Preparation of *N*-Butyl Chitosan and Study of Its Physical and Biological Properties

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Received 11 November 2004; accepted 19 January 2005
DOI 10.1002/app.22212
Published online in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: *N*-Butyl chitosans with different substitution degrees were prepared by solid–liquid heterogeneous reaction, varying the ratio of chitosan to butyl bromide. FTIR and free amino percentage analysis confirmed that the substitution reaction occurred primarily on NH_2 moieties. X-ray diffraction results of the chitosan derivatives showed that the hydrogen bonds were weakened by the incorporation of pendant alkyl. *N*-Butyl chitosan films exhibited a higher elastic modulus, a lower percentage of elongation at break, decreased equilibrium swelling ratios, and slightly increased surface hydrophobicity compared to chitosan film. The results of ELISA indicated that the adsorption amount of fibronectin on butyl chitosan films was much higher than on chitosan film, which was maybe due to the rearrangement of hydrophilicity/hydrophobicity after modification. The MTT assay suggested that the viability and proliferation of osteoblast cells on *N*-butyl chitosan films were higher than that on chitosan film. These results suggest that *N*-butyl chitosan is a promising candidate biomaterial for bone regeneration. J Appl Polym Sci 98: 1016–1024, 2005

Key words: chitosan; alkylation; mechanical property; biocompatibility; osteoblast

INTRODUCTION

Chitosan (CHI) is a natural biopolymer produced by alkaline *N*-deacetylation of chitin, which is a natural polysaccharide occurring widely in the exoskeletons of many crustaceans and in the cell walls of some fungi. Chitosan has become an interesting biomaterial due to its good biocompatibility, biodegradability, low toxicity, and low cost. It has been extensively used for a wide range of applications, such as drug carriers, wound-healing agents, chelating agents, membrane filter for water treatment, and nerve regeneration conduit.[1–5]

Due to its abundant active groups on the main chain, such as amino and hydroxyl groups, chitosan can be easily modified. Many derivatives can be prepared by chemical and physical modification of chitosan, bringing diverse new biological properties to chitosan. Many techniques for modification of chitosan have been attempted, including crosslinking,[6] blending,[7] grafting,[8] and irradiation by γ-ray, and a great deal of progress has resulted: the capability of materials has been enhanced to a large extent, chitosan has been endowed with new characteristics, and thus the application of chitosan has been extended. Chitosan alkylation is one of these grafting modification techniques. During recent years, to overcome the problems of fast drug release, a few studies have been devoted to hydrophobic modification of chitosan with alkyl halides bearing different alkyl chain lengths.[8] Liu et al.[9] examined the use of *N*-(aminoalkyl) chitosan microcapsules as a carrier for gene delivery. It was reported that *N*-(aminoalkyl) chitosan-modified poly(D, L-lactic acid) (PDLLA) films improved the biocompatibility of PDLLA[10].

The use of chitosan for bone tissue engineering as a scaffold material has also been reported[11]. However, chitosan is mechanically weak and still lacks bioactivity, which severely limits its biomedical applications. Therefore, the purpose of this work was to improve the mechanical properties and to strengthen the biological properties of chitosan by modifying chitosan with butyl bromide. In the present study, *N*-butyl chitosans with different substitution degrees were prepared by solid–liquid heterogeneous reaction. Not only were the physicochemical properties of the *N*-butyl chitosans investigated by means of Fourier transform infrared spectroscopy (FTIR), X-ray diffraction, free amino percentage analysis, wettability, swelling ratio, and tensile measurement, but also their biological activity was evaluated by using the adsorption amount

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Contract grant sponsor: Tsinghua-Yue-Yuen Medical Science Fund.

Contract grant sponsor: State Key Basic Research and Development Projects.

of fibronectin (FN) on the surface of films, culture of osteoblast cells, and MTT assay and so on.

The major advantages of this approach are its simplicity and low cost. The results suggest that *N*-butyl chitosan can strengthen the mechanical properties and improve the biocompatibility of materials with osteoblasts. *N*-Butyl chitosan can be utilized as a promising candidate biomaterial for bone regeneration. To date, there are few reports of similar studies.

METHODS

Materials

Chitosan (85.08% deactylated, $M_\eta = 1.8 \times 10^6$, heavy metal content less than 0.1 mg/kg) was purchased from Qingdao Haisheng Bioengineering Company (China) and was prepared from Alaskan crab shell. Human FN, human serum, and rabbit anti-human FN antibody were obtained from the Peking University Health Science Center. HRP-conjugated goat anti-rabbit IgG was purchased from Dingguo Biotechnology Institute. Tissue culture clusters and flasks were purchased from Costar Co. Dulbecco's minimum essential medium (DMEM) was purchased from Gibco Co. and fetal bovine serum (FBS) was obtained from Hangzhou Sijiqing Co. All other reagents were of analytical grade.

