Carbohydrate Polymers 85 (2011) 105–110

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

O,N-carboxymethyl chitosan–zinc complex: A novel chitosan complex with enhanced antimicrobial activity

Ramchandra L. Patale, Vandana B. Patravale*

Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology, Matunga (E), Mumbai 400019, Maharashtra, India

ARTICLE INFO

Article history:
Received 20 July 2010
Received in revised form
25 December 2010
Accepted 1 February 2011
Available online 2 March 2011

Keywords:
Antimicrobial
Chitosan–zinc complex
O,N-carboxymethyl chitosan–zinc complex

ABSTRACT

Chitosan is a biodegradable, biocompatible and non-toxic deacetylated derivative of chitin. It exhibits myriad of biological activities such as antithrombogenesis, anti-tumor activity, antimicrobial activity, wound healing activity and blood compatibility. Due to the presence of amine ($-NH_2$) groups and hydroxyl (–OH) groups, chitosan can be modified to generate various novel derivatives with varying solubility patterns. Among the various derivatives, O,N-carboxymethyl chitosan has been found to elicit broad range of biological activities and proved to be useful in pharmaceutical applications. Chitosan derivatives form co-ordinate complexes with metal ions like zinc and copper which are also known to have antimicrobial activity. In the present investigation, O,N-carboxymethyl chitosan–zinc complex and chitosan–zinc complex were prepared and characterized using FTIR, XRD and DSC. Further, the complexes were evaluated and compared for antimicrobial activities against Gram-positive and Gram-negative bacteria. The studies revealed that the proposed novel O,N-carboxymethyl chitosan–zinc complex exhibited better antimicrobial activity than chitosan–zinc complex.

1. Introduction

Chitosan is a cationic polysaccharide biopolymer mainly composed of β (1–4)-2-amino-2deoxy D-glucopyranose repeating units and includes a small amount (20%) of N-acetyl–D glucosamine residues. It is primarily obtained from the shells of crustatian animals by the deacetylation of chitin. Owing to its unique chemical structure, chitosan exhibits bioadhesivity and is found to be biodegradable and non-toxic. Further, due to the presence of large number of amine groups and hydroxyl groups, it can be easily chemically modified to obtain derivatives of varying solubility as well as form metal complexes (Juang, Wu, & Tsang, 1999).

It has been well established that chitosan has many interesting pharmacological and pharmaceutical properties. There are numerous studies which have elucidated the mechanistic wound healing action of chitosan (Hiroshi et al., 2001; Stone, Wright, Clarke, Powell, & Devaraj, 2000; Ueno et al., 1999b). Due to the excellent wound healing property, researchers have explored topical applications of chitosan (Ueno, Mori, & Fujinaga, 2001a). Chitosan has also been used in veterinary medicine (Senel & McClure, 2004). Further, chitosan has been reported to have immunological activity (Nishimura et al., 1984). Chitosan is also reputed to inhibit hypercholesteremia (Ormrod, Holmes, & Miller, 1998). Chitosan and chitosan blended with other polymers have also been used as a matrix scaffold for the growth of mammalian cells, especially neuronal cells (Aijun et al., 2005; Gingras, Paradis, & Berthod, 2003; Yuan, Zhang, Yang, Wang, & Gu, 2004).

Thus, the purpose of this study was to prepare a chitosan metal complex with enhanced antimicrobial activity which can be used in topical formulations for the treatment of skin infections and wound healing. For this purpose, the authors investigated and compared the antimicrobial activity of chitosan–zinc complex and O,N-carboxymethyl chitosan–zinc complex against *Staphylococcus aureus* and *Escherichia coli*.

2. Materials and methods

Chitosan (MW 40,000–50,000) was provided by Healers Neutraceuticals Pvt. Ltd. (Chennai, India) as a gift sample. Acetic acid was purchased from Merck Ltd. (Mumbai, India). Zinc sulphate, isopropyl alcohol, sodium hydroxide, solid monochloroacetic acid, methanol and ethanol were purchased from s.d. Fine chemicals Ltd. (Mumbai, India) and used without further purification. *E. coli* and *S. aureus* were sub-cultured from the strains available in-house. Soyabean casein digest agar was purchased from HiMedia (Mumbai, India). Distilled water, distilled in the lab was used whenever needed.

* Corresponding author. Tel.: +91 22 33611111x2217; fax: +91 22 33611020.
E-mail address: vbp_muict@yahoo.co.in (V.B. Patravale).

0144-8617/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2011.02.001

2.1. Purification of chitosan

A modified process reported by Gana, Wang, Cochrane, & McCarron (2005) for making protein free chitosan was utilized for making protein free chitosan.

