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Preparation of a series of chitooligomers and their effect on hepatocytes

Meng Tian [a], Feng Chen [a], Dawei Ren [a], Xixun Yu [a], Xiaohua Zhang [a], Rui Zhong [b], Changxiu Wan [a,*]

[a] Department of Biomedical Engineering, College of Polymer Science and Engineering, Sichuan University, Chengdu, Sichuan 610065, China
[b] Institute of Blood Transfusion, Chinese Academy of Medical Sciences, Chengdu, Sichuan 610081, China

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ABSTRACT

A series of chitooligomers with molecular weight ranging from 1.7 ~ 3.8 × 10^3 were prepared by degradation of a high molecular weight chitosan with hydrogen peroxide and selective precipitation in ethanol solutions. The prepared chitooligomers were characterized by gel permeation chromatography, elemental analysis, Fourier transform infrared spectra, ^{13}C nuclear magnetic resonance spectroscopy and X-ray diffraction analysis. Cell culture experiments suggested that the effect of the chitooligomers on the proliferation of L02 hepatocytes was dependent on culture time, namely, at the initial stage of culture there was an inhibitory effect on proliferation of the cells; however, the cultures recovered in cell proliferation and exhibited promotion effect in following days. In the case of chitosan monomer (GlcN), high concentration of GlcN (1 mg/ml) produced a significant suppression in proliferation of L02 cells relative to control, with decreases of 35.2%, 60% and 72.9% on days 1, 3 and 5, respectively. In addition, there was no significant effect of the chitooligomers on the functions of albumin secretion and urea synthesis of the hepatocytes.

1. Introduction

Chitooligomers, a class of chitosans with degree of polymerization <20, are known to have some special biological activities such as antibacterial activity (Jeon, Park, & Kim, 2001), antitumor and immune enhancing effects (Jeon & Kim, 2002; Qin, Du, Xiao, Li, & Gao, 2002; Tokoro et al., 1988). These functions depend not only on their chemical structure but also on the molecular size. In recent years, studies of the effect of chitooligomers on various cells, e.g. polymorphonuclear cells (Usami, Okamoto, Takayama, Shigemasa, & Minami, 1998), macrophages (Okamoto et al., 2003), fibroblasts (Mori et al., 1997), endothelium (Okamoto et al., 2002), osteoblasts (Ohara et al., 2004) and red blood cells (Fernandes et al., 2008), have attracted more and more interest. The study of behaves of polymorphonuclear cells (PMNs), macrophages, endothelium and fibroblasts treated with chitooligomers have been related with the mechanisms of induction of acceleration of wound healing by chitin and chitosan. It has been demonstrated chitooligomers induced chemotactic migration of PMNs and stimulated the induction of interleukin-8 of fibroblast (Mori et al., 1997; Usami et al., 1998). However, to our best knowledge, there is no report on the effect of chitooligomers on hepatocytes.

Chitooligomers can be obtained by enzymatic depolymerization of chitosan (Qin, Wang, Peng, Hu, & Li, 2008) or by chemical depolymerization with acids such as hydrochloric acid (Belamie, Domard, & Giraud-Guille, 1997), nitric acid (Tommeraas, Varum, Christensen, & Smidrod, 2001) and sulfuric acid (Nagasawa, Tohira, Inoue, & Tanoura, 1971). In general, enzymatic methods have advantages over chemical reaction, since enzymes operate under milder conditions and are highly specific; however, their commercial use is limited due to cost and limited availability (Li et al., 2005). In addition, chitooligomers can also be obtained by oxidative depolymerization with oxidants, such as ozone (Yue, He, Yao, & Wei, 2009), sodium nitrite (Allan & Peyron, 1995) and hydrogen peroxide (Chang, Tai, & Cheng, 2001; Qin, Du, & Xiao, 2002; Tian, Liu, Hu, & Zhao, 2004; Wang, Huang, & Wang, 2005). Hydrogen peroxide has long been used in the treatment of chitosan because it is easy to handle, easily available and environmentally friendly. In particular, for cell culture applications, the method does not cause impurities in products. However, it was reported the reaction occurred in a random pattern, resulting in a broad distribution of molecular weight of the chitooligomers (Qin, Du, & Xiao, 2002).

The goal of this work was to prepare a series of chitooligomers with different of molecular weights, and to explore preliminarily the effect of prepared chitooligomers on the hepatocytes. Here, we describe a quick and simple method for the production of a homogeneous series of chitooligomers, varying in molecular weight from 1.7 to 3.8 × 10^3, with low polydispersity. The prepared method involves a simple chemical process and only selective precipitations. Finally, cell proliferation, morphology and functions such as albumin secretion and urea synthesis were employed to determine the effect of the chitooligomers on the hepatocytes.

* Corresponding author. Tel.: +86 28 85405404; fax: +86 28 85405401.
E-mail addresses: wanchangxiu@163.com, tianmong007@gmail.com (C. Wan).

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2. Materials and methods

2.1. Materials

Original chitosan (CS0) with a degree of deacetylation (DD) of 91% was purchased from Zhejiang Aoxing Biotechnology Co., Ltd. (Zhejiang, China). Its viscosity average molecular weight is about 300,000. Chitosan monomer (D-glucosamine hydrochloride), penicillin and streptomycin were supplied by Solarbio (Beijing, China). RPMI-1640 was obtained from Gibco (USA). Fetal bovine serum (FBS) was purchased from HyClone (USA). Urea Nitrogen Kit and Albumin Detection Kit were from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other chemicals were of analytical grade and were used without further purification.