Synthesis of *N*-butyl chitosan

Two grams of CHI was added to 10% sodium hydroxide in isopropanol solution and stirred for 30 min. Then butyl bromide (with molar ratios of 0.5, 0.7, 1, and 3 to the free amino of chitosan) was added dropwise to the mixture and allowed to react at 50°C for 4 h. The obtained precipitate was neutralized with hydrochloric acid and washed with ethanol until the product was neutral (pH 7). The derivatives were obtained after being dried at 50°C and labeled as 0.5-CHI, 0.7-CHI, 1-CHI, and 3-CHI.

Preparation of membrane

One gram of chitosan and 1 g *N*-butyl chitosan were dissolved in 100 mL of 1.2% (v/v) acetic acid solution. After stirring, the solution was filtered through a middle-pore-size nylon cloth to remove the insoluble substance. Chitosan and *N*-butyl chitosan solution was injected into plastic molds or the wells of tissue culture clusters. Solvent was allowed to evaporate at 50°C for 24 h. The films were then soaked in a 1% (w/v) sodium hydroxide solution for 30 min to neutralize the remaining acid. Finally, the films were washed with distilled water and dried.

Study on physical properties

FTIR spectra

Transmission infrared spectra of the chitosan and *N*-butyl chitosan were measured using an FTIR spectrometer (Paragon 1000pc; Perkin-Elmer).

Free amino percentage (FAP) analysis

The FAP analysis of chitosan and *N*-butyl chitosan was used to measure the degree of substitution. The determination of FAP was carried out by acid–base titration[12]. A 0.1*M* sodium hydroxide solution was used as a titrant and methyl orange as an indicator. Then the substitution degrees (SD) of the materials were calculated by eq. (1),

$$\mathrm{SD} = \frac{\mathrm{FAP(c)} - \mathrm{FAP(b)}}{\mathrm{FAP(c)}} \times 100\%, \qquad (1)$$

where FAP(c) and FAP(b) are the free amino percentage of chitosan and *N*-butyl chitosan, respectively.

X-ray diffraction study

X-ray diffraction patterns of chitosan and *N*-butyl chitosan were measured using an X-ray diffractometer (Model No. D8 Advance; Bruker) with Ni-filtered Cu radiation generated at 30 kV and 30 mA as the X-ray source.

Swelling ratio (SR) measurements

Dried films of chitosan and *N*-butyl chitosan were weighed and immersed in phosphate-buffered saline solution (PBS, pH 7.4) for 12 h. The weights of the swollen samples were measured after the surface water was removed with filter paper. Then the SR of the films were calculated by eq. (2),

$$\mathrm{SR} = \frac{W_s - W_d}{W_d} \times 100\%, \qquad (2)$$

where W_d and W_s are the weights of dried and swollen samples, respectively.

Wettability

The static contact angles of chitosan films were measured using a contact angle goniometer (Model No. JY-82; Chengde Experimental Machine Plant, China). Ten microliters of distilled water was dropped onto the surface of the films before measuring. The contact angle data were obtained from the images of water droplets on the film surfaces. All data presented were the mean values of nine independent measurements.

Mechanical properties tests

The mechanical properties of films were evaluated by the tensile test. Samples were soaked in PBS for 12 h before testing and then taken out and wiped with filter paper. The tested specimens were cut to 9×2.5 cm^2. The thickness of each sample was measured at nine different points with a micrometer and the average was taken. The mechanical properties were measured with a universal testing machine (Model No. AG-1; Shimadazu Co.) equipped with a 50*N* initial load cell. The tensile speed was 10 mm/min. The breaking elongation and the elastic modulus were obtained from the stress–strain curves instantaneously recorded using a computer. At least six specimens were measured for chitosan and *N*-butyl chitosan films and the average values were obtained.

Study on biological properties

Fibronectin adsorption

The adsorption of FN on the films was detected by means of enzyme-linked immunosorbent assay (ELISA)[13]. FN solution was diluted to a concentration of 5 μg/mL with PBS. The diluted protein solutions were poured into a 24-well tissue culture cluster precoated with chitosan and *N*-butyl chitosan films, and the cluster was incubated at 4°C for 12 h. After the supernatant was removed, each well was rinsed with the washing buffer (PBS buffer plus 0.05% Tween 20, pH 7.4) three times, and the surfaces were blocked with 1% bovine serum albumin solution for 2 h at room temperature. After the surface was rinsed three times with the washing buffer, the corresponding antibody (diluted 1 : 1000 in PBS) was added and the cluster was incubated for 1.5 h at room temperature. After wells were rinsed, goat antirabbit IgG conjugated to horseradish peroxidase (diluted 1 : 5000 in PBS) was added and incubated for 1 h at room temperature. After further rinsing, 300 μL of substrate solution (containing 25 m*M* citric acid, 50 m*M* sodium dihydrogen phosphate, 2.5 m*M* *o*-phenylenediamine and 10 m*M* hydrogen peroxide, pH 5.0) was added to each well and the wells were placed in the dark at room temperature for 10 min. Finally, 2*M* H_2SO_4 was added to stop the reaction, and the absorbance was measured at 490 nm using an ELISA reader (Model No. 550, Bio-Rad).

