Available online at www.sciencedirect.com
ScienceDirect
Carbohydrate Polymers 67 (2007) 40–45
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Synthesis, biodegradability and cytotoxicity of water-soluble isobutylchitosan

Dong-Hong Li *, Liang-Ming Liu, Kun-Lun Tian, Jian-Chang Liu, Xiao-Qing Fan

State Key Laboratory of Trauma, Burn and Combined Injury, The 2nd Department of Research Institute of Surgery, The Military Medical University, Chongqing 400042, China

Received 15 February 2006; received in revised form 14 March 2006; accepted 10 April 2006
Available online 12 June 2006

Abstract

The objective of the present study was to improve the solubility of chitosan at neutral aqueous solution without altering its bioactivity. Through an alkylation reaction under mild conditions, isobutyl was introduced to chitosan to form isobutylchitosan, which showed a good solubility in neutral aqueous media resulting from the reduced crystallinity of the product. The structure of isobutylchitosan was characterized by IR, ^{1}H NMR spectroscopy, and X-ray diffraction. The degradation of the product was evaluated in vivo using a mouse model, and the cytotoxicity of the product in L929 mouse fibroblasts was monitored using the MTT assay and the release of the cytosolic enzyme lactate dehydrogenase (LDH). It was found that the product exhibited the properties of improved biodegradability, increased MTT values and decreased LDH release. The results suggested that isobutylchitosan might be one of promising and safe biomaterial with potential applications.

Keywords: Isobutylchitosan; Water solubility; Biodegradability; Cytotoxicity

1. Introduction

Chitosan is obtained by extensive deacetylation of chitin, a major structural component of the fungal cell wall and of the exoskeletons of invertebrates, including insects and crustaceans (Jang, Kong, Jeong, Lee, & Nah, 2004). As a natural renewable resource, chitosan has a number of unique properties such as biocompatibility, biodegradability, non-toxicity and excellent film-forming ability, and has been widely used in the fields of medicine, pharmaceuticals, agriculture, functional food, environmental protection and biotechnology for several decades (Kurita, 1998). However, the poor solubility greatly limited the utilization of chitosan, due to the fact that it is insoluble in neutral aqueous solution and many organic solvents except for some acid aqueous solutions. Thus, many attentions have been paid to improve its solubility especially in neutral aqueous media (Hirano, Yamaguchi, & Kamiya, 2002; Chung, Kuo, & Chen, 2005).

Up to now, two kinds of strategies have been developed for the improvement of solubility of chitosan. One was the depolymerization of chitosan, which was mainly achieved by physical, acid-hydrolysis and enzyme methods. Comparatively, physical methods were simple, but the random reactions usually resulted in the variability and unstable solubility of products (Kurita, Ikda, Yoshida, Shimojoh, & Harata, 2002). The acid hydrolysis decomposed chitosan into units of six *N*-acetylglucosamines (Hirano, Konda, & Fuji, 1985), which lost almost all biological and/or chemical activity due to the too low molecular weight of the derivative (Liu, Guan, Yang, Li, & Yao, 2001). Some enzymes such as chitosanase, lysozyme, and papain (Ikeda et al., 1993; Nordtveit, Varum, & Smidstrod, 1996; Terbojevich, Cosani, & Muzzarelli, 1996) could reduce the molecular weight of chitosan and gave the products with higher solubility, but these methods were too expensive. The other was the so-called chemical modification of

* Corresponding author. Tel.: +86 23 68757432; fax: +86 23 68757452.
E-mail address: lqs_cq@yahoo.com.cn (D.-H. Li).

0144-8617/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2006.04.022

chitosan. The direct chemical modification was to introduce some hydrophilic functional groups such as carboxymethyl and sulphonyl etc, to the chitosan to obtain some derivatives with good solubility (Dung, Milas, Rinaudo, & Desbrieres, 1994; Muzzarelli, 1992; Watanabe, Saiki, Matsumoto, & Azuma, 1992). The indirect chemical modification method was to introduce some defects to the crystalline structure of chitosan (Kenji & Yoshito, 1997). Because the poor solubility of chitosan could be, to a large degree, attributed to its strong crystalline structure, introductions of some hydrophobic groups to its chains were found to be an effective way to reduce its crystallinity, thus resulting in the improvement of its solubility.