2.2. Preparation of a series of chitooligomers

CS0 (20 g) was completely dissolved in 800 ml 2% (w/v) acetic acid with a water bath at 70 °C, then 30 ml 30% (v/v) hydrogen peroxide was added to the solution. The resulting solution was stirred and reacted for 2 h. After the reaction, the solution was immersed in an ice bath and neutralized to pH 8.0 with concentrated NaOH to remove high molecular weight chitosan by precipitation. The filtered solution was precipitated with final ethanol concentrations of 50%, 75% and 87.5% (v/v). Each precipitate was centrifuged, washed with ethanol and vacuum dried. COS1, COS2 and COS3 are the products of selectively precipitation with final ethanol concentrations of 50%, 75% and 87.5% (v/v), respectively.

2.3. Characterization

Weight average molecular weight (M_w), number average molecular weight (M_n) and molecular weight dispersion (M_w/M_n) were determined by a gel permeation chromatography (GPC) equipped with a Waters 515 HPLC pump, a Waters 2410 refractive index detector and a OHpak KB-803 HQ column operated at a flow rate of 0.7 ml/min. Each sample with a volume of 10 μl (5 mg/ml) was eluted with a solution of 0.5 M CH_3COOH/0.5 M CH_3COONa at 25 °C. Dextranum standards (National Institute for The Control of Pharmaceutical and Biological Products, China) were used for column calibration and as a relative reference for molecular weight calculation. All data were collected and analyzed using the Empower software.

The elemental analyses were performed with a Vario EL III elemental analyzer. The DD of the sample was calculated by the following equation (Tian et al., 2004; Xu, McCarthy, & Gross, 1996):

$$\mathrm{DD} = \left(1 - \frac{\mathrm{C/N} - 5.14}{1.72}\right) \times 100\%$$

where C/N is the ratio (w/w) of carbon to nitrogen.

Fourier transform infrared spectra (FT-IR) were recorded with KBr pellets on a Nicolet 670 FT-IR Spectrometer.

^{13}C nuclear magnetic resonance spectroscopy (^{13}C NMR) analyses were recorded on a Bruker AV II-400 MHz spectrometer. The products were dissolved in D_2O in the presence of CF_3COOD.

X-ray diffraction (XRD) measurements were carried out on an X' Pert Pro MPD X-ray diffractometer (Philips, Netherlands) and used a Cu Kα target at 40 kV and 40 mA at 25 °C.

2.4. Preparation of chitooligomer solution

Each chitooligomers and chitosan monomer (D-glucosamine hydrochloride) were dissolved with PBS at a concentration of 10 mg/ml. Completely dissolved solution was neutralized to pH ~7.2, and then sterilized with a 0.2 μm filter.

2.5. Cell culture

Normal human hepatocytes, L02, obtained from Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) were used for cell assay. L02 hepatocytes were grown and maintained in RPMI-1640 medium supplemented with 10% FBS, 100 μg/ml penicillin and 100 μg/ml streptomycin at 37 °C in a 5% CO2 atmosphere, with medium changed every day until confluent cell monolayer was formed. The cells in their three passages were used in experiments.

2.6. Cell proliferation assay

L02 hepatocytes were adjusted to a concentration of 5×10^4 cells/ml in the culture medium before the experiment. The cells were seeded in 96 well plates at a density of 5×10^3 cells/well. RPMI-1640 containing 10% FBS, 100 μg/ml penicillin and 100 μg/ml streptomycin in supplement with 1 mg/ml chitooligomer was prepared using the 10 mg/ml chitooligomer solution. This mixture solution was diluted by 10-fold steps with above RPMI-1640 medium to prepare 0.1 and 0.01 mg/ml solutions. 1, 0.1 and 0.01 mg/ml chitosan monomer solutions were also prepared according to above method. After 2 days, the seeded cells were washed with PBS and then treated with different concentrations of chitooligomers or chitosan monomer. As a control, PBS was added to media in place of chitooligomers or chitosan monomer suspension.

The cell proliferation was analyzed by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) mitochondrial reduction. This assay is based on the ability of live cells to reduce a tetrazolium-based compound, MTT, to a purplish formazan product. After 1, 3 and 5 days of culture, the cells were observed under a phase contrast microscope (CKX41, Olympus, Japan) before adding 20 μl of MTT solution (5 mg/ml) to each well and the cells were incubated at 37 °C for 4 h. After removing the culture media, 150 μl of DMSO was added, and the plates were shaken for 10 min. The optical density (OD) of each well was determined using a microplate reader at a wavelength of 490 nm. The OD value is proportionate to the cell numbers.

2.7. Albumin synthesis determination

The L02 cells with a density of 5×10^4 cells/ml were seeded in 6 well plates in RPMI-1640 supplemented with 10% FBS, 100 μg/ml penicillin and 100 μg/ml streptomycin at 37 °C in a 5% CO2 atmosphere. After the cells formed confluent layers, the culture medium was discarded and replaced with the fresh medium containing chitooligomers or chitosan monomer with a concentration of 0.1 mg/ml. The medium was refreshed daily and the collected medium was centrifuged in 14,000 rpm for 10 min. The supernatant was stored at −20 °C for albumin assay. The concentration of albumin remaining in the supernatant was measured by the bromocresol-green method which based on an established method that bromocresol-green forms a coloured complex specifically with albumin (Doumas, Watson, & Biggs, 1971). Levels of albumin were determined using a commercially available kit (Albumin Detection Kit). Known quantities of human albumin were used to establish the standard curve.