MC3T3-E1 cell culture

To study the biocompatibility of *N*-butyl chitosan in bone formation, MC3T3-E1 cells (Japan Riken Cell Collection) were chosen to test the cytocompatibility of the materials. During the experiment, MC3T3-E1 cells were cultured in medium (DMEM–10% FBS containing 100 U/mL penicillin and 100 μg/mL streptomycin). Before the experiment, films were prepared on the surface of a 24-well tissue culture cluster. All the films were sterilized by exposure to UV light (15 W UV sterilamp, Philips) for 2 h, followed by a rinsing step of 30 min in sterile PBS solution. MC3T3-E1 cells were seeded on chitosan and *N*-butyl chitosan films at a density of 3×10^4 cells/cm^2 and then incubated at 37°C and 5% CO_2. The culture medium was replaced every 2 days.

Acquisition of cell images

The films permitted the use of light microscopy to study cell–material interactions in detail. Cells cultured for 1 and 3 days were viewed using an inverted phase contrast microscope (Axiovert 10, Opton). Images from the microscope were acquired using a CCD video camera (CCD color camera, Hitachi) and were subsequently digitized and analyzed using NIH Image software and a Scion image capture board (Scion, Frederick, MD).

MTT assay

An MTT test was performed to quantify the cell viability. Briefly, cells were cultured for 3, 6, and 9 days, and 100 μL MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrasodiuim bromide, 5 mg/mL, Amresco 0793)was added to each well and were incubated at 37°C for 4 h. Then, the MTT solution was removed and the insoluble blue formazan crystal was dissolved in dimethyl sulfoxide. One hundred microliters of solution from each well was aspirated and poured in a 96-well plate for absorbance measurement at 570 nm. The absorbance was directly proportional to cell viability. The absorbance was measured at 570 nm using an ELISA reader (Model No. 550, Bio-Rad).

RESULTS AND DISCUSSION

FTIR spectrometry of chitosan and *N*-butyl chitosan

The IR spectra are shown in Figure 1. There were three significant changes of transmittance peaks areas of *N*-butyl chitosan comparing the FTIR spectra of CHI. The 2860 and 2920 cm^{-1} peaks were attributed to the -CH_2 and -CH_3 stretching. The intensities of these two peaks for *N*-butyl chitosan increased, which confirms that -CH_2 and -CH_3 groups had been introduced into the CHI[14]. The characteristic band of -NH_2 at 1593cm^{-1} decreased after CHI was alkylated, but the C-O band around 1151cm^{-1} didn't change much, indicating that the substitution reaction occurs primarily on NH_2 moieties[15]. The third change was about the new peaks centered at 1460cm^{-1} (-CH_3 bending) for