Briefly, chitosan (10 gm) was dispersed in 100 ml of 1 M sodium hydroxide. This was heated to 70 °C and stirred for 2 h at a speed of 1500 rpm. Thereafter, the solution was filtered and the residue was washed with water, followed by ethanol. The resultant residue was dried for 18 h at 45 ± 5 °C. Subsequently, the residue (7 gm) was dissolved in 500 ml of 0.1 M acetic acid, and the pH was adjusted to 4 using conc. hydrochloric acid. The acidified solution was filtered using a 100-mesh sieve. The obtained filtrate was treated with 1 M sodium hydroxide to attain the pH of 8–9. The resultant residue was washed with distilled water until the washings were neutral, followed by ethanol. Finally, the residue was heated up to 60 °C and then dried at room temperature until dry.

2.2. Preparation of chitosan–zinc complex

The complex was prepared as described by Wang, Du, & Liu (2004). Chitosan was dissolved in acetic acid (1% w/v). During stirring, required amount of zinc sulphate (in a ratio of 4:1) was added into the solution. For complexation, the pH of the solution was increased to 7.0 by adding liquid ammonia solution. Thereafter, the mixture was refluxed at 80 °C for 3 h with stirring. After it was cooled to room temperature, the mixture was poured into acetone. The resulting white precipitate was filtered and washed repeatedly with ethanol and finally dried to constant weight.

2.3. Preparation of O,N-carboxymethyl chitosan

O,N-carboxymethyl chitosan was prepared by modified Hayes's method (Hayes, 1986). To 20.0 g of a commercial chitosan product, 200 ml of isopropyl alcohol was added and the resulting slurry stirred in a 1 l flask at 25 °C. To the stirred slurry, over a period of 20 min, 50.4 ml of 10 N aqueous sodium hydroxide solution was added in six equal portions at 4 min intervals. The alkaline slurry was stirred for an additional 45 min, then 24.0 g of solid monochloroacetic acid added in five equal portions at 5 min intervals over a period of 20 min. The reaction mixture was then stirred continuously for 3 h at a temperature of 60 °C. Following which, 17 ml of cold distilled water was added to the mixture and pH was adjusted to 7.0 by addition of glacial acetic acid. The reaction mixture was then filtered and the residue was added to 300 ml of a 70% (v/v) methanol/water mixture in which it was stirred to wash the product. The solid product was again filtered, and then dispersed in 300 ml of anhydrous methanol in which it was stirred to wash it. The washed product was finally filtered, collected and dried overnight in an oven at 60 °C.

2.4. Preparation of O,N-carboxymethyl chitosan–zinc complex

O,N-carboxymethyl chitosan was dissolved in distilled water to give a 2% (w/v) solution. To this solution, zinc sulphate was added in different proportions. Then the mixture was centrifuged. Ethanol was added to the supernatant solution in a quantity sufficient to precipitate complex. The resulting precipitate was dried overnight at 60 °C to obtain O,N-carboxymethyl chitosan–zinc complex.

2.5. Effect of temperature and zinc ion concentration on the yield of O,N-carboxymethyl chitosan–zinc complex

2.5.1. Effect of temperature

The reaction was carried out in a controlled temperature water bath at different temperature conditions in the range of 25–60 °C; to study the effect of temperature on the yield of O,N-carboxymethyl chitosan–zinc complex.

2.5.2. Effect of zinc ion concentration

The reaction was carried out at different concentrations of zinc sulphate in the range of 0.05 M–5 M at constant temperature of 50 °C; to study the effect of zinc ion concentration on the yield of O,N-carboxymethyl chitosan–zinc complex.

2.6. Characterization of chitosan–zinc complex, O,N-carboxymethyl chitosan and O,N-carboxymethyl chitosan–zinc complex

Chitosan–zinc complex, O,N-carboxymethyl chitosan and O,N-carboxymethyl chitosan–zinc complex were characterized by Fourier transform infrared spectroscopy (FT-IR), X-Ray diffraction (XRD) and Differential scanning calorimetry (DSC). The solubility of above materials was also studied.

The FT-IR spectra were obtained by using FT-IR spectrophotometer (Perkin Elmer). The samples were mixed uniformly with potassium bromide at 1:5 (sample:KBr) ratio respectively. The KBr discs were prepared by compressing the powders (mixture of sample and KBr) at pressure of 5 tons for 5 min in a hydraulic press. The discs were scanned in the range of 500–4000 cm^{-1} to obtain FT-IR spectra.

Powder XRD patterns were obtained at room temperature using Rigaku Miniflex diffractometer, with Cu Kα target tube, NaI detector, variable slits, a 0.050 step size, operated at a voltage of 30 kV, 15 mA current, at 2θ/min scanning speed, and scanning angles ranged from 0 to 80° (2θ).

The DSC measurements were performed on a Differential scanning colorimeter (Perkin Elmer coupled to Pyris-6 Software) with a thermal analyzer. Accurately weighed samples were placed and sealed in aluminum pans, before heating under nitrogen flow (20 ml/min) at a scanning rate of 10 °C/min from 35 °C to 250 °C. An empty aluminum pan was used as a reference.