2.8. Urea synthesis assay

To assess the urea synthesis function of the L02 cells treated with chitooligomers, the culture medium was replaced with fresh medium containing 5 mm NH_4Cl. The cells were cultured in this medium for 120 min, before the medium was again replaced with the normal medium. The collected medium was tested for urea production using a Urea Nitrogen Kit.

2.9. Statistical analysis

Statistical analysis was performed with SPSS (v12.0). Quantitative data were presented as means ± standard deviation (SD). A student's *t*-test was performed to determine the statistical significance between experimental groups. A value of $p < 0.05$ was considered to be statistically significant.

3. Results and discussions

3.1. Molecular weight, polydispersity and DD of the prepared chitooligomers

The preparation of chitooligomers by degradation reaction with hydrogen peroxide has long been studied. In acid solution, chitosan was dissolved and the degradation reaction was homogenous. During the degradation reaction in our experiment, the solution viscosity dropped sharply at the beginning of the reaction (0 ~ 10 min), then decreased slowly. After reaction 2 h, there was almost no water-insoluble chitosan precipitation in the reaction solution when the pH value of the solution was adjusted to 8.0 by addition of a concentrated aqueous NaOH. Therefore, we limited the reaction time to 2 h to obtain chitooligomers. However, the reaction occurred in a random pattern, resulting in a broad distribution of molecular weight of the product. To reduce the degree of polydispersity of the chitooligomer, a selective precipitation in ethanol solutions was used.

The M_w and polydispersity (M_w/M_n) obtained by GPC measurements and DD values calculated from elemental analysis are given in Table 1. The molecular weight of the chitooligomers is in the order of COS1 > COS2 > COS3 and all the three products have similar polydispersity (~1.4). By GPC analyses of the products, it can be seen that chitooligomers with M_w up to 3.8×10^3 were soluble in 50% ethanol solution and up to 2.7×10^3 only in 75% ethanol solution. In the 87.5% ethanol solution, the M_w of the soluble chitooligomer appeared to be lower than 1.7×10^3. The DD of the product was decreased with the decrease of the molecular weight, which coincided with the previous studies reported by Qin, Du, and Xiao (2002). The decrease of the DD, resulting from the decrease of the mass ratio of N/C, suggested the loss of nitrogen was occurred in the degradation. As described by Qin, Du, and Xiao (2002), when chitosan was treated with hydrogen peroxide, the scission of glycosidic bond was predominantly from H-abstraction at C-1 and C-2 that both led to the deamination. Furthermore, oxidative scission might be produce diverse termini, which may partially destroy the end residues of the chitooligomers. Therefore, "chitooligomers" in this work should be "partial oxided chitooligomers".

From these results, it can be seen that oxidative degradation of chitosan combined with a selective ethanol precipitation appears to be a quick and simple method to obtain chitooligomers with low polydispersity.

3.2. FT-IR spectral analysis

The FT-IR spectra of original chitosan and the chitooligomers were shown in Fig. 1. The characteristic absorptions of the original chitosan are similar to that of chitosans with high deacetylation degree reported previously (Brugnerotto et al., 2001; Tian et al., 2004). The absorption band at 3430 cm^{-1} is attributed to the N–H and O–H stretching vibrations. The peaks at 2877 and 2920 are assigned to C–H stretching vibrations, and the peak at 1650 is assigned to amide I. The bands at 1602 and 599 correspond to $-NH_2$ bending vibrations. The band at 1381 is corresponds to C–H bending and C–H stretching vibrations, and the band in the range 1155–898 are assigned to the characteristics of its polysaccharide structure.

Compared with the FT-IR spectrum of the original chitosan, the spectra of the chitooligomers exhibited most of the characteristic absorption bands of the original chitosan. However, some differences are as follows: the N–H and O–H stretching vibrations in the spectra of the chitooligomers moved toward lower wavenumbers (~3370), which indicates that the intermolecular and intramolecular hydrogen bonds of chitosan were weakened after degradation (Wang et al., 2005). The amide I band in the spectra of the chitooligomers were weakened, which was agreement with the results of the elemental analysis that the loss of nitrogen was occurred in the degradation reaction. Qin, Du, and Xiao (2002) studied the effect of hydrogen peroxide treatment on the molecular weight and structure of chitosan. They observed a new band at 1735 cm^{-1} when further degradation with hydrogen peroxide, which assigned to the carboxylic group. However, no absorption band at this wavenumber in the spectra of the chitooligomers was observed in our study, which might be due to the samples in the study of the above researchers were in the hydrochloric salts.

