic acid and lactic acid. The pK_a of chitosan is 6.3. Sodium tripolyphosphate was supplied by Loba Chemie, Mumbai, India. Glacial acetic acid and hydrochloric acid were of analytical grade. Millipore Gradient A10 water (Molsheim, France) was used throughout the experiments.

Preparation of cross-linked chitosan

Chitosan (60 mg) was dissolved in 20 mL acetic acid (2% vol/vol) to obtain chitosan solution. TPP (0.1%) was added to chitosan solution with mild stirring until an opalescent

Corresponding Author: Varsha B. Pokharkar, Department of Pharmaceutics, Poona College of Pharmacy, Bharati Vidyapeeth Deemed University, Erandwane, Pune-411038, Maharashtra State, India. Tel: 91-20-2543 7237; Fax: 91-20-2543 9383; E-mail: vbpokharkar@yahoo.co.in

suspension was obtained. The pH of TPP was adjusted from the original pH 9 to 3 using 0.1 N hydrochloric acid. The opalescent suspension of cross-linked chitosan particles was subjected to freeze-drying using a freeze dryer (Jouan/Heto Lyopro 3000, Copenhagen, Denmark).

Characterization

Conductivity Studies:

The conductivity studies were performed to monitor the cross-linking reaction. The change in conductivity was measured after each mL of addition of TPP. The conductivity of chitosan suspension was measured on a pico conductivity meter (LabIndia, Navi Mumbai, India).

pH Studies (Titrimetric Studies):

Change in pH of the chitosan solution was recorded on a pH meter (Toshniwal Instrument Manufacturers Pvt Ltd, Ajmer, India) after addition of each mL of TPP.

Scanning Electron Microscopy With Energy Dispersive X-ray Analysis:

Cross-linked chitosan samples were coated with a thin gold-palladium layer by a sputter coater unit (VG-Microtech, Uckfield, UK) and the surface topography was analyzed with a Cambridge Stereoscan 440 scanning electron microscope (SEM; Leica, Cambridge, UK) operated at an acceleration voltage of 20 kV. The elemental analysis was performed using energy dispersive x-ray analysis (EDAX).

Swelling Index:

The freeze-dried product was weighed initially (Wd) and immersed in 250 mL deionized water at ambient temperature for 24 hours. The swollen weight (Ws) was obtained by gently removing the surface water with blotting paper. Swelling index (SI) was then calculated using the following formula:

$$SI = \left(\frac{Ws - Wd}{Wd}\right) \times 100\% \qquad (1)$$

Fourier-transform Infrared Spectroscopy:

Fourier-transform infrared (FTIR) spectra of the chitosan and cross-linked chitosan were obtained on a JASCO V5300 FTIR (Tokyo, Japan). The pellets were prepared on a KBr-press (Spectra Lab, Mumbai, India). The spectra were scanned over the wave number range of 4600 to 400 cm^{-1}.

X-ray Diffraction:

The x-ray diffraction (XRD) patterns of chitosan and cross-linked chitosan were recorded on an x-ray diffractometer (PW 1729, Philips, Eindhoven, Netherlands). The samples were irradiated with monochromatized Cu Kα radiation (1.542 Å) and analyzed between 2 and 50° (2 θ). The voltage and current used were 30 kV and 30 mA, respectively. The range and the chart speed were 2×10^3 CPS and 10 mm/^{o} (2 θ), respectively.

Inductively Coupled Plasma-Atomic Emission Spectrometry:

The phosphorus content of the cross-linked chitosan was determined on an inductively coupled plasma–atomic emission spectrometer (ICP-AES; ICAP 9000, Shimadzu, Tokyo, Japan). It was dissolved in 0.1 N HCl and further diluted with water. The measured concentration of phosphorus in aqueous solution was converted into actual phosphorus content taking into consideration the dilution factor.

Differential Scanning Calorimetry:

Thermograms of chitosan and cross-linked chitosan were obtained using a Mettler-Toledo DSC 821^{e} instrument equipped with an intracooler (Mettler-Toledo, Greifensee, Switzerland). Indium standards were used to calibrate the differential scanning calorimetry (DSC) temperature and enthalpy scale. The samples were hermetically sealed in aluminum pans and heated at a constant rate of 20°C/min, over a temperature range of 0 to 550°C. Inert atmosphere was maintained by purging nitrogen at the flow rate of 100 mL/min.