In this paper, we reported a new approach to the improvement of the solubility of chitosan in the neutral aqueous media by a mild and convenient alkylation reaction, on the basis of which the bioactivities of the product such as the biodegradability and the cytotoxicity were investigated.

2. Materials and methods

2.1. Synthesis of isobutylchitosan

In a typical synthesis, two grams of the chitosan powder (85% deacetylated, Zhejiang, China) was alkalized at room temperature for 80 min using 8 g of sodium hydroxide in 40 mL of isopropanol. After the alkalization process, the temperature of the mixture was raised to a range of 45–85 °C, 6 mL of 2-bromobutane was then added drop-wise to the mixture with stirring. The suspension was further stirred at the same temperature for 3–5 h, the slurry was then neutralized with 15% hydrochloric acid. After that, a precipitate formed with the addition of acetone, and it was filtered and washed with 85% methanol till there was no Br^- in the filtrate. After being washed with absolute ethanol twice, the precipitate was dried under reduced pressure to afford a yellowish powder.

2.2. Characterization of isobutylchitosan

Infrared spectra were recorded in a KBr disk using a Nicolet 5DXC FT-IR (Perkin-Elmer Co., USA) spectrometer. The ^1H NMR spectrum was recorded on an INOVA-600 spectrometer (Varian Inc., America) at 600 MHz at 25 °C, using D_2O as the solvent. X-ray diffraction patterns were recorded on a Shimadzu XD-3A powder diffractometer (Japan). The viscosities of isobutylchitosan were determined by the Ubbelohde type viscometer at 25 ± 1 °C in an aqueous solution containing 0.1 M CH_3COOH and 0.2 M CH_3COONa. The viscosity-average molecular weights were determined according to literature (Nishimura, Nishi, & Tlkure, 1986). The degrees of substitution (DS) were evaluated similar to that in literature (Kim & Lee, 1993). Briefly, 50 mg of the product was added to 0.3 M of hydrochloride solution under stirring till it dissolved completely. The average number of substitutions per pyranose unit was calculated by titrating potentiometrically with 0.1 M of sodium hydroxide aqueous solution. To determine the degree of deacetylation (DD) of isobutylchitosan, 20 mg of the product was dissolved in 10 mL of 0.1 M acetic acid aqueous solution, and the solution was then quantitatively transferred into a 50-mL volumetric flask, and made up to 50 mL with deionized water. After that, 5 mL of the solution was withdrawn, followed by addition of one drop of 1% toluidine blue used as indicator. Potassium polyvinyl sulfate solution was then successively added to the solution till the titration end point was reached Chung et al., 2005.

2.3. Preparation of film of chitosan and isobutylchitosan

The film of chitosan was prepared according to the method reported by Mochizuki, Sato, Ogawara, and Yamashita (1989) by casting 1.5% (w/v) chitosan aqueous solution containing 1% acetate acid on a Petri dish and drying at room temperature. The film was immersed in an aqueous solution containing 3% NaOH and 50% ethanol to neutralize the acid, and was then washed several times with distilled water. The thickness of the dried film was approximately 40–60μm.

The film of isobutylchitosan was easily prepared by dissolving it in distilled water to afford a 1.5% (w/v) aqueous solution, which was poured on a Petri dish and dried at room temperature. The thickness of the dried film could be controlled in the range of 40–60μm.

2.4. In vivo degradation

The films of both chitosan and isobutylchitosan were cut into the size of 5 mm × 5 mm, dried to constant weight, and then sterilized with ultraviolet radiation prior to implantation. A pouch was made in the thigh muscle of mice; the films soaked in sterile phosphate-buffered saline (PBS, pH 7.4) were inserted into the pouch. The pouch and the skin incision were closed with silk suture. After 1, 2, 4 and 6 weeks, the mice were killed in batches, and the films were retrieved, rinsed with distilled water, dried to constant weight. The extent of in vivo degradation was calculated by $(W_0–W_1)/W_0 \times 100\%$, where W_0 and W_1 were the weights of the films before and after implanting, respectively.