2.7. Evaluation of antimicrobial activity

In-vitro antimicrobial activity of O,N-carboxymethyl chitosan–zinc complex and chitosan–zinc complex was evaluated using *S. aureus* and *E. coli* as representative bacteria by agar cup method. Mupirocin was used as the positive control. All the cultures were maintained on soyabean casein digest agar medium by periodic subculturing. The inocula for both microorganisms were prepared from a 24-h old growth of organism on soyabean casein digest agar slant. With the help of sterile nichrome wire loop, the growth of the organism on slant was aseptically transferred to a tube containing sterile normal saline solution. The contents of the tube were then shaken properly so as to get uniform cell suspension of the organism. Optical density of the inoculum was adjusted to 0.4 on the photoelectric colorimeter by using sterile normal saline, before using it as an inoculum. The concentration of *S. aureus* in inocula was equivalent to 4.6×10^{15} cfu/ml. The concentration of *E. coli* in inocula was equivalent to 5.2×10^{15} cfu/ml.

One ml of the inoculum was mixed with the 20 ml of molten soyabean casein digest agar, then poured into sterile petri-plate (9 cm in diameter) and allowed to solidify. The wells were formed by punching a sterile stainless steel borer onto the plate and removing the agar. Each test sample (0.5 ml of 2% (w/v) solution of O,N-carboxymethyl chitosan–zinc complex and 0.5 ml 2% (w/v) solution of chitosan–zinc complex in 0.3% hydrochloric acid and 100 μl of Mupirocin stock solution) was placed in each well. The petriplates were kept in the dark conditions at room temperature for 24 h.

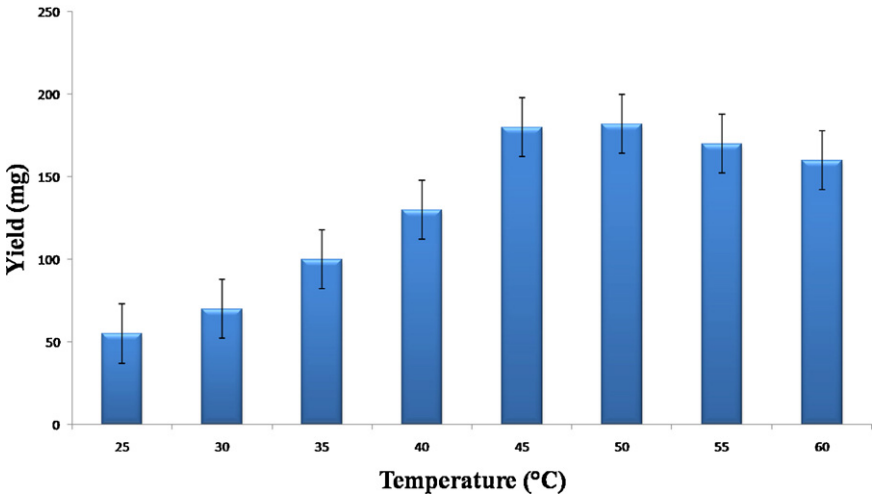

Fig. 1. Effect of temperature on the yield of O,N-carboxymethyl chitosan–zinc complex.

After incubation, the inhibition zone diameter around each well was measured.

3. Results and discussion

3.1. Purification of chitosan

Chitosan being a product from natural origin is often contaminated with proteins. Thus, there was a need to purify chitosan before using it for analytical purposes. Initial treatment with sodium hydroxide was given to remove fatty residues from chitosan. It was followed by dissolving chitosan in acid. Chitosan contains a large number of glucosamine units, and is soluble only in an acidic medium. Thus, it was decided to select the acid very judiciously taking into consideration factors such as strength, economy and handling safety. Among the acids, acetic acid and lactic acid emerged as possible candidates. For further study, acetic acid was selected because it is a weak acid, cost effective and safe to handle. After precipitating out the chitosan from acid by addition of sodium hydroxide, the residue was washed until the pH was neutral and dried at 60 °C overnight to get a pale yellow granular powder of chitosan.

3.2. Effect of temperature and zinc ion concentration on the yield of O,N-carboxymethyl chitosan–zinc complex

3.2.1. Effect of temperature

The influence of the reaction temperature on chelation of Zinc to O,N-carboxymethyl chitosan is shown in Fig. 1. It was observed that temperature had a pivotal role in the complexation of metal and chitosan derivative. As shown in the Fig. 1., the absorption of Zn^{2+} ions appeared to increase as the temperature was increased to 50 °C. Correspondingly, the yield of water soluble product also increased, which was slightly accelerated from 45 °C to 50 °C. Thereafter, there was marked decreased in the overall yield of the product. This observation could be explained by the desorption and dechelation phenomenon. It has been reported that apparently at lower temperature (<50 °C), the formation of O,N-carboxymethyl chitosan–zinc complexes is favoured. While high temperature (>50 °C) favours the desorption and dechelation (David, Hon, & Tang, 2000). Hence, in agreement with the aforementioned theory, it could be concluded for increased yield of O,N-carboxymethyl chitosan–zinc complex, the temperature has to be optimum, preferably at 50 °C.