RESULTS AND DISCUSSION

Chitosan with a pK_a of 6.3 is polycationic when dissolved in acid and presents $-NH^+_3$ sites. Sodium tripolyphosphate ($Na_5P_3O_{10}$) dissolved in water dissociates to give both hydroxyl and phosphoric ions. Since the cross-linking of chitosan would be dependant on the availability of the cationic sites and the negatively charged species, it was expected that the pH of TPP would play a significant role in the same. pH would bring about a change on the extent and type of cross-linking. Hence in the present study, 2 different pH conditions namely pH 3 and pH 9 were used for obtaining the cross-linked particles. When the pH of TPP was adjusted to 3, only phosphoric ions were present. However, at pH 9, both OH^- and phosphoric ions were present and may compete with each other to interact with the $-NH^+_3$ of chitosan. The conductivity studies were performed to detect the relative changes in the ionic species with the change in pH. This in turn would reflect the nature of cross-linking with the change in the pH of TPP. The

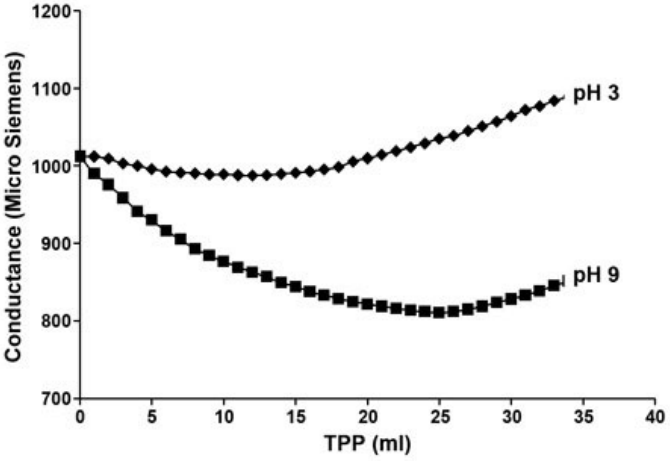

Figure 1. Effect of pH of TPP on conductivity during cross-linking of chitosan with tripolyphosphate.

conductivity curve is illustrated in Figure 1. Initially, when chitosan is solubilized in 2% acetic acid, (pH 3.05) the free amino groups get hydrated. Addition of TPP at lower pH (pH 3) leads to a decrease in conductivity up to 13 mL of TPP addition. When TPP at pH 3 containing $P_3O^{5-}_{10}$ ions is added to the chitosan solution it interacts with the $-NH^{+}_{3}$ amino groups, leading to a decrease in the conductivity. When a further amount of TPP is added, all the amino groups get saturated with $P_3O^{5-}_{10}$ ions and the conductivity rises after 13 mL addition of TPP. After the equivalence point, there is an increase in conductance because of the increase in H^+ ions. The process is predominantly ionic cross-linked. When TPP at higher pH (pH 9) was added to chitosan solution, the decrease in conductance was observed up to addition of 26 mL of TPP. As stated above, in TPP at higher pH (pH 9) both the OH^- and phosphoric ions are present, which compete with each other to interact with the $-NH^{+}_{3}$ sites of chitosan. The OH^- ions are linked to the

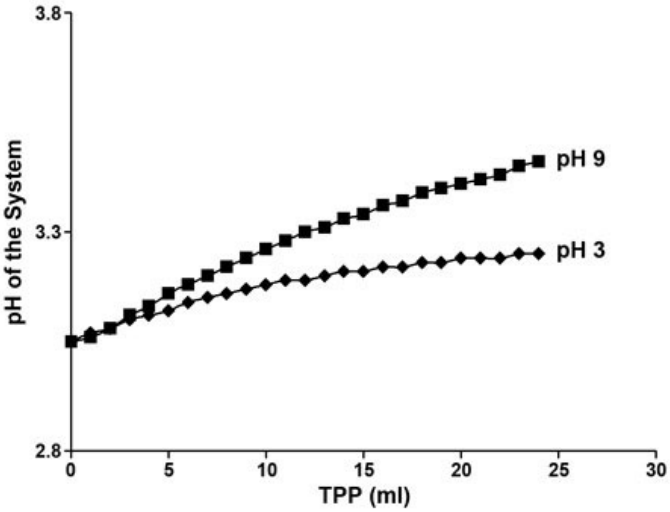

Figure 2. Titration curve: effect of pH of TPP on pH of the system.