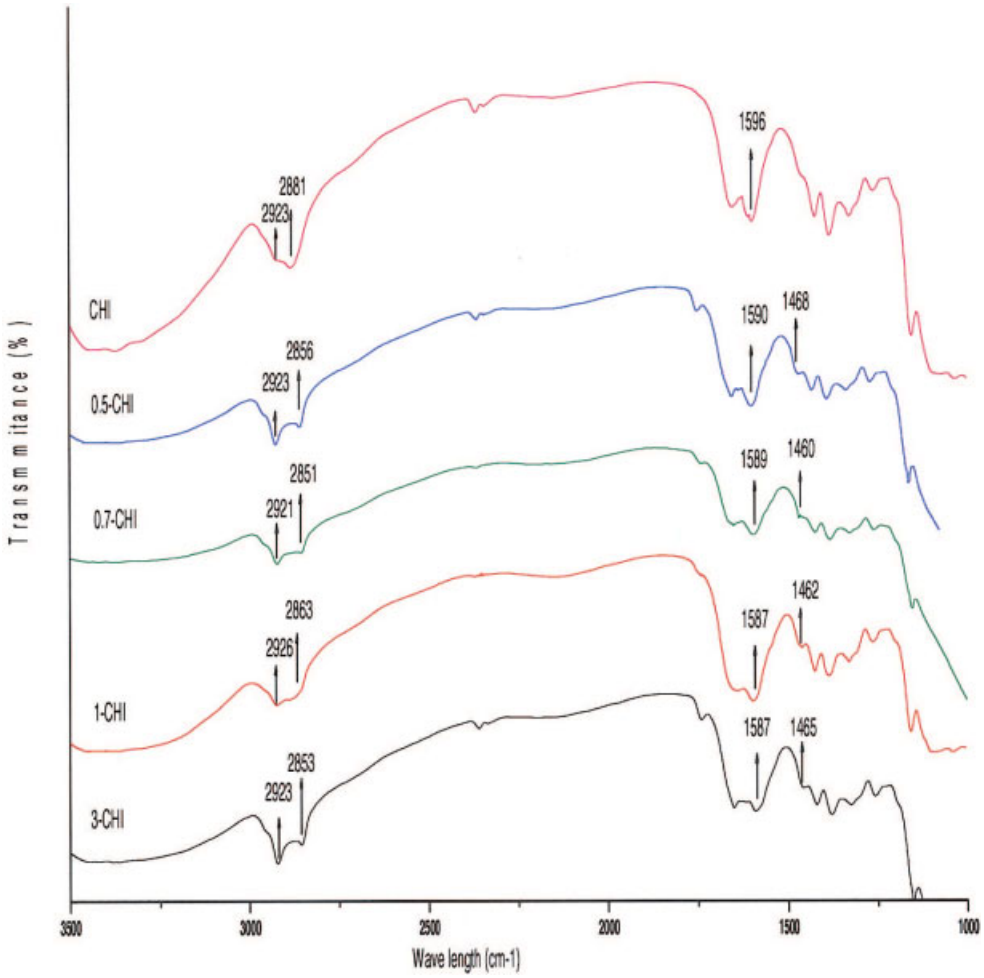

Figure 1 IR spectra of chitosan and *N*-butyl chitosan.

N-butyl chitosan, which further suggests that alkyl groups had been introduced into the CHI.

FAP and substitution degree (SD)

Results are shown in Table. I. The FAP of *N*-butyl chitosan decreased with the increase in the amount of butyl bromide. Since FAP mainly represents the free amino percentage of chitosan molecules, it may be concluded that the main reaction group is -NH_2 moieties and the substitution degree increases with increases in the reaction amount of butyl bromide. Thus, SD increased. But it did not increase in accord with the molar ratio of butyl bromide to amino of chitosan, which may mean that the reaction is controlled by many factors other than the amount of reactant added, including temperature, time, and so on. Although we could not define the reacting condition precisely, we still could obtain the typically physical and biological results of *N*-butyl chitosan with rising SD.

TABLE I
FAP, SD, and SR of Chitosan and *N*-Butyl Chitosan

Materials	CHI	0.5-CHI	0.7-CHI	1-CHI	3-CHI
FAP (%)	85.08	74.27	57.63	31.48	13.39
SD (%)	0	12.71	32.26	63.00	84.26
SR (%)	219.23	207.07	198.54	156.34	140.15

X-ray diffraction pattern

Figure 2 shows X-ray diffraction patterns of chitosan and *N*-butyl chitosan with different substitution degrees. The X-ray diffraction pattern of CHI shows that two typical diffraction peaks appeared at 10 and 20°, respectively. As for *N*-butyl chitosan, the intensities of diffraction peaks markedly decreased and even disappeared as the substitution degree increased. Therefore, it is reasonable to consider that the incorporation of alkyl side chains into CHI lessens the hydrogen binding interaction in CHI, which maybe further confirmed that the main reaction group is -NH_2. Similar explanations have been discussed in blending chitosan with other materials[7].

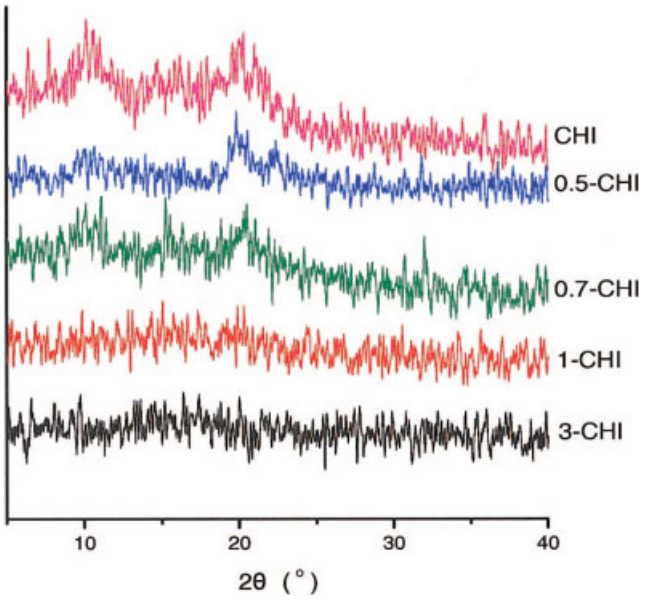

Figure 2 X-ray diffraction patterns of chitosan and *N*-butyl chitosan.

Determination of SR

As shown in Table I, the SR decreased with the increase in the substitution degree. The swelling behavior of a material refers to the case when water molecules enter the material and combine with the hydrophilic groups in material molecules. Consequently, the content of hydrophilic groups and the intermolecular force are the two main factors that influence the material's swelling property[16,17]. On one hand, the increase of the substitution degree lessens the intermolecular hydrogen bonds and reduces the compactness of the network. This will raise the swelling ratio. On the other hand, the hydrophobicity of the network is enhanced upon increasing the amount of the butyl chain. The increased hydrophobicity will result in a decrease of the swelling ratio. In the experiment, the SR of *N*-butyl chitosan was smaller than that of CHI. Hence, hydrophobicity was predominant. Furthermore, the higher the substitution degree is, the more evident the hydrophobicity is.