3.2.2. Effect of zinc sulphate concentration

The influence of the zinc sulphate concentration on the yield of complex of O,N-carboxymethyl chitosan with zinc is shown in following Fig. 2. It was observed that, yield of water soluble O,N-carboxymethyl chitosan–zinc complex increases up to 2 M zinc sulphate and decreases thereafter.

This behavior could be explained by the simple fact that the increase in the metal ionic concentration tends to decrease in the complex capacities. Since it has been observed that with increase in ionic strength, not only there is a decrease in ion activity but also increase in concentration of competitive anions in the metal ions used (Udhaybhaskar, Iyengar, & Rao, 1990). For instance, the influence of ionic concentration resulted in the decrease in yields of Cr^{6+}-chitosan (Udhaybhaskar et al., 1990), Cu^{2+}-and Ni^{2+}-chitosan beads and Nr^{2+}-N-carboxymethyl chitosan (Riccardo, M, Weckx, & Filippini, 1989). Similarly, in this case, highest chelation was observed at 2 M concentration of zinc sulphate solution with subsequent decrease at higher concentration. Thus, variation in ionic concentrations demonstrated pronounced effect on formation of water soluble complex, indicating that the formation is governed by electrostatic attraction mechanism. As reiterated earlier, beyond 2 M concentration, the desorption rate was greater than the com-

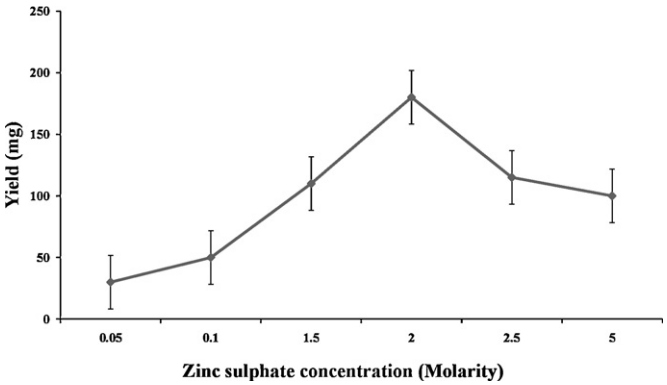

Fig. 2. Effect of zinc ion concentration on the yield of O,N-carboxymethyl chitosan–zinc complex.

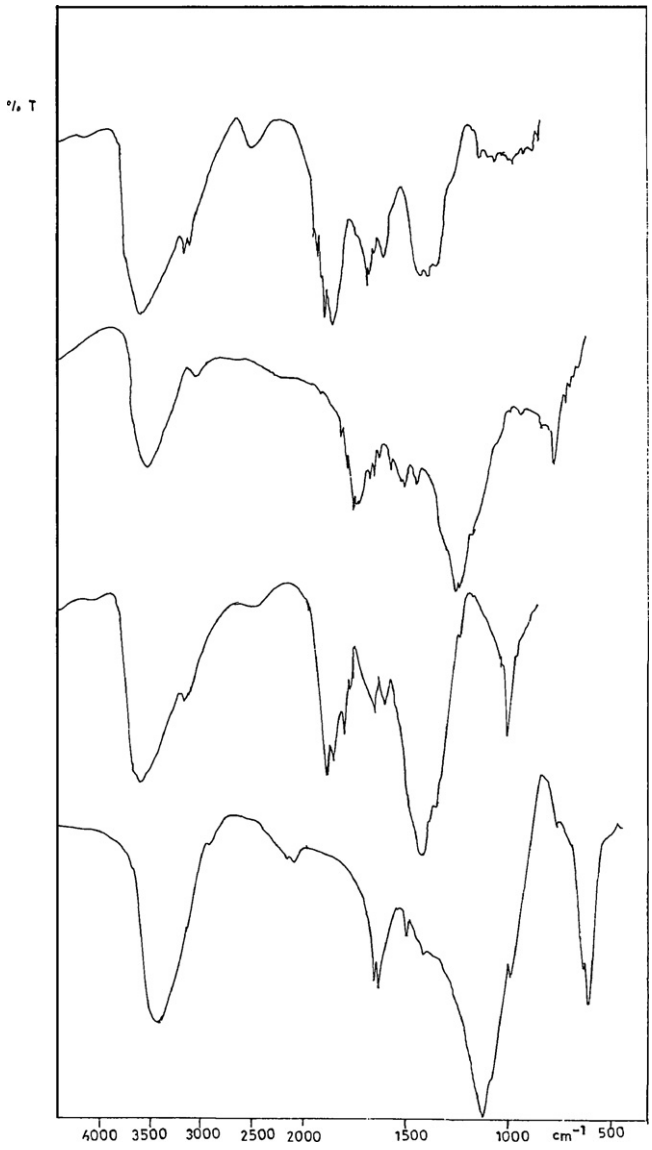

Fig. 3. FT-IR spectrum of (a) chitosan, (b) O,N-carboxymethyl chitosan, (c) chitosan–zinc complex and (d) O,N-carboxymethyl chitosan–zinc complex.

plexing rate, consequently, the complexing rate was lower. (David et al., 2000).