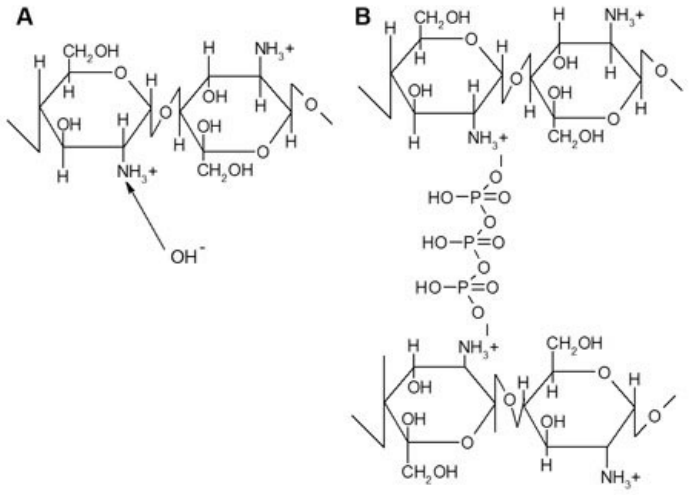

Figure 3. Interaction of chitosan with TPP (a) deprotonation, (b) ionic cross-linking.

amino groups by deprotonation. These results are supported by the pH change in the system. The pH of the system was monitored after addition of TPP at different pH (TPP pH 3 and 9). In spite of addition of TPP at higher pH, a very gradual Figure 2), whereas the pH of the system remained unchanged in the case of cross-linking with lower pH of TPP (pH 3). Thus, cross-linking was effected by deprotonation at higher pH of TPP, while the chitosan–TPP complex at lower pH was formed by the ionic interaction between positively charged chitosan and negatively charged phosphoric ions. The mechanism of cross-linking of chitosan with TPP could be either by deprotonation or ionic interaction as shown in Figure 3.

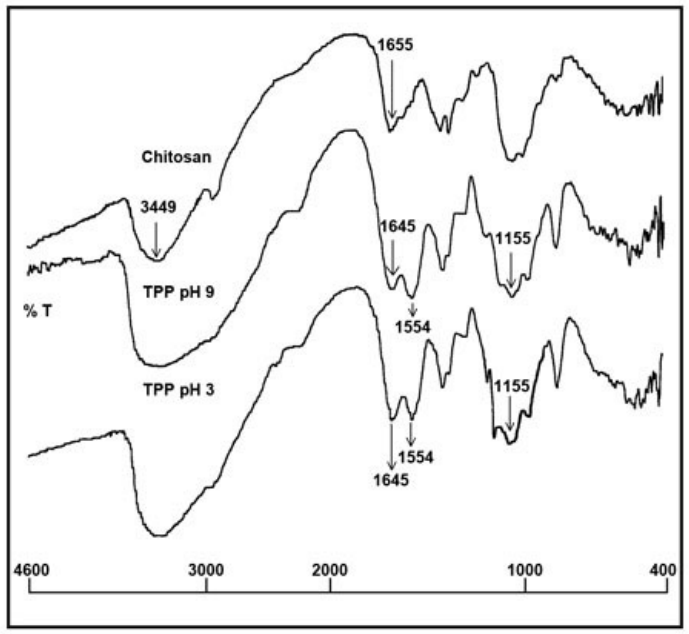

Figure 4. IR spectra of chitosan and chitosan cross-linked with TPP at pH 9 and pH 3.