2.5. MTT assay

Extracts of chitosan and isobutylchitosan films were obtained by immersing the fragments of the films into the RPMI-1640 culture medium (Hyclone, Logan, Utah, USA) at a concentration of 0.2 g/mL at room temperature for 24 h without shaking. After being filtered with a 0.22 μm sterile membrane, fetal calf serum (Hyclone, Logan, Utah, USA) was added to the filtrate at a proportion of 10%. All extracts must be used within 24 h.

The L929 cells cultured for two days were digested by 0.25% trypsinase (Sigma) and then transferred to the wells

of 96-well culture plates at a quantity of 1×10^6 cells/well. After the cells were cultured for 24 h at 37 °C, the culture medium was removed, and 100 μL of the medium with different concentration of extract was added to the wells. Totally, there were five groups, two with the extracts of chitosan at the concentration of 0.2 g/mL and 0.1 g/mL, respectively, and the other two with the extracts of isobutylchitosan at the concentration of 0.2 g/mL and 0.1 g/mL, respectively. One incubated with culture medium without extract used as control group. After 2, 4 and 7 days, the medium was aspirated and 20 μL sterile filtered (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT, Sigma) stock solution in phosphate-buffered saline pH 7.4 was added to each well. After 6 h of incubation, unreacted dye was removed by aspiration, and 150 μL of dimethylsulfoxide (Sigma) was added to each well and vibrated for 10 min. The absorbance of the sample at 490 nm was determined in a 450 micro-plate reader (BIO-RAD, USA). The spectrophotometer was calibrated to zero absorbance with culture medium without cells. The relative cell viability (%) related to control was calculated by [A]test/[A]control × 100%. For each concentration triplet of determination was run.

2.6. LDH assay

A total of 5×10^4 L929 cells/well were seeded in 12-well cell culture plates. After 24 h incubation, cells were washed with phosphate-buffered solution and were then incubated with the extracts of chitosan and isobutylchitosan in different concentration (0.2 and 0.1 g/mL), respectively. One hundred microliter per well samples were collected at predetermined points and centrifuged at 1500 rpm for 10 min. The LDH content in these supernatants was assayed by utilizing a commercial kit according to the manufacturer's protocol. Control experiments were performed with 0.1%(w/v) Triton X-100 and set as 100% cytotoxicity. LDH release was calculated by the following equation (Shirui et al., 2005)

$$\mathrm{LDH}(\%) = \frac{[\mathrm{A}]\mathrm{sample} - [\mathrm{A}]\mathrm{medium}}{[\mathrm{A}]_{100\%} - [\mathrm{A}]\mathrm{medium}} \times 100\%,$$

where [A]sample, [A]medium, $[\mathrm{A}]_{100\%}$ denoted the absorbance of the sample, medium control and Triton X-100 control, respectively. All experiments were run in triplicate.

3. Results and discussion

Chitosan has both reactive amino and hydroxyl groups that can be used to chemically alter its properties under mild reaction conditions. Fig. 1 showed the FTIR spectra of chitosan and isobutylchitosan. Both spectra exhibited the absorption peaks at 1155, 1097, 1019, and 892 cm^{-1}, which could be assigned to the saccharide moiety. When an alkyl chain attached to chitosan, new peaks at 1425 (-CH_2, vs) and 662 cm^{-1} (CH_2 rocking in the methylene chain) appeared, and the signal intensity of peak at 2886 cm^{-1} increased. In addition, the characteristic absorption peak at 1524 cm^{-1} almost disappeared, representing a decrease of $-NH_2$ group content, which implied that the substitution took place mainly on the N atoms under this experimental condition. The ^{1}H NMR spectrum of isobutylchitosan was shown in Fig. 2. Compared to the ^{1}H NMR spectrum of chitosan (Lee, Hong, Hajiuchi, & Yang, 2005), in the spectrum of isobutylchitosan, except the inherent signals of chitosan at 2.04 (*N*-acetyl), 3.0–4.0 (CH), 5.0 ppm (OH), the important signal of the protons of CH_3 at 1.1 ppm confirmed the introduction of the isobutyl group into chitosan. Fig. 3 showed the X-ray powder diffraction patterns of chitosan and isobutylchitosan. The pattern of chitosan showed the characteristic peak at $2\theta = 10°$ due to the presence of (001) and (100) and that at $2\theta = 20°$ caused by the presence of (101) and (002) (Kim & Lee, 1993). For isobutylchitosan, the peak at $2\theta = 10°$ disappeared, and most importantly, the characteristic peak at $2\theta = 20°$ was much wider and weaker than that of chitosan. This result indicated that isobutylchitosan was of poor crystallinity compared to chitosan. The poor crystallinity of isobutylchitosan was attributed to the deformation of the strong hydrogen bond in the chitosan