3.3. Characterization of chitosan–zinc complex, O,N-carboxymethyl chitosan and O,N-carboxymethyl chitosan–zinc complex

3.3.1. Chitosan–zinc complex

3.3.1.1. Fourier transform infrared spectroscopy (FT-IR) studies. The spectra of chitosan and chitosan–zinc complex are shown in Fig. 3a and c respectively. The FT-IR spectrum of chitosan–zinc complex exhibits many alterations from that of chitosan. The major differences are: the wide peak at 3448.2 cm^{-1}, corresponding to the stretching vibration of $–NH_2$ group and –OH group, shifted to lower frequency (3411 cm^{-1}). The peak at 1617 cm^{-1} is assigned to the bending vibration of $–NH_2$ group shifted to higher wavenumber (1628 cm^{-1}). It indicated that the $–NH_2$ group and –OH group were involved in complexation; the band (1089.6 cm^{-1}) assigned to the second –OH group showed a significant shift to lower wavenumber, which was enhanced with increasing zinc content. It suggested that the second –OH group got involved in complexation.

Table 1
Solubility of different concentrations of O,N-carboxymethyl chitosan in distilled water.

Concentration (% w/w)	Solubility
0.5	Soluble
0.0	Soluble
1.5	Soluble
2.0	Soluble but slightly viscous
2.5	Soluble with high viscosity
3.0	Not completely soluble

3.3.1.2. X-ray diffraction studies. The diffractograms of chitosan and chitosan–zinc complex are shown in Fig. 4a and b respectively. The chitosan chelated with zinc sulphate showed more numerous and sharper X-ray diffraction bands that untreated chitosan. The pattern revealed formation of new crystalline phase.

3.3.2. O,N-carboxymethyl chitosan

3.3.2.1. Solubility studies. The solutions of different concentrations of O,N-carboxymethyl chitosan were prepared in distilled water. The concentration of the solution and ease of handling with increase in concentration is given in the Table 1. Chitosan is insoluble in water and is only soluble in acidic medium. It has been often reported that the solubility is the main hindrance in the utilization of chitosan for different biomedical purposes. In comparison to the chitosan, the developed derivative, O,N-carboxymethyl chitosan was soluble in distilled water up to 2% (w/v). The solution was found to be transparent, pale yellow and slightly viscous.

3.3.2.2. FT-IR studies. The spectrum of O,N-carboxymethyl chitosan is depicted in Fig. 3b. In particular, the spectrum of O,N-carboxymethyl chitosan shows the bands at 1603 and 1414 cm^{-1}, this can be attributed to the carboxylation. Additionally, the formation of O,N-carboxymethyl chitosan is also confirmed by the intensification of the bands at 1072 and 1323 cm^{-1} corresponding to C–O–C stretching. The band at 3421 cm^{-1} becomes wider and weaker, which suggests that the carboxylation occurred on some of both the amino and primary hydroxyl sites of the glucosamine units of the chitosan structure.

3.3.2.3. X-ray diffraction studies. The diffractogram is given in Fig. 4c. Chitosan shows characteristic sharp peak at 20° (Fig. 4a). In O,N-carboxymethyl chitosan sharpness of that peak decreases and it shows another characteristic peak at around 33° (Fig. 4c). It indicates the formation of O,N-carboxymethyl chitosan.

3.3.2.4. Differential scanning calorimetry (DSC). The DSC thermogram of O,N-carboxymethyl chitosan is shown in Fig. 5b. As compared to chitosan (Fig. 5a), shift in the endothermic peak maxima to a higher temperature was occurred in O,N-carboxymethyl chitosan; this can be attributed to carboxymethylation.