3.3. ^{13}C NMR analysis

The ^{13}C NMR spectrum of the original chitosan was shown in Fig. 2. According to previous reports (Qin et al., 2003; Saito, Mamizuka, Tabeta, & Hirano, 1981), the signals at 55.9, 60.1, 70.2, 74.9, 76.6 and 97.7 ppm assigned to C-2, C-6, C-3, C-5, C-4 and C-1, respectively. In comparison with the ^{13}C NMR spectrum of the original chitosan, the signals assigned to C-1 of the COS1, COS2 and COS3 shifted to 98.8, 100.1 and 101.1 ppm, respectively. Similarly, the signals of C-2, C-3 and C-4 of the chitooligomers were shifted to the downfield. However, the signals of C-5 and C-6 of the chitooligomers were not changed significantly in comparison with those of the original chitosan. (Fukamizo, Ohtakara, Mitsutomi, & Goto, 1991) analyzed the structure of two partially deacetylated chitotri-

Table 1
The M_w, polydispersity and DD values of the prepared chitooligomers.

Sample	M_w ($\times 10^{-3}$)	Polydispersity (M_w/M_n)	N/C	DD (%)
COS1	3.8	1.44	0.170	55.9
COS2	2.7	1.47	0.166	48.8
COS3	1.7	1.36	0.162	39.0

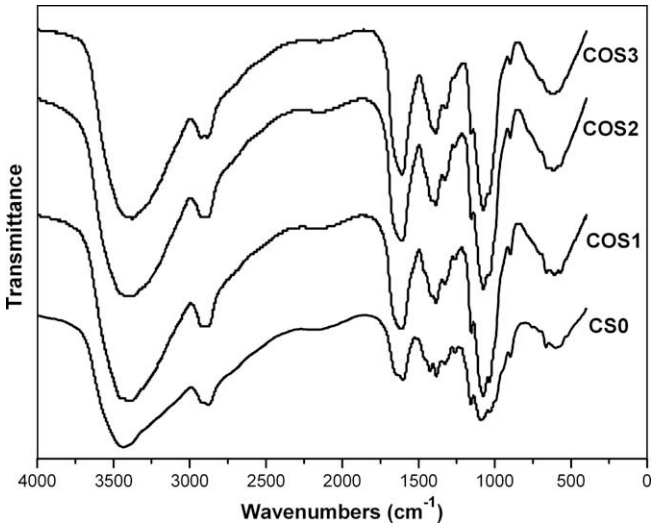

Fig. 1. FT-IR spectra of original chitosan and prepared chitooligomers.

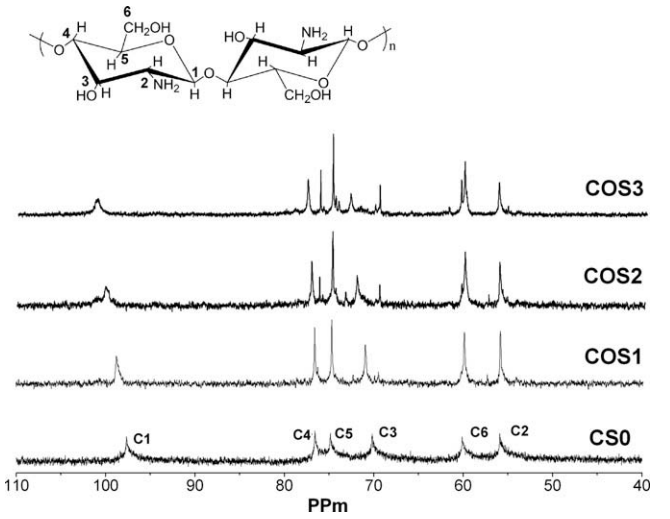

Fig. 2. ^{13}C NMR spectra of original chitosan and prepared chitooligomers.

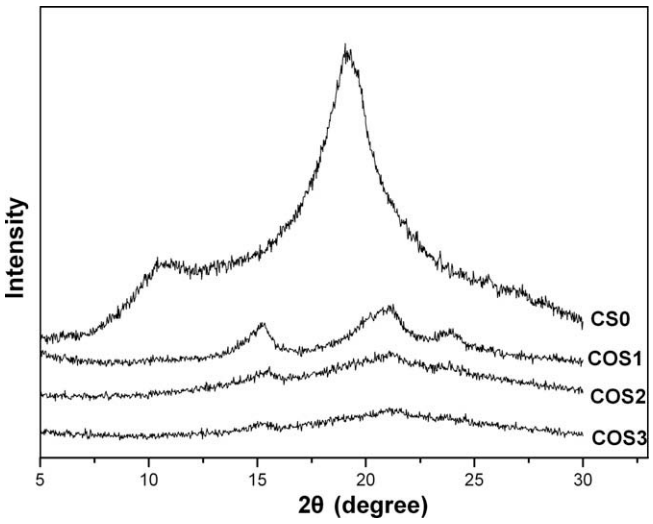

Fig. 3. XRD patterns of original chitosan and prepared chitooligomers.

saccharides by comparing the NMR spectra of these compounds with those of chitotriose [$(GlcNAc)_3$] and chitosatriose [$(GlcN)_3$]. The results showed that, with the decrease of the DD of the chitotrisaccharide, the chemical shifts of C-1, C-2, C-3 and C-4 were changed in a complicated manner, while the values of C-5 and C-6 were not changed significantly. In particular, most of the signals of C-1, C-3 and C-4 were shifted to the downfield with the decrease of the DD. Here, the change of the chemical shifts of the chitotrisaccharides with different DD was similar to that of our prepared chitooliogmers. Although the prepared chitooligomers have different molecular weight, their DDs were also decreased with the decrease of the molecular weight. Furthermore, it can be seen that there was only 0.7 ppm change in chemical shift between the signals of C-1 of our original chitosan and $(GlcN)_3$, both of which have a DD > 90%. Thus, it was seems that the DD of the chitooligomer caused a greater effect on the chemical shifts of C-1, C-2, C-3 and C-4 than the molecular weight. The reasons why the chemical shift changed have been suggested to be involved with the conformation change of the carbons at the glycosidic linkage, resulting from a different of electrostatic repulsion among the ammonium groups of the glucosamine residues due to their positive charges when DDs of chitosans was different (Fukamizo et al., 1991). For COS3 and COS4, there were more signals between the signals of C-4 and C-6 than that in the spectrum of the original chitosan and COS1. However, these peaks cannot fully be explained at present, which was also observed in others studies and was not explained completely (Qin et al., 2008; Saito et al., 1981).