Wettability

The static water contact angles on the films are shown in Figure 3, in which the contact angles for all the materials were less than 90°, and the contact angle increased with the increase of substitution degree. A higher contact angle indicates higher hydrophobicity. Therefore, *N*-butyl chitosan increased hydrophobicity, which was due to the incorporation of the butyl group. Such an effect will be expounded further in the discussion of the ELISA experiment.

Mechanical properties

The elastic modulus (E) and percentage of elongation at break (ε_B) of *N*-butyl chitosan and chitosan films in a wet state are plotted in Figure 4. As can be seen from Figure 4, chitosan derivatives have a higher elastic modulus than chitosan. 1-CHI and 3-CHI have the highest elastic modulus among them. On the other hand, the ε_B of *N*-butyl chitosan decreased greatly compared with that of chitosan film. In sum, the rigidity of *N*-butyl chitosan increased, which is advantageous in bone tissue engineering. The reason may be that after incorporation of butyl groups, the hydrophobicity of the network was enhanced and the swell-

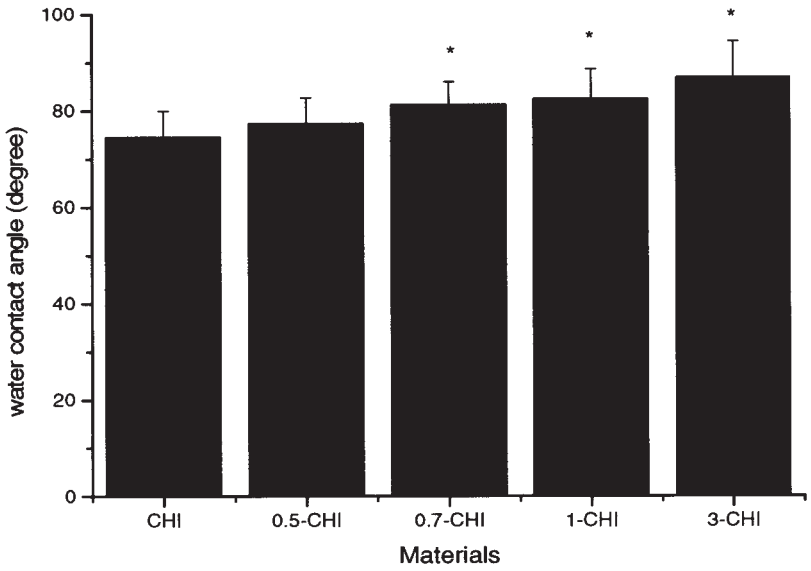

Figure 3 Water contact angle of chitosan and *N*-butyl chitosan. *$P < 0.05$ relative to CHI.