3.3.3. O,N-carboxymethyl chitosan–zinc complex

3.3.3.1. FT-IR studies. The spectrum of O,N-carboxymethyl chitosan–zinc complex is depicted in Fig. 3d. A broad and strong band appearing at approximately 3434 cm^{-1} is related to vibration of the bound water molecule. The appearance of the bands at 1321 cm^{-1} is associated with–OH in plane and 1026 cm^{-1} due to –OH out of plane deformation vibrations suggests that the –OH groups in O,N-carboxymethyl chitosan do not participate in complex formation with zinc ions. The presence of C–N characteristic bands near 2300 cm^{-1} suggests that neither –NH nor $–NH_2$ is involved in the complex formation. The bands around 1630 and

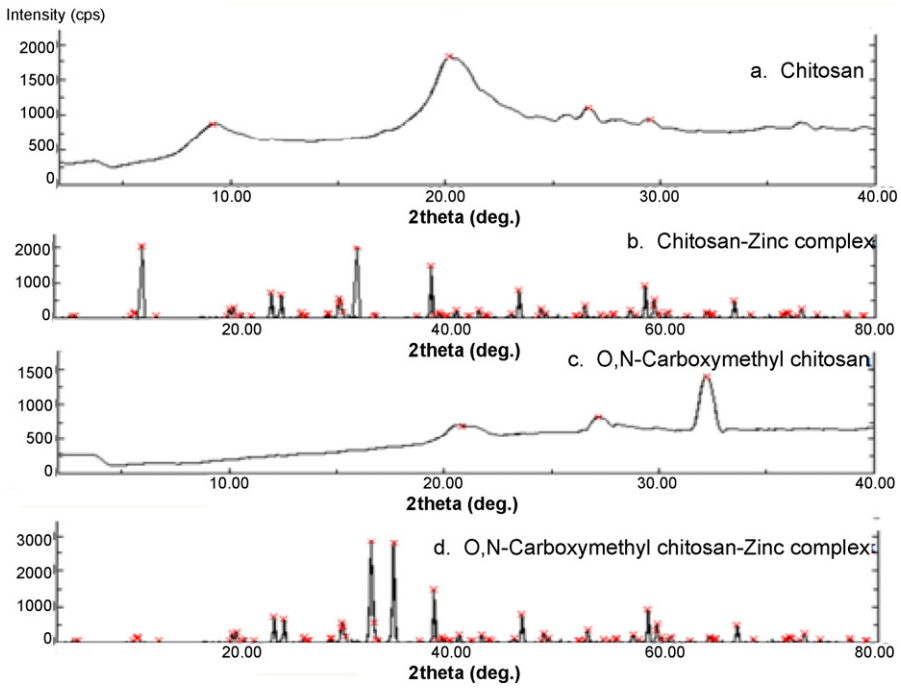

Fig. 4. X-ray diffractogram of (a) chitosan, (b) chitosan–zinc complex, (c) O,N-carboxymethyl chitosan and (d) O,N-carboxymethyl chitosan–zinc complex.

1450 cm^{-1} are attributed to bound form of carboxylic groups in the complex which is in its ionized form. A medium intensity of Zn–O absorption band at 621 cm^{-1} and no band appearing at about 500 cm^{-1} imply that complexation took place by metal oxygen binding only.

3.3.3.2. X-ray diffraction studies. Powder XRD patterns were obtained at room temperature using Rigaku Miniflex diffractometer, with Cu Kα target tube, NaI detector, variable slits, a 0.05° step size, operated at a voltage of 30 kV, 15 mA current, at 2θ/min scanning speed, and scanning angles ranged from 0 to 80° (2θ).

As compared to XRD pattern of O,N-carboxymethyl chitosan (Fig. 4c), diffractogram of O,N-carboxymethyl chitosan–zinc complex (Fig. 4d) showed sharper peaks indicating formation of new crystalline phase.

3.3.3.3. Differential scanning calorimetry (DSC). The DSC thermogram of O,N-carboxymethyl chitosan–zinc complex is shown in Fig. 5c. Complexation is usually associated with lowering in the energy of entire system and the system achieves stability. Stable system requires more energy to again bring up change in it. The shift in the DSC thermogram's endothermic peak towards higher temperature indicates that complexation has occurred and more energy was required to achieve that peak.

3.4. Antimicrobial activity

The zone of inhibition and comparative antimicrobial activity of chitosan–zinc complex and O,N-carboxymethyl chitosan–zinc against *S. aureus* complex is given in Fig. 6. The zone of inhibition and comparative antimicrobial activity of chitosan–zinc complex and O,N-carboxymethyl chitosan–zinc complex against *E. coli* is given in Fig. 7.

O,N-carboxymethyl chitosan–zinc complex exhibited greater *in vitro* antimicrobial activity compared to chitosan–zinc complex against *S. aureus* and *E. coli*. The possible explanation for the enhanced antimicrobial activity of the O,N-carboxymethyl chitosan–zinc complex could be the enhanced solubility of O,N-