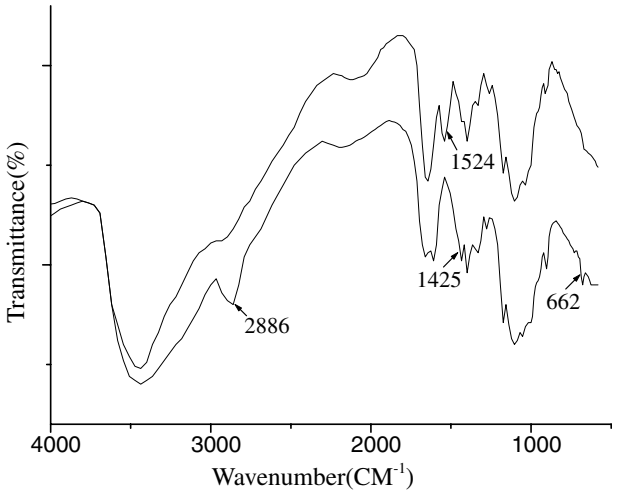

Fig. 1. FTIR spectra of chitosan (up) and isobutylchitosan (down).

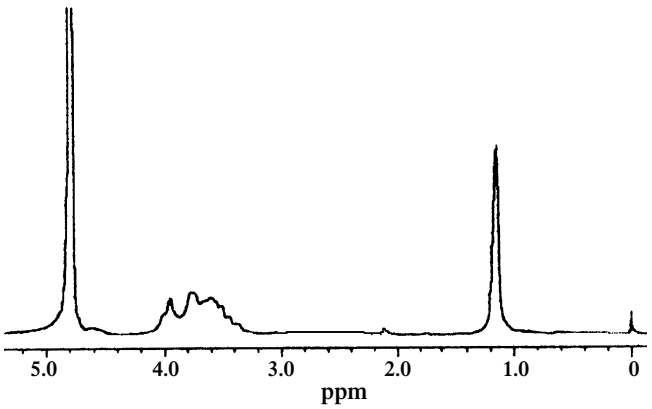

Fig. 2. ^{1}H NMR spectrum of isobutylchitosan in D_2O at 25 °C.

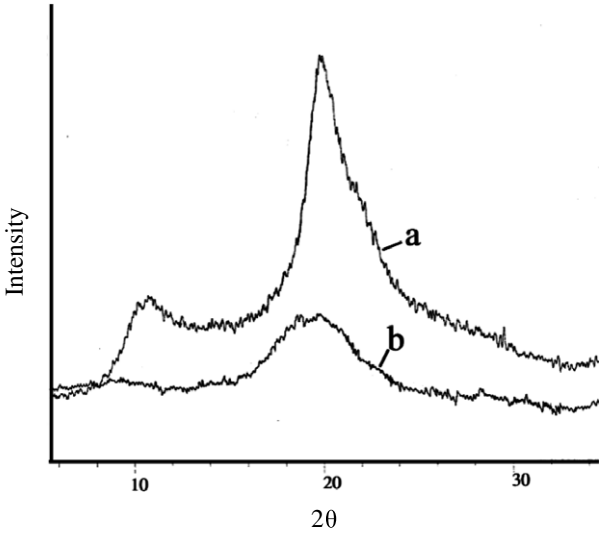

Fig. 3. X-ray powder diffraction patterns of (a) chitosan, (b) isobutylchitosan.

backbone with the substitution of isobutyl group on the N and O atoms of chitosan.