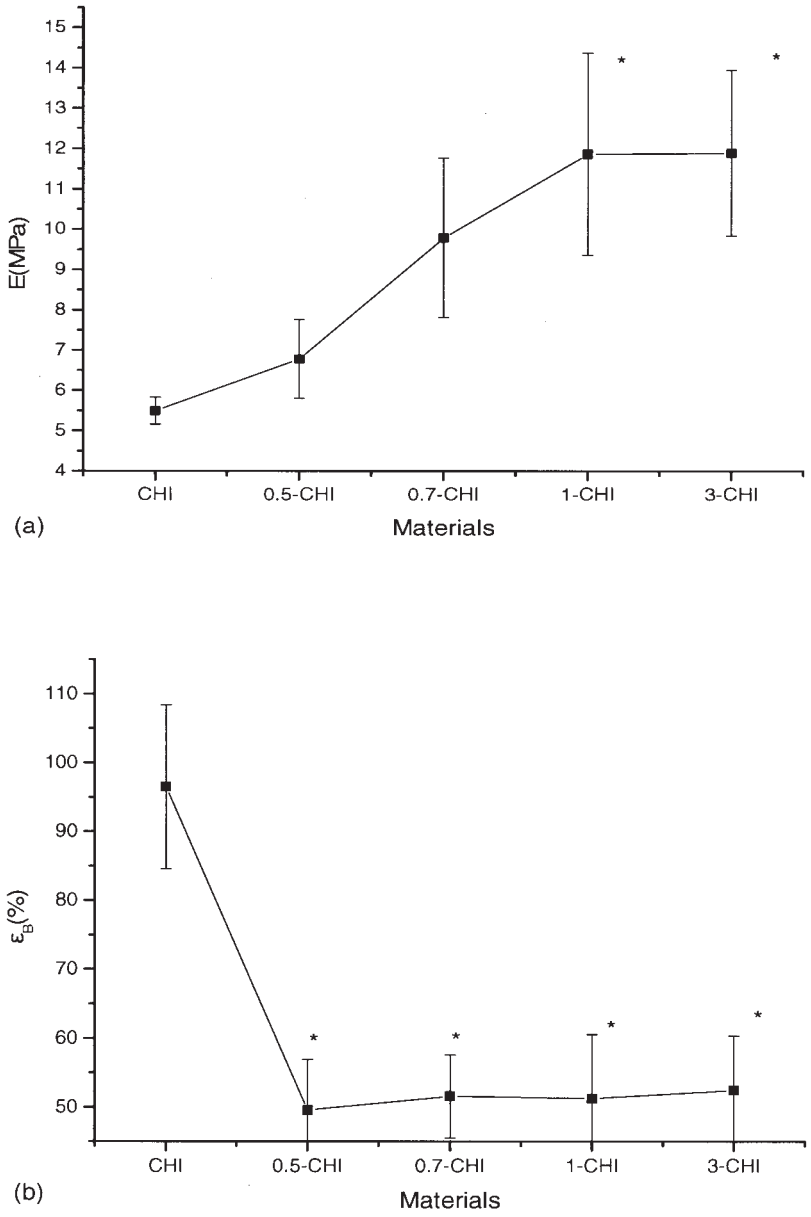

Figure 4 Elastic modulus (a) and percentage of elongation at break ε_B (b) of chitosan and *N*-butyl chitosan. *$P < 0.05$ relative to CHI.

ing ratio decreased (which is in agreement with the results described under the section Determination of SR). Free water can act as an effective plasticizer for the polymer and can influence the mechanical properties of the swollen films[18,19]. Lower free water content led to higher E and lower ε_B in *N*-butyl chitosan films compared with chitosan film.

Adsorption of FN on films

The results of FN adsorption on chitosan and *N*-butyl chitosan are shown in Figure 5: Chitosan film had the lowest A_{490}; 0.5-CHI and 3-CHI films had moderate values; 0.7-CHI and 1-CHI had the highest A_{490}.

The serum FN adsorption experiment exhibited a similar order among different materials, although their absorbencies were not equal to that of the sole FN adsorption experiment due to the difference of concentration and the competition of other proteins. Therefore, the results of the ELISA experiment can be used to describe the relationship between biomaterials and fibronectin, one of the adhesive ECM molecules. The connection of FN adsorption and cell growth will be discussed in later results.

There are several factors affecting the adsorption of proteins on a material's surface, such as the topological structure of the material's surface, hydrophilicity/hydrophobicity, and electrostatic attractive forces[20,21].

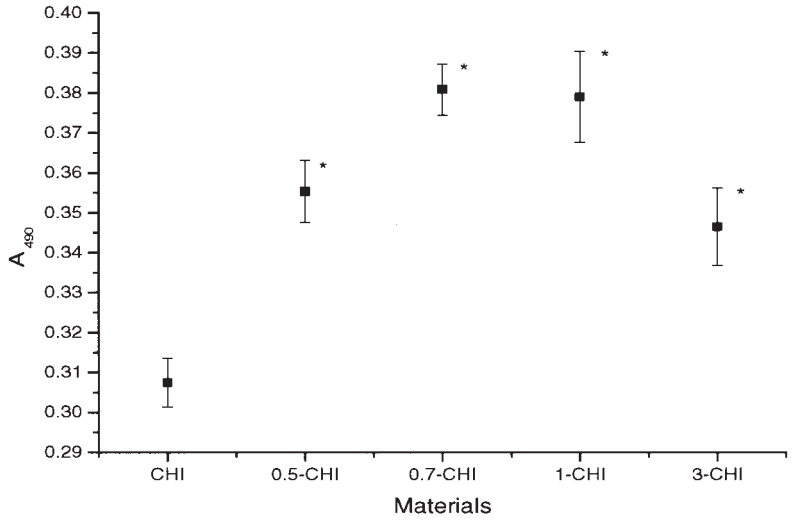

Figure 5 Adsorption of FN on chitosan and *N*-butyl chitosan. *$P < 0.05$ relative to CHI.

It has been confirmed that the introduction of hydrophobic side chains into chitosan can facilitate protein adsorption[22], which is in accord with the results of this experiment. But the amount of FN adsorption did not increase with the increase in the hydrophobicity; the maximum of FN adsorption was achieved in the middle substitution, 0.7-CHI and 1-CHI.

As for this phenomenon, a possible reason may be that some regions of the molecule are linked with the hydrophobic butyl group, but others retain their original state after modification, which modulates the distribution of hydrophilicity/hydrophobicity regions on the surface of chitosan. As proteins have both hydrophobic and hydrophilic regions, the adsorption of the proteins onto a surface should be selective; an area with appropriate proportions of hydrophilicity to hydrophobicity would be the most favored region. From this point of view, 0.7-CHI and 1-CHI should have the most appropriate adsorption region distribution. Second, with regard to 3-CHI, the steric hindrance is increased, kinetically lowering the contacting opportunity and decreasing the amount of FN adsorption, but the whole adsorption amount is still higher than that of chitosan. Other characteristics of materials, such as topological structure, may also affect the adsorption of proteins on materials, which requires further investigation.