Table 1. EDAX Analysis of Chitosan Cross-linked With TPP at pH 3 and pH 9

Elements	Chitosan Cross-linked With TPP at pH 3 (wt%)	Chitosan Cross-linked With TPP at pH 9 (wt%)
C	40.77	41.93
N	11.71	14.40
O	38.97	38.17
P	8.54	5.50

The FTIR spectra of chitosan and cross-linked chitosan are shown in Figure 4. A characteristic band at 3449 cm^{-1} is attributed to $–NH_2$ and –OH groups stretching vibration and the band for amide I at 1655 cm^{-1} is seen in the infrared spectrum of chitosan. Whereas in the FTIR spectra of cross-linked chitosan the peak of 1655 cm^{-1} disappears and 2 new peaks at 1645 cm^{-1} and 1554 cm^{-1} appears. The disappearance of the band could be attributed to the linkage between the phosphoric and ammonium ions. The cross-linked chitosan also showed a peak for $P = O$ at 1155 cm^{-1}. Xu et al,[6] Knaul et al,[12] and Wang et al[13] observed similar results in their study of formation of chitosan nanoparticles and chitosan film treated with phosphate.

The swelling studies of the cross-linked chitosan were performed to relate the cross-linking density. At pH 3, phosphoric ions were predominantly involved in cross-linking, which led to introduction of a greater number of phosphate groups. The swelling index obtained for cross-linked chitosan at pH 3 was 668.85%, which was distinctly higher than the 157.65% obtained for cross-linked chitosan at pH 9. It indicated that cross-linking density of the product obtained at pH 3 was higher. The phosphorus content of the cross-linked chitosan was detected by ICP. The phosphorus content of chitosan cross-linked with TPP at pH 3 was 1.145 ppm, which was found to be higher than chitosan cross-linked with TPP at pH 9 where the phosphorus content determined was 0.473 ppm. The elemental analysis by EDAX as shown in Table 1 also revealed the higher wt% of phosphorus in chitosan cross-linked at lower pH of TPP (pH 3). The scanning electron microphotographs of cross-linked chitosan at TPP pH 3 and pH 9 are shown in Figure 5. It can be noted that the chitosan cross-linked at higher pH (pH 9) was more porous. Loose and open structures with more porosity obtained at higher pH could be attributed to its lower cross-linking density.