3.4. XRD analysis

Fig. 3 showed the XRD patterns of the original chitosan and the chitooligomers. For original chitosan, there were two characteristic peaks at $2\theta = 10.4$ and 20°, which corresponded to the pattern of the "L-2 polymorph" (Saito & Tabeta, 1987). COS1 and COS2 exhibited three main peaks at $2\theta = 15.3$, 21.1 and 23.8°, which is referred to as the "annealed polymorph" according to previous report (Ogawa, 1991; Ogawa, Hirano, Miyanishi, Yui, & Watanabe, 1984). COS3 displayed a characteristic peak at 15.2° and a very weak peak at 21°. Compared with the original chitosan, the main difference of the chitooligomers was the appearance of the peak at 15.3° and the disappearance of the peak at 10.4°, indicating the absence of water molecules in the unit cell of the chitooligomers. This phenomenon was similar to the conversion from the "tendon polymorph" to the "annealed polymorph" observed by Ogawa (1991) when heating an aqueous suspension of chitosan. In addition, it can be seen from our results that, with the decrease of the molecular weight, the peak at 15.3, 21.1 and 23.8° tend to be broad and weak, suggested that the crystallinity of the chitooligomer was decreased with the decrease of the molecular weight. The crystallinity was in the order COS1 > CS0 > COS2 > COS3. Here, observation of a higher crystallinity of COS1 compare to CS0 might be due to the recrystallization of the short chain chitosan, which was also observed by other reseachers (Li et al., 2005; Qin et al., 2003).

3.5. The effect of chitooligomers on hepatocytes

3.5.1. Cell proliferation study

The effect of the chitooligomers and chitosan monomer with different concentrations on the proliferation of L02 hepatocytes was shown in Fig. 4. After 1 day of culture (Fig. 4a), it was found there was a statistically significant ($p < 0.05$) decrease in the optic density in all chitooligomer groups except 0.1 mg/ml COS2 and 0.1 mg/ml COS3 groups compared with the control, indicated the growth of L02 was inhibited. However, with the culture prolonged, the cultures recovered in cell proliferation for the chitooligomer groups in the following 3 days and there was no statistically significant in the optic density in all chitooligomer groups compared with the control (Fig. 4b). Finally, after 5 days of culture (Fig. 4c), all chitooligomer groups exhibited acceleratory effect on the proliferation of L02 hepatocytes ($p < 0.05$) except 1 mg/ml COS1 group. In the case of chitosan monomer (GlcN), GlcN showed an inhibition in cell proliferation after 1 day culture, especially in the concentration of 1 mg/ml, which exhibited a significant reduction of cell proliferation, with decrease of 35.2% compared with the control. On days 3 and 5, both the GlcN groups in concentration 0.1 and 0.01 mg/ml recovered in cell proliferation whereas high concentration group (1 mg/ml) produced a significant suppression in cell proliferation, with decreases of 60% and 72.9% respectively in comparison with the control, indicated GlcN showed cytotoxicity for L02 at a concentration of 1 mg/ml.

3.5.2. Morphology observation

The morphology of L02 cultured with chitooligomers and chitosan monomer was also observed by phase contrast microscopy. As shown in Fig. 5, the groups of chitooligomers and the control as well as the GlcN (0.1 and 0.01 mg/ml) exhibited similar cell morphology at all time points. On day 1, L02 were round or polygonal in shape. The cells formed a monolayer arrange from 3 to 5 days of