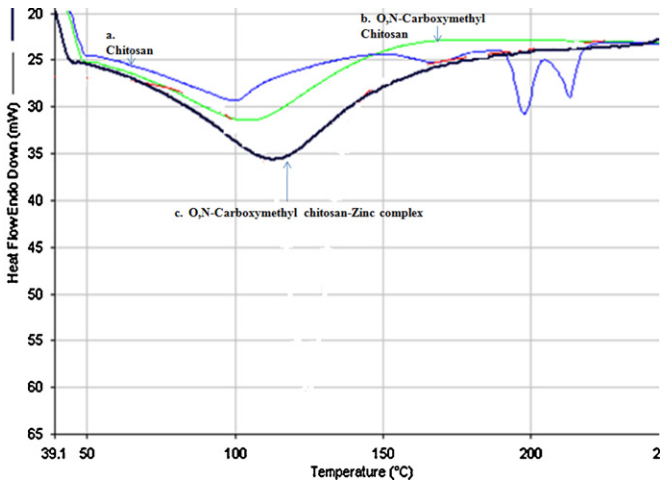

Fig. 5. DSC thermogram of (a) chitosan, (b) O,N-carboxymethyl chitosan and (c) O,N-carboxymethyl chitosan–zinc complex.

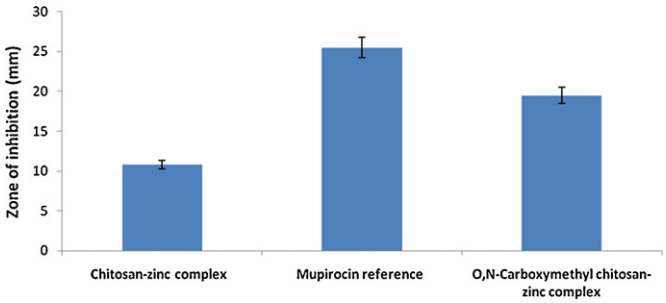

Fig. 6. Comparative antimicrobial activity of O,N-carboxymethyl chitosan–zinc complex and chitosan–zinc complex against *Staphylococcus aureus*.

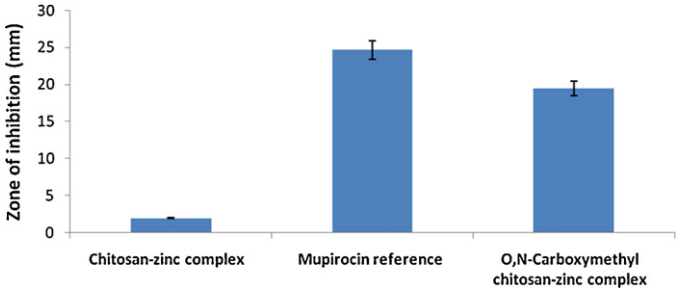

Fig. 7. Comparative antimicrobial activity of O,N-carboxymethyl chitosan–zinc complex and chitosan–zinc complex against *Escherichia coli*.

carboxymethyl chitosan–zinc complex in the aqueous solution. Therefore, higher diffusion may have occurred and higher concentration was available at the site action. Whereas, chitosan–zinc complex was present in the suspension form and was insoluble in water and sparingly soluble in 0.3% HCL, so its diffusion was very less resulting in low antimicrobial activity.

Although, the exact mechanism of the antimicrobial action of chitosan derivatives is still unknown, researchers have proposed putative mechanisms. The prominent being the positive charge of the group at C-2 results in a polycationic structure which is expected to interact with the predominantly anionic components (lipopolysaccharides, proteins) of the microorganism's surface. This interaction results in great alteration of the structure of outer membrane causing release of major proportion of proteinaceous material from the cells (Helander, Lassila, Ahvenainen, Rhoades, & Roller, 2001). Further, it is believed that when carboxymethyl group is introduced along the molecular chain, the presence of molecular chains with hydrophilic ends and forming weak interaction between hydrophilic ends and chitosan enhances the antimicrobial activity (Du, Sun, Fan, Chen, & Yang, 2006). Additonally, chitosan derivatives with low molecular weight can enter into the intracellular parts of the cells, combine with DNA, restrain mRNA from combining with protein, and as a result destroy the transcribe of DNA (Sudharshan, Hoover, & Knorr, 1992).

Moreover, as reported by Du et al. (2006), when chitosan is chelated with zinc ions, the positive charge on the amino group of carboxymethyl chitosan is greatly potentiated contributing to the easier complexation with anionic components of cell surface, and exhibited higher inhibitory activities.

Considering the aforesaid possible theories, it can be concluded that developed novel O,N-carboxymethyl chitosan–zinc complex could be easily touted as an effective antimicrobial polymer having multifaceted mechanism of action.

4. Conclusion

A novel O,N-carboxymethyl chitosan–zinc complex was successfully prepared and characterized by FT-IR, DSC and XRD. The effect of temperature and zinc ion concentration on the yield of the complex was studied and it was observed that these two factors significantly affected the yield of the complex. The comparative antimicrobial activity evaluation of O,N-carboxymethyl chitosan–zinc complex and chitosan–zinc complex was carried out against *S. aureus* and *E. coli*. It was observed that the developed novel O,N-carboxymethyl chitosan–zinc complex exhibited superior antimicrobial activity than chitosan–zinc complex.