MC3T3-E1 cell culture on the materials

Micrographs of MC3T3-E1 cells cultured for 1 day on the chitosan and *N*-butyl chitosan films are shown in Figure 6 (the results of 0.7-CHI and 3-CHI are not shown in this paper). The micrographs of a 1-day culture reflect the status of MC3T3-E1 cell attachment and spreading. On chitosan films, most cells still kept a spherical shape and only a few cells began to extend. However, on *N*-butyl chitosan films, quite a number of cells changed from spherical to flat, assuming a fibroblast-like morphology. This means that most cells had finished attachment and were in the process of spreading. Figure 7 (the results of 0.7-CHI and 3-CHI are not shown in this paper) showed micrographs of MC3T3-E1 cells cultured for 3 days on biomaterials. On chitosan films, the amount of cells bearing fibroblast-like morphology increased, but there were still many round cells because most spherical cells could not continue growing on the materials. However, on the *N*-butyl chitosan, the amount of flat-shaped cells increased and the spreading was more uniform.

The MTT assays on days 3, 6, and 9 were used as a measure of relative cell proliferation. MTT was a pale-

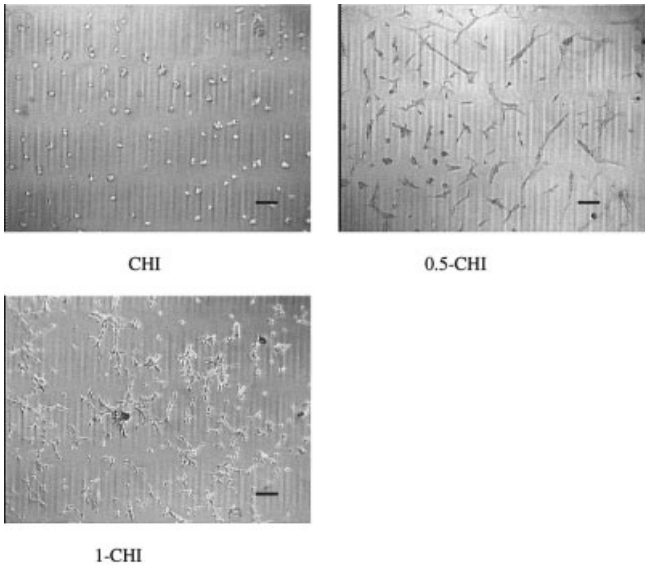

Figure 6 Photomicrographs of MC3T3-E1 cells cultured for 1 day on chitosan and *N*-butyl chitosan. Bar = 100 μm.

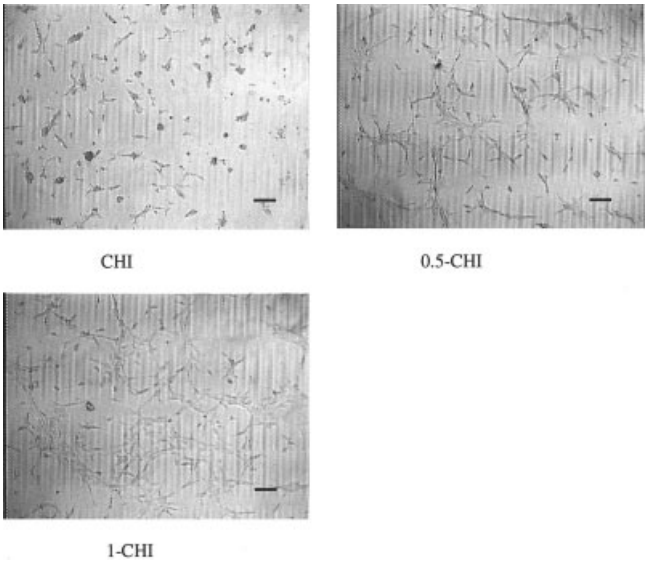

Figure 7 Photomicrographs of MC3T3-E1 cells cultured for 3 days on chitosan and *N*-butyl chitosan. Bar = 100 μm.

yellow substrate (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrasodiuim bromide) that was reduced by living cells to a dark blue formazan. Thus, this assay could be used to measure the proliferation of MC3T3-E1 cells. As shown in Figure 8, the increased absorbance on all surfaces at the end of the culture period showed that proliferation occurred on all films. Compared to chitosan films, osteoblast proliferation was significantly greater on *N*-butyl chitosan films after 6 or 9 days of culturing. In particular, the cell viabilities of 0.7-CHI and 1-CHI were the greatest, which is the same as the results of FN adsorption on films. Successful cell attachment is beneficial to cell proliferation. Therefore, we can conclude that the cell attachment and proliferation on the surfaces of biomaterials was significantly improved by *N*-butyl modification.

