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Preparation of biodegradable chitin/gelatin membranes with GlcNAc for tissue engineering applications

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Abstract

Chitin is a natural biopolymer have been used for several biomedical applications due to its biodegradability and biocompatibility. By using the calcium solvent system, chitin regenerated hydrogel (RG) was prepared by using α-chitin. And also, the swelling hydrogel (SG) was prepared by using β-chitin with water. Then, both RG and SG were mixed with gelatin and *N*-acetyl-D-(+)-glucosamine (GlcNAc) at 120 °C for 2 h. The chitin/gelatin membranes with GlcNAc were also prepared by using RG and SG with GlcNAc. The prepared chitin/gelatin membranes with or without GlcNAc were characterized by mechanical, swelling, enzymatic degradation, thermal and growth of NIH/3T3 fibroblast cell studies. The stress and elongation of chitin/gelatin membrane with GlcNAc prepared from RG was showed higher than the chitin/gelatin membranes without GlcNAc. But, the chitin/gelatin membranes prepared from SG with GlcNAc was showed higher stress and elongation than the chitin/gelatin membranes without GlcNAc. It is due to the crosslinking effect of GlcNAc. The chitin/gelatin membranes prepared from SG showed higher swelling than the chitin/gelatin membranes prepared from RG. In contrast, the chitin/gelatin membranes prepared from RG showed higher degradation than the chitin/gelatin membranes prepared from SG. And also, these chitin/gelatin membranes are showing good growth of NIH/3T3 fibroblast cell. So these novel chitin/gelatin membranes are useful for tissue engineering applications.

Keywords: Chitin hydrogel; Chitin/gelatin membranes; GlcNAc; Swelling and degradation studies; Thermal properties; NIH/3T3 fibroblast cell

1. Introduction

Chitin, a natural abundant polysaccharide, is known to be the β-1,4-glycan of *N*-acetyl-D-glucosamine (GlcNAc). Chitin is also known to be biodegradable polymer in nature and in the animal body (Hirano et al., 1990; Sashiwa, Saimoto, Shigemasa, Ogawa, & Tokura, 1990) and to be a low toxicity. Due to its low toxicity, the chitin is useful for variety of biomedical applications (Nishimura et al., 1985; Okamoto et al., 1993). However, chitin is difficult to dissolve in common organic solvents due to its rigid crystalline structure (Austin, 1975; Delacruz et al., 1992; Kaifu, Nishi, & Tokura, 1981; Minke & Blackwell, 1978; Tamura, Sawada, Nagagama, Higuchi, & Tokura, 2006; Tamura et al., 2004; Tamura, Nagahama, & Tokura, 2006). The outer skeletal chitin consists of α-chitin and squid pen consists of β-chitin. α-Chitin has been proposed to form a much tighter crystalline structure than β-chitin. Although several research work have been reported about the chitin solubility (Tokura, Nishi, & Noguchi, 1979). Recently, the calcium solvent system was found to be a good solvent to dissolve the chitin in the mild conditions (Nagahama, Higuchi, Jayakumar, Furuike, & Tamura, in press-a; Nagahama et al., in press-b; Tamura, Sawada, et al., 2006; Tamura, Nagahama, et al., 2006; Tokura, Nishimura, Sakairi, & Nishi, 1996). It has also been found that the chitin hydrogel also prepared by using this solvent system (Jayakumar & Tamura, in press; Nagahama et al., in press-b; Tamura, Sawada, et al., 2006; Tamura, Nagahama, et al., 2006).

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Gelatin is also biocompatible protein, and when it takes in living body, it shows low antigenecity and very high bioabsorptivity. The three-dimensional gel network of gelatin is composed of microcrystallites interconnected with amorphous regions of randomly coiled segments and it has the characteristics, such as heat reversibility (Achet & He, 1995; Arvanitoyannis, Nakayama, & Aiba, 1998). The predominant property of gelatin would be the Sol–Gel transition under aqueous condition. The membranes between chitosan and gelatin having so many biomedical applications have been reported (Kolodziejska, Piotrowska, Bulge, & Tylingo, 2006). However, chitin/gelatin membranes were not well known. A variety of porous materials have been used to produce three-dimensional cell composites by allowing individual cells to attach on the scaffold surface, promoting cell growth and maintaining the differentiated cell phenotypes (Dasdia, Bazzaco, Ferrero, Campanelli, & Dolfine, 1998; Grande, Halberstadt, Naughton, Schwartz, & Manji, 1997). For a tissue to be successfully regenerated, sufficient cell propagation and appropriate differentiation must be achieved in the three-dimensional cellular composite. Nonwoven fabrics are widely used as scaffolds for tissue engineering application (Aigner et al., 1998; Bhat, 1995; Ma, Li, Yang, & Kniss, 1999). However, nonwoven fibrous matrices currently used in tissue engineering have a relatively large porosity, and pore size, in the range of several hundred micrometers, and have not been structurally optimized for specific application (Grande et al., 1997; Organ & Vacanti, 1997). So there is need for a reliable method that can be easily used to modify the microstructure of nonwoven fibrous matrix to be used as a membranes for tissue engineering applications.