Acknowledgements

Authors are thankful to Healers Neutraceuticals Pvt. Ltd. for gift sample of Chitosan and Glenmark Pharmaceuticals Pvt. Ltd. for the gift sample of Mupirocin. University Grant Commission (UGC) for awarding junior research fellowship and contingency.

References

Aijun, W., Qiang, A., Wenling, C., Chang, Z., Yandao, G., Nanming, Z., et al. (2005). Fiber-based chitosan tubular scaffolds for soft tissue engineering: fabrication and *in vitro* evaluation. *Tsinghua Science Technology*, *10*(4), 449–453.

David, N., Hon, S., & Tang, L. G. (2000). Chelation of chitosan derivatives with zinc ions. I. O,N-carboxymethyl chitosan. *Journal of Applied Polymer Science*, *77*, 2246–2253.

Du, Y., Sun, L., Fan, L., Chen, X., & Yang, J. (2006). Preparation, characterization and antimicrobial activity of quaternized carboxymethyl chitosan and application as pulp-cap. *Polymers*, *46*, 1796–1804.

Gana, Q., Wang, T., Cochrane, C., & McCarron, P. (2005). Modulation of surface charge, particle size and morphological properties of chitosan–TPP nanoparticles intended for gene delivery. *Colloids Surfaces B*, *44*, 65–73.

Gingras, M., Paradis, I., & Berthod, F. (2003). Nerve regeneration in a collagen chitosan tissue-engineered skin transplanted on nude mice. *Biomaterials*, *24*, 1653–1661.

Hayes (1986). N,O-carboxymethyl chitosan and preparative method therefor. *US Patent Office*, Patent. No. 4619995.

Helander, I. M., Lassila, N., Ahvenainen, R., Rhoades, J., & Roller, S. (2001). Chitosan disrupts the barrier properties of the outer membrane of gram-negative bacteria. *International Journal of Food Microbiology*, *71*, 235–244.

Hiroshi, U., Fumio, N., Masaaki, M., Masahiro, O., Tsuyoshi, K., & Toru, F. (2001). Evaluation effect of chitosan for the extracellular matrix production by fibroblast and the growth factors production by macrophages. *Biomaterials*, *22*, 2125–2130.

Juang, R. S., Wu, F. C., & Tsang, R. L. (1999). Adsorption removal of copper (II) using chitosan from simulated rinse solutions containing chelating agents. *Water Research*, *33*(10), 2403–2409.

Nishimura, K., Nishimura, S., Nishi, N., Saiki, I., Tokura, S., & Azuma, I. (1984). Immunological activity of chitin and its derivatives. *Vaccine*, *2*(1), 93–99.

Ormrod, D. J., Holmes, C. C., & Miller, T. E. (1998). Dietary chitosan inhibits hypercholesterolemia and atherogenesis in the apolipoprotein E-deficient mouse model of atherosclerosis. *Atherosclerosis*, *138*, 329–334.

Riccardo, A. A., Muzzarelli, Weckx, M., & Filippini, O. (1989). Removal of trace metal ions from industrial waters, nuclear effluents and drinking water, with the aid of cross-linked N-carboxymethyl chitosan. *Carbohydrate Polymers*, *11*(*4*), 293–306.

Senel, S., & McClure, S. J. (2004). Potential applications of chitosan in veterinary medicine. *Advanced Drug Delivery Reviews*, *56*, 1467–1480.

Stone, C. A., Wright, H., Clarke, T., Powell, R., & Devaraj, V. S. (2000). Healing at skin graft donor sites dressed with chitosan. *British Journal of Plastic Surgery*, *53*, 601–606.

Sudharshan, N. R., Hoover, D. G., & Knorr, D. (1992). Antibacterial action of chitosan. *Food Biotechnology*, *6*, 257–272.

Udhaybhaskar, P., Iyengar, L., & Rao, P. (1990). Hexavalent chromium interaction with chitosan. *Journal of Applied Polymer Science*, *39*(*3*), 739–747.

Ueno, H., Mori, T., & Fujinaga, T. (2001). Topical formulations and wound healing applications of chitosan. *Advanced Drug Delivery Reviews*, *52*, 105–115.

Ueno, H., Yamada, H., Tanaka, I., Kaba, N., Matsuura, M., Okumura, M., et al. (1999). Accelerating effects of chitosan for healing at early phase of experimental open wound in dogs. *Biomaterials*, *20*, 1407–1414.

Wang, X., Du, Y., & Liu, H. (2004). Preparation, characterization and antimicrobial activity of chitosan–Zn complex. *Carbohydrate Polymers*, *56*, 21–26.

Yuan, Y., Zhang, P., Yang, Y., Wang, X., & Gu, X. (2004). The interaction of Schwann cells with chitosan membranes and fibers *in vitro*. *Biomaterials*, *25*, 4273–4278.