The degree of deacetylation of chitosan typically affects its physical, chemical and even biological properties or activities (Chung, Wang, Chen, & Li, 2003). Hence, it is necessary to determine the degree of deacetylation of chitosan derivatives. Table 1 showed the effects of reaction temperature and time on the degree of deacetylation, degree of substitution, viscosity-average molecular weight and viscosity of isobutylchitosan. As the reaction temperature and time increased, both the degree of deacetylation and degree of substitution increased, while both the viscosity average molecular weights and viscosity decreased. In addition, it was found that the amount and the addition manner of 2-bromobutane affected the DD and DS of isobutylchitosan as well (the results didn't show in the Table 1). The addition drop-wise and a higher concentration of 2-bromobutane made for increasing of DD and DS of derivatives. These results demonstrated that the DD and DS of the products were strongly controlled by various factors such as reaction temperature, time and reactant, etc. Since colloid titration was used to determine the numbers of free amine groups, compared to the high value of degree of deacetylation of chitosan (85%), the lower values of deacetylation of isobutylchitosan indicated that the isobutyl group mainly substituted on the $-NH_2$ of chitosan. Further, the low M_η value of isobutylchitosan, compared to that of unmodified chitosan, implied that the chitosan chain degraded during alkalization and alkylation.

Table 1
The effects of temperature, reaction time on the properties of isobutylchitosan

Reaction conditions		DD (%)	DS (%)	M_η ($\times 10^{-4}$)	Viscosity (mL/g)
Temperature(°C)	Time(h)				
45	4	33.2	41.6	22.1	372
55	4	38.1	45.4	16.7	281
65	4	47.8	55.8	11.6	195
75	4	50.2	59.1	7.4	125
85	4	56.5	64.6	5.9	106
65	3	42.5	50.9	13.1	221
65	5	51.2	61.5	6.8	115
65	6	53.8	65.2	6.5	104

The biodegradability is one of the most important properties of biomaterials. The biodegradation can reduce the accumulation of the implanted foreign materials in the body, and result in a lighter foreign-body reaction during their contact with a living structure. The biodegradation of the films of chitosan and isobutylchitosan implanted in the thigh muscle of mice were shown in Fig. 4. Compared to chitosan, isobutylchitosan was readily degraded and cleared by mouse bodies. At the first week, chitosan just was degraded 3.47%, while isobutylchitosan was degraded up to 27.37%. After six weeks, the degradation percent of the films of chitosan and isobutylchitosan were 24.21% and 65.31%, respectively. The better biodegradability of isobutylchitosan maybe attributed to the decrease of the molecular weight of isobutylchitosan and the deformation of the strong hydrogen bond in the chitosan backbone. On other hand, as described by Kenji and Yoshito (1997), the chitosan, which was free of acetamide groups couldn't be catalyzed hydrolysis in vivo, on the contrary, the chitin and its 69% deacetylation derivative were readily degraded in vivo. The results implied that the biodegradability of chitosan was related to the amount of the amino group in the molecules of chitosan. Therefore, in our experiment, with the substitution of amino group by isobutyl group, the amount of the amino group in the molecules of chitosan decreased, which resulted the increase of the rate of degradation of isobutylchitosan.

Generally, the in vitro cytotoxicity of biomaterials was evaluated by determining the cell viability. MTT and LDH assays were two methods commonly used for this purpose. The MTT assay was selected to determine detrimental intracellular effects on mitochondria and metabolic activity. The colorimetric MTT test, based on the selective ability of viable cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide into purple formazan relied on intact metabolic activity and was frequently used for screening of cytotoxicity (Mosmann, 1983). By contrast, LDH test provided the information of the effect of biomaterial after short incubation times, and reflectd the damage/leakage of plasma membranes. It had been found that the changes in metabolic activity were superior indicators of early cell injury, and effected on membrane integrity were indicative of more serious damage, leading to cell death. Fig. 5 showed the cytotoxicity of chitosan and isobutylchitosan by MTT assay. It could be seen that both the relative cell viability obtained from chitosan and isobutylchitosan were higher than that obtained from the control. Fig. 6 showed that all values of LDH release were lower than zero, regardless of the concentration of chitosan and isobutylchitosan, which meaning both chitosan and isobutylchitosan