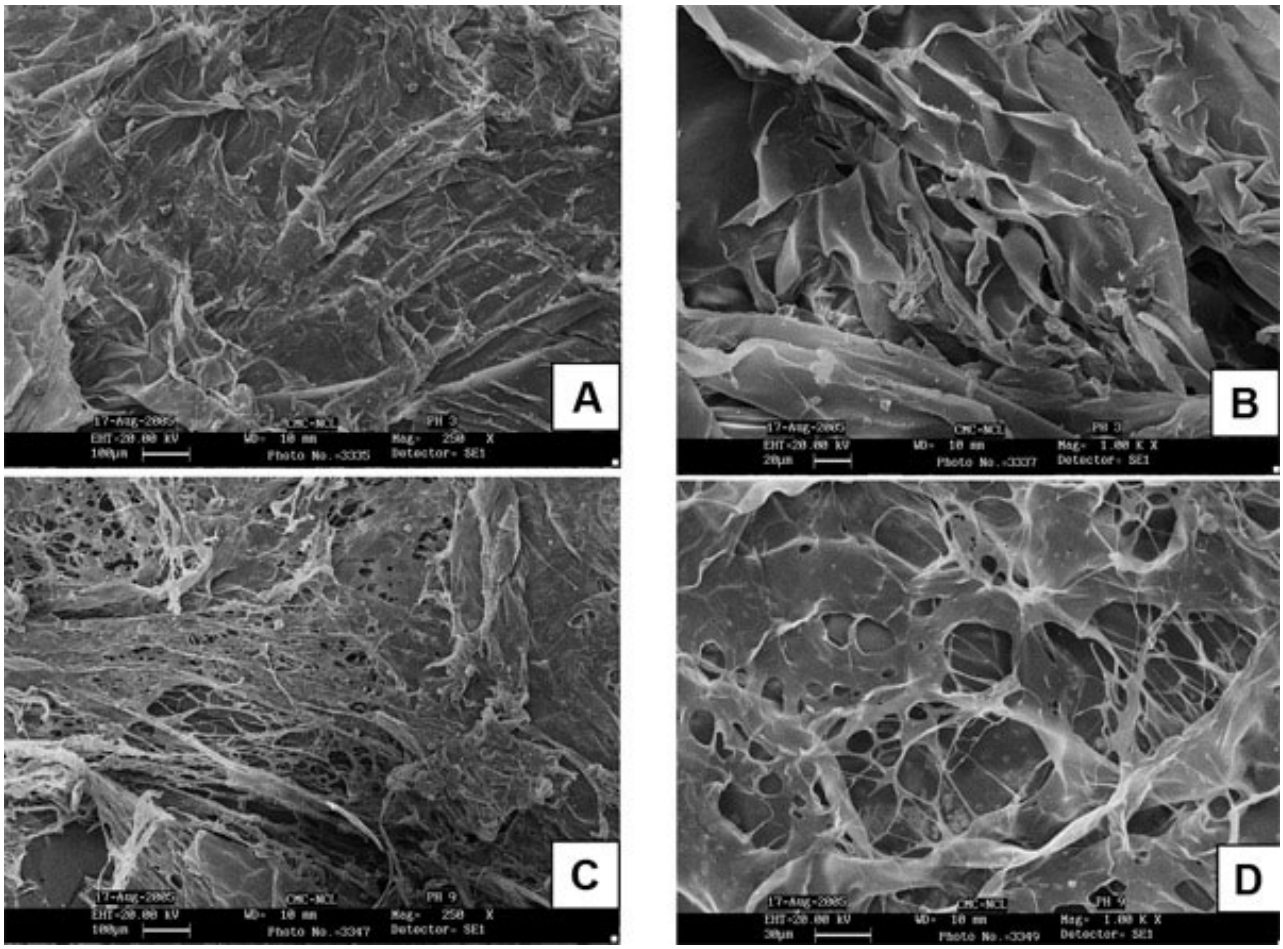

Figure 5. Scanning electron microphotographs of chitosan cross-linked with (a) TPP at pH 3, ×250, (b) TPP at pH 3, 1 KX, (c) TPP at pH 9, ×250, (d) TPP at pH 9, 1 KX.

XRD spectra of chitosan (Figure 6) showed two prominent crystalline peaks at 10° (2θ) and 20° (2θ). In the case of cross-linked chitosan (Figure 6) there was significant decrease in the intensity of characteristic peaks of chitosan, which was in agreement with the study reported by Wan et al[14] The distinct differences in the diffraction patterns of chitosan and cross-linked chitosan could be attributed to modification in the arrangement of molecules in the crystal lattice. In chitosan cross-linked with TPP at pH 3 more suppressed peaks at 10° (2 θ) and 20° (2 θ) were observed, which might be due to amorphization.

DSC studies were performed to understand the behavior of the cross-linked chitosan on application of thermal energy. Polysaccharides usually have a strong affinity for water and in solid state these macromolecules may have disordered structures that can be easily hydrated. The hydration properties of polysaccharides depend on primary and supramolecular structures. The endotherm related to evaporation of water is expected to reflect the molecular changes brought in after cross-linking. Thus, chitosan and cross-linked chitosan at different pH had varied water-holding capacity. In chitosan the bound water molecules are associated with hydrophilic hydroxyl groups. The thermogram of chitosan showed endotherm at 102.24°C with the enthalpy of fusion (ΔH) 210 J/g (Figure 7). The water-holding capacity of cross-linked chitosan with TPP at pH 3 was found to be more (ΔH 439.56 J/g) compared with that of plain chitosan and chitosan cross-linked with TPP at pH 9 (ΔH 273.20 J/g). As discussed earlier, the cross-linking of chitosan with TPP at different pH modifies the crystalline nature of chitosan. The hydrophilicity of cross-linked chitosan is higher at pH 3, which might be responsible for its increase in the water-holding capacity. On the basis of these results it can be stated