Chitin and its derivatives are currently used in various fields such as: treating water, biomedical, cosmetic, agricultural and food industries (Jayakumar, Nwe, Tokura, & Tamura, 2007; Jayakumar, Prabaharan, Reis, & Mano, 2005; Jayakumar, Reis, & Mano, 2006; Sashiwa, 2005). It also shows some biological activities such as immunological, antibacterial, wound healing, drug delivery and have been proposed for tissue engineering applications (Jayakumar et al., 2007, 2006; Jiang, Nair, & Laurencen, 2006; Rinki, Dutta, & Dutta, 2007; Verma, Verma, Ray, & Ray, 2007; Verma, Verma, & Ray, 2005; Wang et al., 2006). In this study, the RG and SG with gelatin was mixed with GlcNAc according to Maillard reaction, which produces browning compounds due to the interactions between carbonyl group, reducing sugar and amino compounds. This reaction generally occurs when they are used in food industry (Friedman, 1996; Mao, Zhao, Yin, & Yao, 2003) and was just beginning to use for modification of polysaccharide, chitosan (Chung, Tsai, & Li, 2006; Tanaka, Huang, Chiu, Ishizaki, & Taguchi, 1993; Ueshima & Kawai, 2007). In this paper we are describing about the preparation of chitin/gelatin membranes with or without GlcNAc were prepared from two different chitin hydrogels. And also, we are reporting in this paper about the surface morphology, swelling, enzymatic degradation, mechanical, thermal and growth of NIH/3T3 fibroblast cell studies of the prepared chitin/gelatin membranes with or without GlcNAc in detail.

2. Experimental

2.1. Materials

α- and β-chitin were received from KYOWA TECNOS Co. Ltd. NIH Swiss mouse embryo fibroblast NIH/3T3 cell line was purchased from Invitrogen, Japan. The fibroblast culture medium was composed with Dulbecco's Modified Eagle's Medium (DMEM, Gibco–BRL, Rockville, MD, USA) and 10% fetal bovine serum (FBS, Gibco–BRL). Trypsin–EDTA (0.5% trypsin with EDTA-4Na), antibiotic agent and penicillin–streptomycin were purchased from Gibco–BRL. The trypan blue (0.4%, 100 ml) was purchased from Mp Biomedicals, Inc, France. Gelatin, calcium chloride dihydrate, GlcNAc and the other chemicals and lysozyme were purchased from Wako Chemical Co. (Japan) and used without any further purification. The Ca solvent system has been prepared according to our previous reported method (Tamura, Sawada, et al., 2006; Tamura, Nagahama, et al., 2006).

2.2. Preparation of RG with gelatin

Twenty grams of α-chitin powder was suspended in 1 L of Ca solvent and then refluxed for 6 h with constant stirring, followed by filtration to remove the insoluble material. A 50 ml of chitin solution was added dropwise into 500 ml of distilled water under vigorous stirring for 3 h at room temperature. Then, the precipitate was collected by centrifugation followed by several washing with distilled water and then homogenized by Wearing Blender. The obtained homogenized gel was dialyzed against distilled water until no calcium ion was detected in the outer solution. The water content obtained in RG was 97.0% (w/w). Gelatin of 0.13 g was added into the RG solution. The chitin/gelatin solution was agitated at 60 °C till dissolution of gelatin.

2.3. Preparation of SG with gelatin

Ten grams of β-chitin powder was suspended in 20 ml of distilled water and agitated by Warring Blender for 30 s at room temperature. The same procedure was repeated at several times by the stepwise addition of distilled water until the homogeneous gel was formed. The water content obtained in SG was 99.0% (w/w). Gelatin of 0.13 g was added into the SG solution. The chitin/gelatin solution was agitated at 60 °C till dissolution of gelatin.

2.4. Preparation of RG or SG with GlcNAc

The 0.25 g of RG or SG was mixed with as dry weight and suspended in water. Gelatin of 0.13 g was added into

the RG or SG solution. These chitin/gelatin solution was agitated at 60 °C till dissolution of gelatin. Moreover, GlcNAc of 20% (w/w), according to dry weight of chitin, was added into the chitin/gelatin solution. These suspensions were treated with autoclave at 120 °C for 2 h.