Cell attachment, spreading, and growth are important to judge the biocompatibility of a biomaterial. This process is influenced by many surface properties of biomaterials, including surface composition, wettability, domain composition, and morphology. The process of cell attachment usually occurs in two steps. The first is the random cell adsorption on the surface of biomaterials, which is mainly directed by physical and chemical interaction between materials and cells. The second step is specific cell adsorption on the surface. ECM adhesive molecules, like laminin and fibronectin, are concerned with the specific attachment and proliferation of cells on a material's surface[23]. Therefore, the results of the cell culture can be explained by ECM adhering molecular adsorption.

It has been demonstrated previously that the distribution of hydrophilicity/hydrophobicity regions on the surface of chitosan was regulated after *N*-butyl modification. Among the derivatives, 0.7-CHI and 1-CHI had the best proportions of hydrophilicity to hydrophobicity. Therefore, compared to chitosan, FN adsorption on *N*-butyl chitosan was increased. The result of FN adsorption was basically consistent with the result of MTT. Increased FN adsorption on *N*-butyl chitosan film is one possible mechanism to explain the

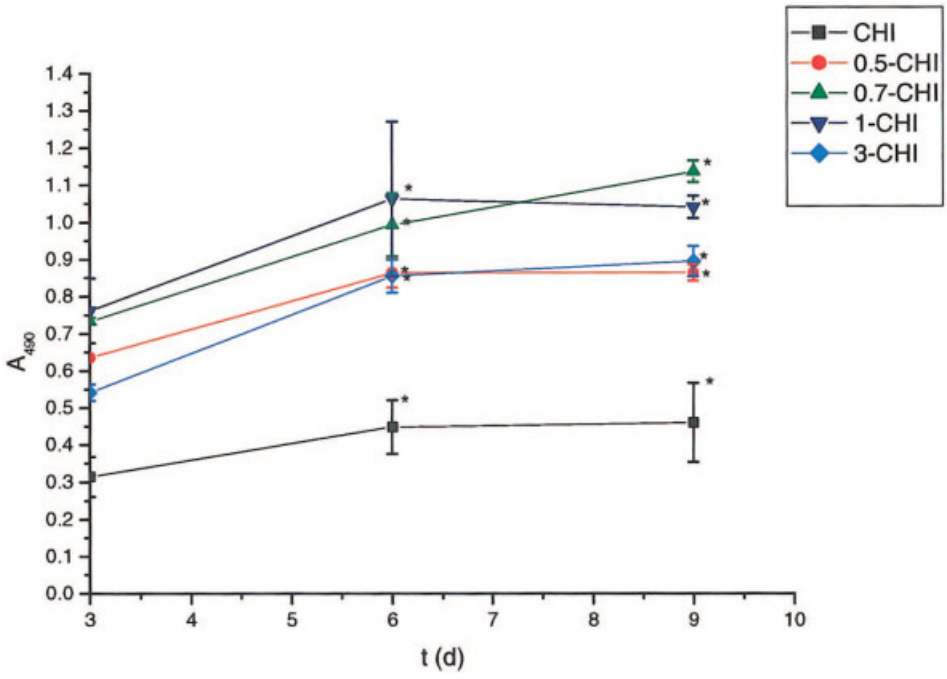

Figure 8 Growth of MC3T3-E1 cells on chitosan and *N*-butyl chitosan. *$P < 0.05$ relative to CHI.

improved MC3T3-E1 cell affinity. FN is an adhesive ECM glycoprotein that is involved in many important physiological processes, such as promoting cell adhesion, proliferation, and migration in the body. FN has also proven to be critical to the expression of the osteoblastic phenotype[24]. By specifically binding FN, *N*-butyl chitosan films can stimulate attachment and proliferation of MC3T3-E1 cells.

CONCLUSIONS

Butyl bromide was used to modify chitosan, achieving *N*-butyl chitosan with different substitution degrees. This study thoroughly analyzed the physical properties and MC3T3-E1 cell affinity of *N*-butyl chitosan. FTIR, free amino percentage analysis, and X-ray diffraction results confirmed that the substitution reaction occurred primarily on NH_2 moieties. The static contact angles experiment and the swelling ratio experiment showed the increase hydrophobicity of *N*-butyl chitosan. Compared with chitosan films, *N*-butyl chitosan films exhibited a higher elastic modulus, which is advantageous in bone tissue engineering. *N*-Butyl chitosan films are more favorable for MC3T3-E1 cell attachment and proliferation, and MTT assay indicated the cell viability of *N*-butyl chitosan films was greater than that of chitosan film. The results obtained from ELISA indicated the adsorption amount of fibronectin on butyl chitosan films was much higher than on chitosan film, which can explain the improved osteoblast cell affinity. In conclusion, considering its improved mechanical properties and favorable osteoblast cell affinity, especially 1-CHI (SD = 63.00%), *N*-butyl chitosan is a promising candidate biomaterial for bone regeneration.

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