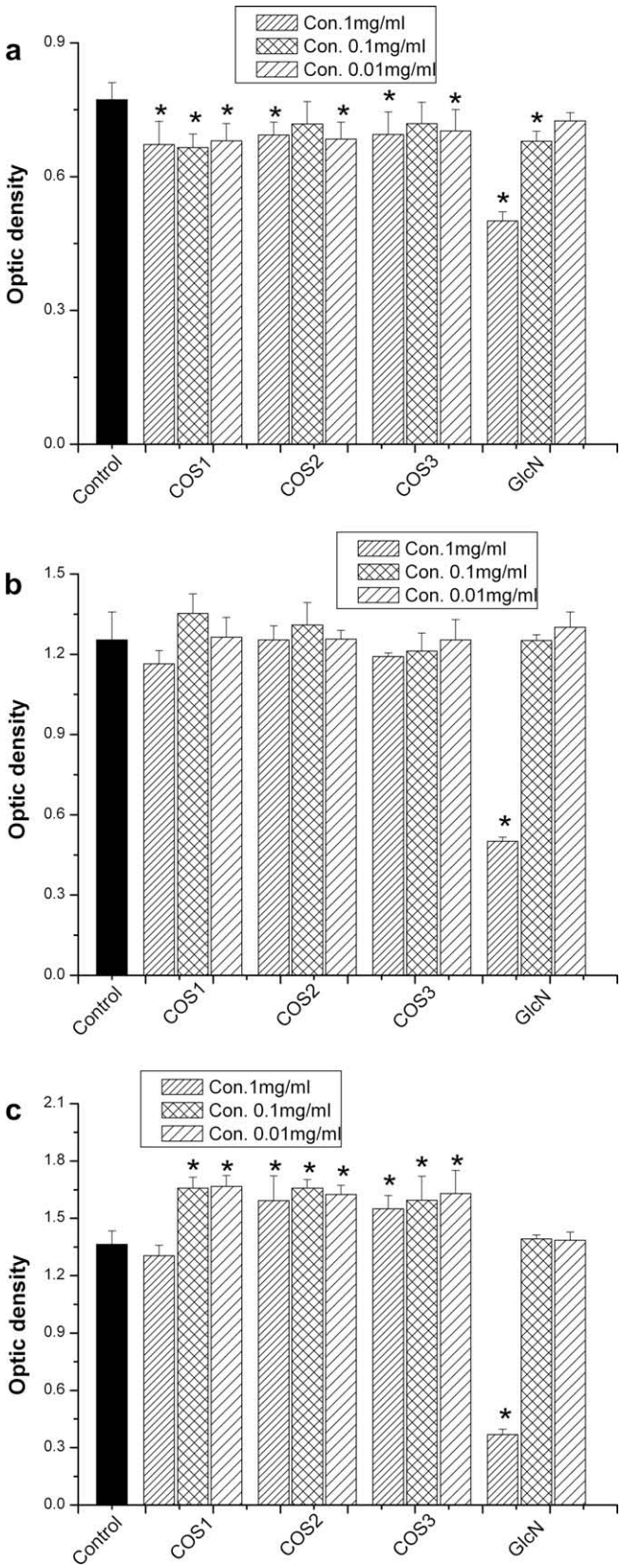

Fig. 4. MTT assay for proliferation of L02 hepatocytes cultured with chitooligomers at various periods (a), 1 day of culture; (b), 3 days of culture; (c) 5 days of culture. Error bars represent means ± SD for $n = 4$. $p < 0.05$ (compared to control group).

culture, and reached confluency after 5 days. The GlcN group in concentration of 1 mg/ml, however, displayed different cell morphology with the above groups. With the culture prolonged, more cells in round shape were observed and more and more cells were died on the culture plate, which consistent with the results of the MTT measurements.

3.5.3. Cellular function of hepatocytes

Hepatocyte function was evaluated by albumin secretion level and urea synthesis level as a function of culture time. The effect of the chitooligomers on the albumin secretion level was shown in Fig. 6. The results were determined from albumin concentration in the medium. After 1 day of culture, hepatocytes cultured in the chitooligomers and chitosan monomer contained media expressed similar levels of albumin secretion function (0.17 ~ 0.18 mg/ml). The albumin secretion levels increased rapidly on day 2, and were maintained at high levels until day 4, followed by a 17% reduction on day 5 compared to that of day 3. The increase of albumin secretion on days 2, 3 and 4 may be due to result from the proliferation of the cells, however, during 5 days of culture, no significant difference of the albumin secretion level was observed among all groups at the same time point.

The effect of the chitooligomers on the urea synthesis level showed the same tendency as a function of time. As shown in Fig. 7, all groups showed lower urea synthesis level (1.7 ~ 1.8 mg/l) on day 1, and then exhibited similar levels of urea synthesis function for the following 3 days. On day 5, all groups showed a sharply decrease of urea synthesis level, with decrease of 24% compared with the level of day 3. Similar to the results of the albumin secretion levels, there was no significant difference of urea synthesis level among all groups at the same time point.

4. Discussion

Chitooligomers are known to have some special biological activities. The effect of chitooligomers on various cell types, e.g. osteoblasts, macrophages, fibroblasts, endothelium and red blood cells, has been widely studied in recent years. It has been shown that chitooligomers exhibited effects on cell activity which were dependent on different targeted cells and concentration and molecular weight of chitooligomer. Ngo, Kim, and Kim (2008) examined the effects of the chitosan oligosaccharides (NA-COS; M_w 229.21–593.12 Da) on human myeloid cells (HL-60) and mouse macrophages (Raw 264.7) and the results showed the NA-COS had inhibitory effect on myeloperoxidase activity in HL-60 and oxidation of DNA and protein in Raw 264.7 and the cell viability of both cells was not affected by NA-COS even at a high concentration (1 mg/ml) after 2 days of culture. In contrast, Pae et al. (2000) reported the chitooligomer with 3 ~ 10 saccharide residues was able to inhibit proliferation of HL-60 cells and induce these cells to differentiate. It was found that after 4-day treatment with 0.1 mg/ml of chitooligomer, the growth of HL-60 was inhibited by 6%. But at a high dose of chitooligomer (1 mg/ml), the inhibition rate was 56%. After 6 days the cell number steadily declined when cells cultured in a dose of 0.5 mg/ml chitooligomer. Okamoto et al. (2002) studied the effects of GlcNAc1–GlcNAc6 and GlcN1–GlcN6 on fibroblasts (3T6) and vascular endothelium (HUVECs) and they found migratory activity of HUVECs was reduced by GlcN1–GlcN6. In a proliferation assay, the results were similar to that of Ngo et al. (2008) which showed no proliferation effect on two cells after 1 day of culture. However, Ohara et al. (2004) observed the alkaline phosphatase (ALP) activity was significantly high compared with the control when osteoblasts (NOS-1) cultured in a 0.005% (w/v) chitooligosaccharide (molecular weight <1000 Da) solution for 3 days. Furthermore, they studied the gene expression in osteoblasts cultured with the chitooligosaccharide and found a super-low concentration of chi-