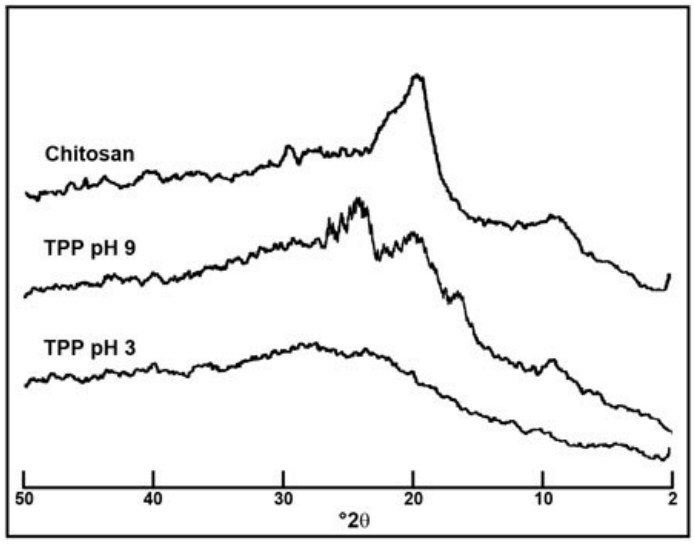

Figure 6. X-ray diffraction patterns of chitosan and chitosan cross-linked with TPP at pH 9 and pH 3.

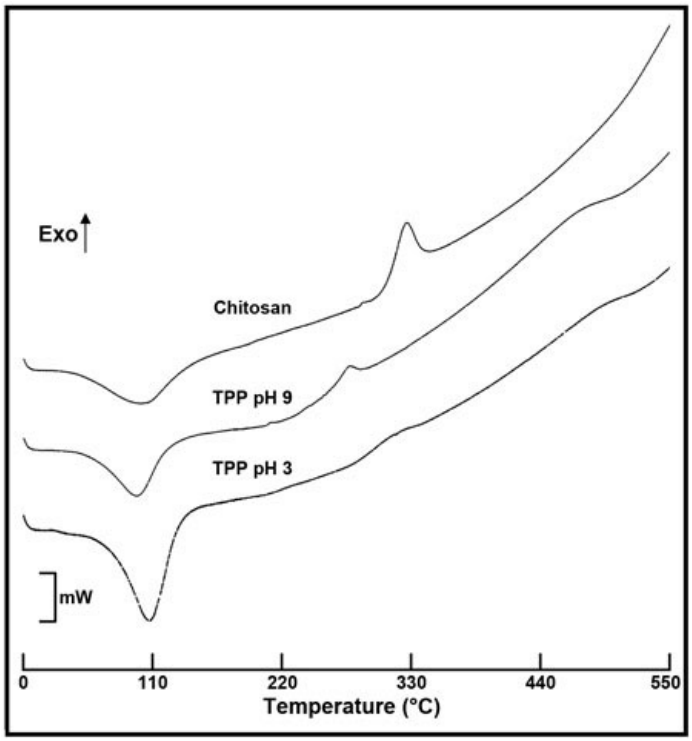

Figure 7. DSC thermograms of chitosan and chitosan cross-linked with TPP at pH 9 and pH 3.

that increase in the polar groups and reduction in crystalline domains caused an increase in the water-holding capacity of cross-linked chitosan. These results are in agreement with the work done by Kittur et al[15] where they studied the thermal behavior of chitin and chitosan and their carboxymethyl derivatives showing that the increase in the N-deacetylation and carboxymethylation of chitosan led to increase in the water-holding capacity. The second thermal event observed was an exotherm due to the decomposition of the polymer. Owing to the differences in the chemical characteristics, changes in the exothermic peak of chitosan and cross-linked chitosan were also observed. The presence of free unsubstituted amine groups showed higher ΔH values.[15] As the free amine groups in cross-linked chitosan with TPP pH 9 are more, the ΔH value found was 31.11 J/g as compared with that of cross-linked chitosan at TPP pH 3 where the energy required was 19.50 J/g. Characterization of cross-linked chitosan by XRD, DSC, ICP, and EDAX provided the evidence of reduction in crystallinity and increase in hydrophilicity after cross-linking with TPP pH 3.