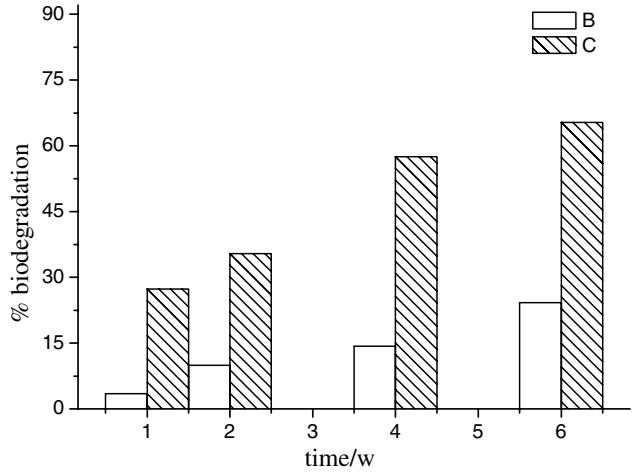

Fig. 4. Biodegradation ability of chitosan (B) and isobutylcitosan (C) in mice.

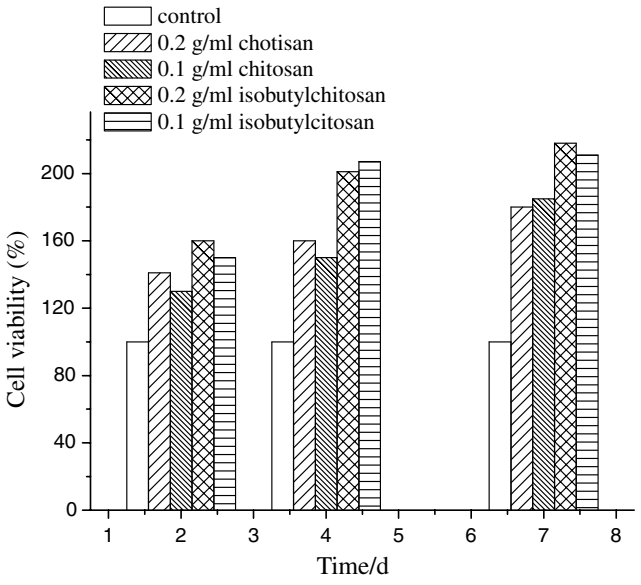

Fig. 5. Cytotoxicity of the chitosan and isobutylchitosan by MTT assay. Each point represents the mean ± SD of three experiments.

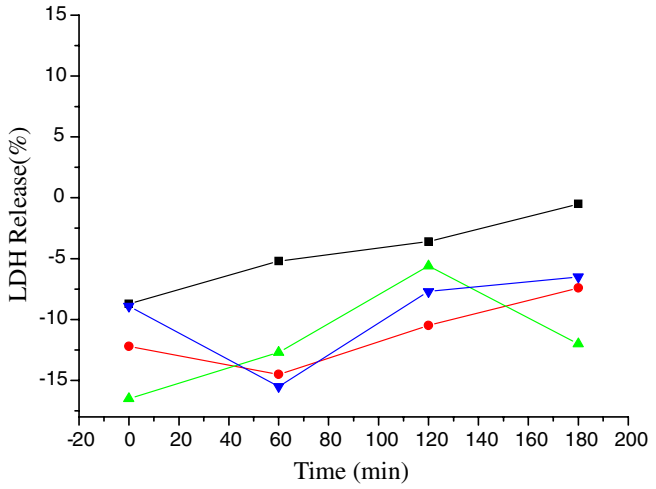

Fig. 6. Effect of the chitosan and isobutylchitosan on LDH release. Each point represents the mean ± SD of three experiments. (■)0.1 g/ml chitosan, (●)0.2 g/ml chitosan, (▲)0.1 g/ml isobutylchitosan, (▼)0.2 g/ml isobutylchitosan.