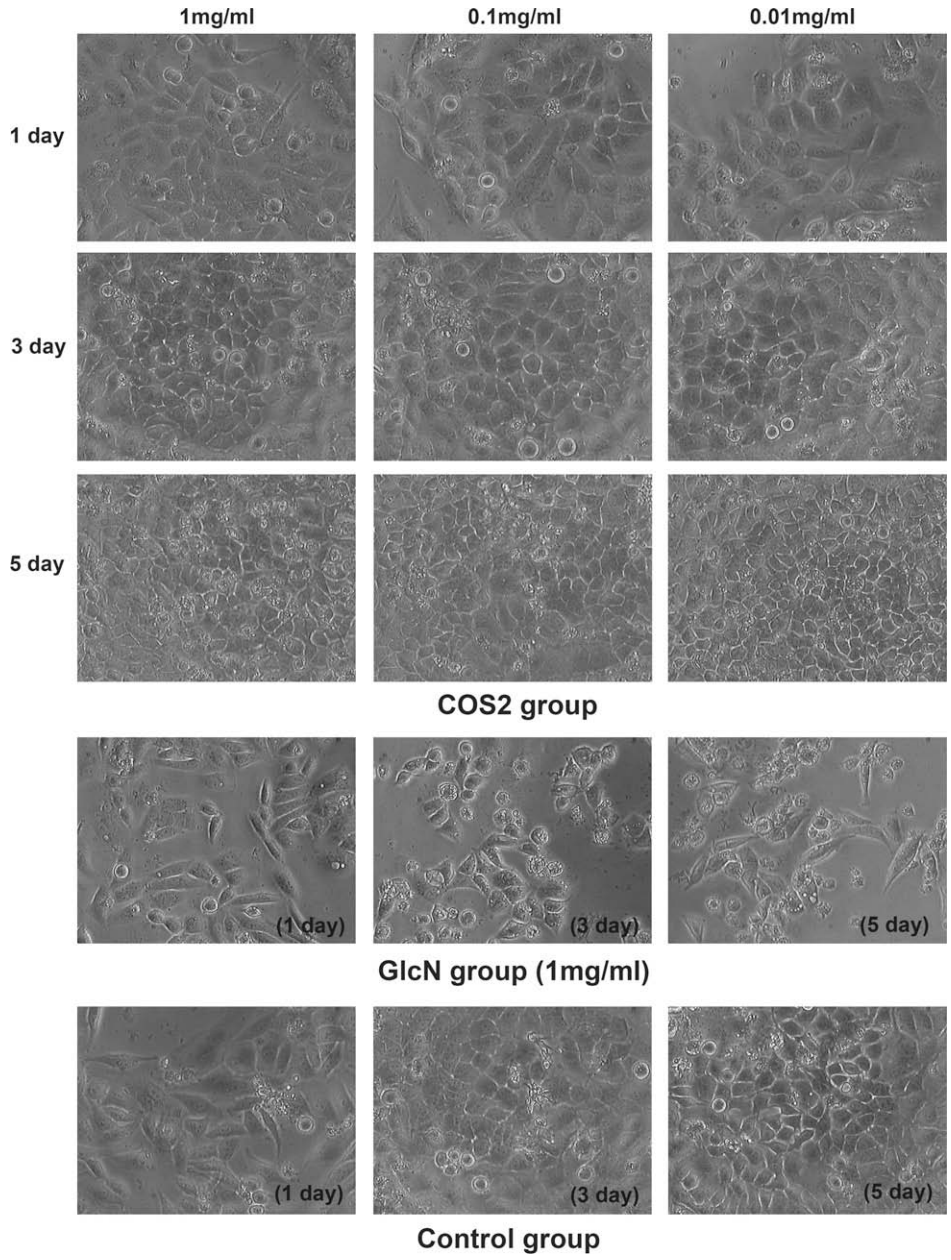

Fig. 5. Phase-contrast microscopy photographs of the L02 hepatocytes.