Summary and Conclusions

The ionotropic gelation method for formation of cross-linked chitosan particles can be easily modified from ionic cross-linking to deprotonation by adjusting the pH of TPP. Chitosan was cross-linked ionically with TPP at lower pH and by deprotonation mechanism at higher pH. The swelling behavior of cross-linked chitosan appeared to depend

on the pH of TPP. The ionically cross-linked chitosan showed higher swelling ability. Thus the nature of cross-linked chitosan can be tailor made to obtain the desired properties in terms of cross-linking density, crystallinity, and hydrophilicity.

ACKNOWLEDGMENTS

The authors are thankful to National Chemical Laboratory, Pune, India, and Marine Chemicals and Cochin for providing the facility of SEM and gift sample of chitosan respectively. D.R.B. is thankful to Fair and Lovely Foundation for providing financial support in form of a Project Saraswati Scholarship.

REFERENCES

1. Ilium L. Chitosan and its use as a pharmaceutical excipient. *Pharm Res.* 1998;15:1326–1331.

2. Felt O, Buri P, Gurny R. Chitosan: a unique polysaccharide for drug delivery. *Drug Dev Ind Pharm.* 1998;24:979–993.

3. van der Lubben IM, Verhoef JC, Borchard G, Junginger HE. Chitosan for mucosal vaccination. *Adv Drug Deliv Rev.* 2001;52:139–144.

4. van der Lubben IM, Verhoef JC, Borchard G, Junginger HE. Chitosan and its derivatives in mucosal drug and vaccine delivery. *Eur J Pharm Sci.* 2001;14:201–207.

5. van der Lubben IMV, Kersten G, Fretz MM, Beuvery C, Verhoef JC, Junginger HE. Chitosan microparticles for mucosal vaccination against diphtheria: oral and nasal efficacy studies in mice. *Vaccine.* 2003;21:1400–1408.

6. Xu Y, Du Y. Effect of molecular structure of chitosan on protein delivery properties of chitosan nanoparticles. *Int J Pharm.* 2003;250:215–226.

7. Calvo P, Remunan-Lopez C, Vila-Jato CL, Alonso MJ. Novel hydrophilic chitosan- polyethylene oxide nanoparticles as protein carriers. *J Appl Polym Sci.* 1997;63:125–132.

8. Calvo P, Remunan-Lopez C, Vila-Jato CL, Alonso MJ. Chitosan and chitosan/ethylene oxide-propylene oxide block copolymer nanoparticles as novel carriers for proteins and vaccines. *Pharm Res.* 1997;14:1431–1436.

9. Janes KA, Alonso MJ. Depolymerized chitosan nanoparticles for protein delivery: preparation and characterization. *J Appl Polym Sci.* 2003;88:2769–2776.

10. Akbuga J, Durmaz G. Preparation and evaluation of cross-linked chitosan microspheres containing furosemide. *Int J Pharm.* 1994;111:217–222.

11. Berger J, Reist M, Mayer JM, Felt O, Peppas NA, Gurny R. Structure and interactions in covalently and ionically crosslinked chitosan hydrogels for biomedical applications. *Eur J Pharm Biopharm.* 2004;57:19–34.

12. Knaul JZ, Hudson SM, Creber KAM. Improved mechanical properties of chitosan fibres. *J Appl Polym Sci.* 1999;72:1721–1731.

13. Wang X, Ma J, Wang Y, He B. Structural characterization of phosphorylated chitosan and their applications as effective additives of calcium phosphate cements. *Biomaterials.* 2001;22:2247–2255.

14. Wan Y, Creber KAM, Peppley B, Bui VT. Synthesis, characterization and ionic conductive properties of phosphorylated chitosan membranes. *Macromol Chem Physiol.* 2003;204:850–858.

15. Kittur FS, Harish Prashanth KV, Udaya Sankar K, Tharanathan RN. Characterization of chitin, chitosan and their carboxymethyl derivatives by differential scanning calorimetry. *Carbohydrate Polymers.* 2002;49:185–193.