did not induce but inhibited LDH release. The results indicated that both chitosan and isobutylchitosan stimulated the cells proliferation. The higher cell viability of isobutylchitosan was due to its higher virtual concentration in the culture medium, resulting from its better solubility in an aqueous neutral medium. Our results were similar to those reported by Prasitsilp's group (2000) and Graeme's group (2001), but different from that reported by Mori et al. (1997). As described by Prasitsilp et al., a high degree of deacetylation in chitosan was more favorable for supporting cell growth, proliferation and attachment, which could be attributed to the electropositive nature of the amino group permitting interactions between chitosan and cells. However, as reported by Mori et al., chitosan appeared to, exhibit an inhibitory effect for the cells growth due to the interaction of chitosan with growth factors thereby immobilizing the growth factors. Therefore, the effect of chitosan and its derivatives on cell proliferation is also governed by factors other than the content of amino group in the molecules. This requires further studies.

4. Conclusion

The isobutylchitosan could be synthesized though an alkylation reaction under basic condition. The reaction temperature, time and the concentration of reactants were found to be crucial factors that affected on the DD, DS, M_η and viscosity of isobutylchitosan. As the reaction temperature and time increased, both the degree of deacetylation and degree of substitution increased, while both the viscosity average molecular weights and viscosity decreased. The improved solubility of isobutylchitosan in neutral aqueous solution could be attributed to the reduction of crystallinity of chitosan as the introduction of isobutyl group on the N and O atoms of chitosan. Compared to chitosan, isobutylchitosan had a better biodegradability and the same non-cytotoxicity. The results suggested that the water-soluble isobutylchitosan might be a promising and safe biomaterial, which can be used in many fields.

Acknowledgement

The authors thank to the Chongqing Science and Technology Council, for the partial financial support to this work.

References

Chung, Y. C., Kuo, C. L., & Chen, C. C. (2005). Preparation and important functional properties of water-soluble chitosan produced through Maillard reaction. *Bioresource Technology, 96*, 1473–1482.

Chung, Y. C., Wang, H. L., Chen, Y. M., & Li, S. L. (2003). Effect of abiotic factors on the antibacterial activity of chitosan against waterborne pathogens. *Bioresource Technology, 88*(3), 179–184.

Dung, P. I., Milas, M., Rinaudo, M., & Desbrieres, J. (1994). Water soluble derivatives obtained by controlled chemical modifications of chitosan. *Carbohydrate Polymer, 24*(3), 209–215.

Graeme, I. H., Peter, W. D., Paul, A. G., Frank, C. H., Michael, D., & Edward, J. W. (2001). The effect of chitin and chitosan on the proliferation of human skin fibroblasts and keratinocytes in vitro. *Biomaterials, 22*, 2959–2966.

Hirano, S., Konda, Y., & Fuji, K. (1985). Preparation of acethlated derivatives of modified chito-oligosaccharides by the depolymerization of partially *N*-acetylated chitosan with nitrous acid. *Carbohydrate Research, 144*(2), 338–346.

Hirano, S., Yamaguchi, Y., & Kamiya, M. (2002). Novel *N*-saturated-fatty-acyl derivatives of chitosan soluble in water and in aqueous acid and alkaline solutions. *Carbohydrate Polymer, 48*(2), 203–207.

Ikeda, I., Sugano, M., Yoshida, K., Sasaki, E., Iwamoto, Y., & Hatano, K. (1993). Effects of chitosan hydrolysates on lipid absorption and on serum and liver lipid concentration in rats. *Journal of Agriculture Food Chemistry, 41*(2), 431–439.

Jang, M. K., Kong, B. G., Jeong, Y., Lee, C. H., & Nah, J. W. (2004). Physicochemical characterization of α-chitin, β-chitin, and γ-chitin separated from natural resources. *Journal of Polymer Science Part A, 42*(14), 3423–3432.