tooligosaccharide could modulate the activity of osteoblasts through mRNA levels and the genes concerning cell proliferation and differentiation could be controlled by chitooligosaccharide. Their research is the first report demonstrating that chitooligosaccharide directly affects osteoblastic proliferation at the gene level. In our present work, it was found for the first time, that the effect of the chitooligomers on the proliferation of L02 cells was dependent on culture time, namely, at the initial stage of culture there was an inhibitory effect on proliferation of L02 cells; however, the cultures recovered in cell proliferation and exhibited promotion effect in following days. Although the mechanisms underlying the effects are not completely known, some reasons have been suggested in recent years (Mori et al., 1997; Pae et al., 2000; Qin et al., 2006). First, when chitosan is dissolved in solution, the amino groups in the *N*-deacetylated subunits are conferring a high positive charge density and thus it behaves as a polycation at acidic or neutral pH. The charged chitosan can ionically interact with the negative charged molecules, for example, it has been shown to bind mammalian cells by interacting with surface glycoproteins (MacLaughlin et al., 1998). Therefore, a possible interaction of the positive charge of chitosan with cell surface may lead a change in the ionic environment of the cell membrane which is of importance in maintaining cell integrity and the numerous functions necessary for cell growth and differentiation. Some experiments confirmed that the interaction of the positive charge of chitosan with cells affected the viability of the cells (Huang, Mendis, Rajapakse, & Kim, 2006). The second reason is chitosan may absorb some substances in the culture medium which are involved in cell activity, owing to its well known property of protein absorption. In the case of GlcN, it was observed that high concentration of GlcN (1 mg/ml) showed a significant suppression in proliferation of L02 cells relative to control. This phenomenon is similar to that reported by Mori et al. (1997); however, the reason remains not yet clear.

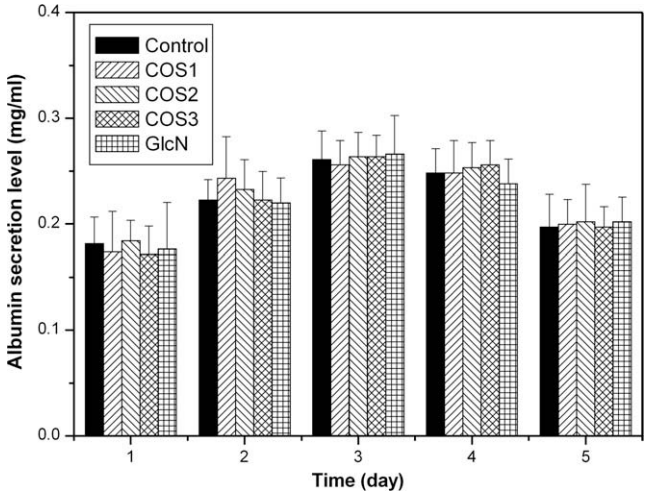

Fig. 6. Albumin secretion levels of hepatocytes cultured with chitooligomers at various time points. Error bars represent means ± SD for $n = 3$.

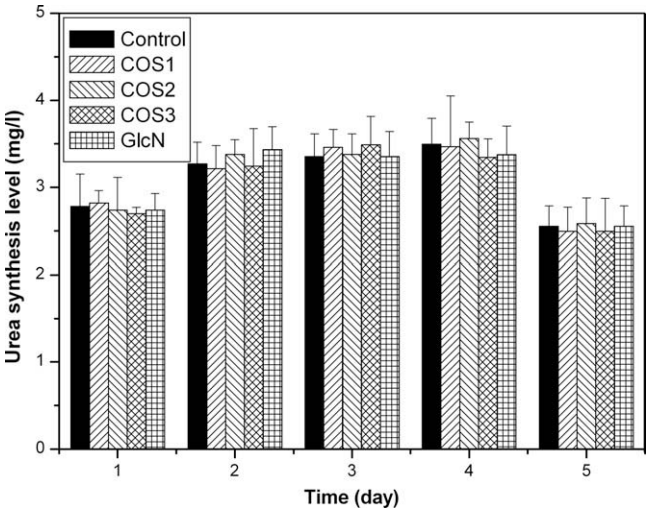

Fig. 7. Urea synthesis levels of hepatocytes cultured with chitooligomers at various time points. Error bars represent means ± SD for $n = 3$.

Albumin secretion and urea synthesis are often regarded as main indicators of liver-specific functions of hepatocytes. When looking at the results in our study, it was shown there was no statistical significance between the groups of chitooligomers and the control at any time points, suggested the chitooligomers had no significant effect on the functions of albumin secretion and urea synthesis of L02 hepatocytes. In general, hepatocytes are anchorage-dependant cells and have the tendency to form aggregates spontaneously in vitro. When enzymatically isolated from the liver and cultured in monolayer or suspensions, they rapidly lose adult liver morphology and differentiation functions. Recently study on maintenance of liver-specific functions of hepatocytes was focused on introduction of galactose moieties as asialoglycoprotein-receptor (ASGP-R) ligands to improve the hepatocytes anchorage and the interaction of hepatocytes with scaffold materials (Cho et al., 2006). It was reported that hepatocytes attached onto galactosylated surface were changed from spreading to round shapes with an increase of galactose moieties concentration and maintained the differentiated functions with promoting spheroids formation, which was shown to maintain a tissue-like cytological structure and sustain higher levels of many differentiated functions than hepatocytes cultured as monolayers (Yang, Goto, Ise, Cho, & Akaike, 2002). In this work, cell spreading was observed and no spheroids formed in all groups indicated the hepatocytes exhibited low levels of liver-specific functions.

5. Conclusions

The present research showed that oxidative degradation of chitosan with hydrogen peroxide combined with a selective ethanol precipitation appears to be a quick and simple method to obtain chitooligomers with low polydispersity. The prepared chitooligomers were found to have effects on the proliferation of L02 hepatocytes which were dependent on culture time. In the case of chitosan monomer (GlcN), high concentration of GlcN produced a significant suppression in proliferation of L02 cells compared to the control. In addition, there was no significant effect of the chitooligomers on the functions of albumin secretion and urea synthesis of the hepatocytes.

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