Kenji, T., & Yoshito, I. (1997). In vitro and in vivo degradation of films chitin and its deacetylated derivatives. *Biomaterials, 18*(7), 567–575.

Kim, J. H., & Lee, Y. M. (1993). Synthesis and preparation of diethylaminoethyl chitosan. *Polymer, 34*(9), 1952–1957.

Kurita, K. (1998). Chemistry and application of chitin and chitosan. *Polymer Degradation Stability, 59*(2), 117–120.

Kurita, K., Ikda, H., Yoshida, Y., Shimojoh, M., & Harata, M. (2002). Chemoselective protection of amino groups of chitosan by controlled phthaloylation: facile preparation of a precursor useful for chemical modifications. *Biomacromolecules, 3*(1), 1–4.

Lee, M. Y., Hong, K. J., Hajiuchi, T., & Yang, J. W. (2005). Synthesis of chitosan-based polymeric surfactants and their adsorption properties for heavy metals and fatty acids. *International Journal of Biological Macromolecules, 36*, 152–158.

Liu, X. F., Guan, Y. L., Yang, D. Z., Li, Z., & Yao, K. D. (2001). Antibacterial action of chitosan and carboxymethylated chitosan. *Journal of Applied Polymer Science, 79*(7), 1324–1335.

Mochizuki, A., Sato, Y., Ogawara, H., & Yamashita, S. (1989). Pervaporation separation of water/ethanol mixtures through polysaccharide membranes, II. The permselectivity of chitosan membrane. *Journal of Applied Polymer Science, 37*, 3375–3384.

Mori, T., Okumura, M., Mausuura, M., Ueno, K., Tokura, S., Okamoto, Y., Minami, S., & Fujinaga, T. (1997). Effects of chitin and its derivatives on the proliferation and cytokine production of fibroblasts in vitro. *Biomaterials, 18*, 947–951.

Mosmann, T. (1983). Rapid colorimetric assays for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods, 65*, 55–63.

Muzzarelli, R. A. A. (1992). Modified chitosan carrying sulfonic acid groups. *Carbohydrate Polymer, 5*(3), 461–475.

Nishimura, N. S., Nishi, N., & Tlkure, S. (1986). Bioactive chitin derivatives: activation of mouseperitoneal macrophages by O-(carboxymethyl)chitins. *Carbohydrate Research, 146*, 251–258.

Nordtveit, R. J., Varum, K. M., & Smidstrod, O. (1996). Degradation of partially N-acetylated chitosans with hen egg white and human lysozyme. *Carbohydrate polymer, 29*(2), 163–167.

Prasitilp, M., Jenwithisuk, R., Kongsuwan, K., Damrongchai, N., & Watts, P. (2000). Cellular response to chitosan in vitro: the importance of deacetylation. *Journal Material Science Material Medical, 11*, 773–778.

Shirui, M., Xintao, S., Florian, U., Matthias, W., Xiulan, X., & Thomas, K. (2005). Synthesis, characterization and cytotoxicity of poly(ethylene glycol)-graft-trimethyl chitosan block copolymers. *Biomaterials, 26*, 6343–6356.

Terbojevich, M., Cosani, A., & Muzzarelli, R. A. A. (1996). Molecular parameters of citosans depolymerized with the aid of papain. *Carbohydrate Polymer, 29*(1), 63–68.

Watanabe, K., Saiki, I., Matsumoto, Y., & Azuma, I. (1992). Abtimetastatic activity of neocarzinostatin incorporated into controlled release gels of CM-chitin. *Carbohydrate Polymer, 17*(1), 29–33 .

Dong-Hong Li, Female, 43 ages, Ph.D., Associate Professor. Graduated and got B.S degree from the Chemistry Department of Southwest Normal University in 1983, obtained M.S degree from Sichuan University in 1990, and obtained Ph.D. degree from Sichuan University in 1997. As a visiting scholar, worked in the group of professor V. V. Yam, the head of the Chemistry Department of Hongkong University from Oct. 2002 to Mar. 2005. From 1990, has been working in the Second Department of Research Institute of Surgery, the Third Military Medical University.