2.5. Preparation of chitin/gelatin membranes

These chitin hydrogel (RG and SG) solution with gelatin or GlcNAc with heat treatment as 0.25 g of dry weight was filtered through a saran and paper filter to remove the broad water. Resultant chitin/gelatin membranes were dried under 1 t pressure at room temperature for a day".

2.6. Swelling studies

The swelling studies of the chitin/gelatin membranes were carried out by the following method. The membranes were cut into 2 cm × 2 cm length and measured the weight (W_0). Then, the chitin/gelatin membranes were immersed in phosphate buffered saline (PBS, pH 7.2) at 37 °C. After predetermined time, the samples were removed and the weight (W_1) were measured. The swelling rate was calculated as following: swelling ratio (R) = (W_1/W_0).

2.7. Enzymatic degradation studies

The enzymatic degradation behavior of chitin/gelatin membranes was studied by the following method. The membranes were cut into 2 cm × 2 cm length and measured the weight (W_0). Then, the degradation buffer was prepared from PBS (pH adjusted to 5.2) with acetic acid and added 0.01% (w/v) lysozyme. The samples were immersed in the degradation buffer and incubated at 37 °C for 7 and 14 days. After predetermined time, the samples were removed and washed with water, dried and then measured the weight (W_1). The degradation rate was calculated from these weight as following: degradation rate % (w/w) = $(W_0 - W_1)/W_0 \times 100$.

2.8. In vitro NIH/3T3 fibroblast cell studies

The prepared chitin/gelatin membranes (0.5 × 0.5 cm) were sterilized by autoclave in 2 ml distilled water for 15 min at 121 °C. After sterilization, the distilled water was completely removed from the sterilized medium with the help of micropipette. The sterilized membranes were used for growth of fibroblast NIH/3T3 cell. Cells were grown on chitin/gelatin membranes and studied the cell attachment and viability. Each chitin/gelatin membrane was inoculated with 150 μl of cell solution (6×10^4 cells/ml). The cells were allowed to attach in static condition at 37 °C in CO_2 incubator for 4 h. After cell attachment, chitin/gelatin membranes were washed with 1× PBS to remove unattached cells and then added 5 ml of DMEM medium and incubated at 37 °C in a humidified 5% CO_2 environmental incubator for 7 days. For cell viability, specimens were washed three times with 1× PBS and incubated at 37 °C with 1 ml of 2 μg/ml Fluorescein Diacetate (FDA, Wako Pure Chemicals, Japan) in phosphate buffered saline (PBS) for 15 min to stain viable cells green. The samples were viewed under a laser scanning fluorescence microscopy (Carl Zeiss Laser Scanning Microscopy, Axiovert 200 M, LSM5PASCAL, Germany).

2.9. Measurements

The surface morphology of the chitin/gelatin membranes were studied by scanning electron microscope (SEM, JEOL JSM-6700 microscope). Tensile strength and elongation of the membranes were measured by ORIENTEC Universal testing machine STA-1150 RTC. The samples for tensile strength were cut in the following shape, 5 mm of wide and 10 mm of length, and measured more than 10 times at 3.0 mm/min rate. The thermogravimetric (TGA) and differential thermal analysis (DTA) were measured by SII TG/DTA6200 (EXSTAR 6000) at heating rate of 10 °C/min in N_2 atmosphere over a temperature range of 25–600 °C.

3. Results and discussion

3.1. Preparation of chitin membranes

The chitin/gelatin membranes were prepared by using two different chitin hydrogels RG and SG were mixed with gelatin and GlcNAc with heat treatment. The preparation data of these chitin/gelatin membranes were shown in Table 1. The chitin/gelatin membranes except s-1 and s-3 were modified with GlcNAc and heat treatment. After that, these hydrogels were fabricated for the preparation of chitin/gelatin membranes. The chitin membranes were treated with GlcNAc and heat treatment were showed slightly brown colour. These chitin membranes except s-2 were not brittle and kept in the form membranes.

3.2. Morphology studies

The SEM images of the chitin/gelatin membranes were shown in Fig. 1. It was found that surface morphology of the chitin/gelatin membranes prepared from RG and SG (s-1 and s-3) was relatively smooth and homogeneous. This may be due to the preparation of chitin/gelatin membranes

Table 1
Preparation data of chitin/gelatin membranes

Sample No.		s-1	s-2	s-3	s-4
RG	g	0.25	0.25		
SG	g			0.25	0.25
Gelatin	g	0.13	0.13	0.13	0.13
GlcNAc	% (w/w) [a]		20.00		20.00

The hydrogel with GlcNAc, s-2 and s-4, was heated at 120 °C for 2 h.
[a] The concentration was calculated from the weight of chitin.

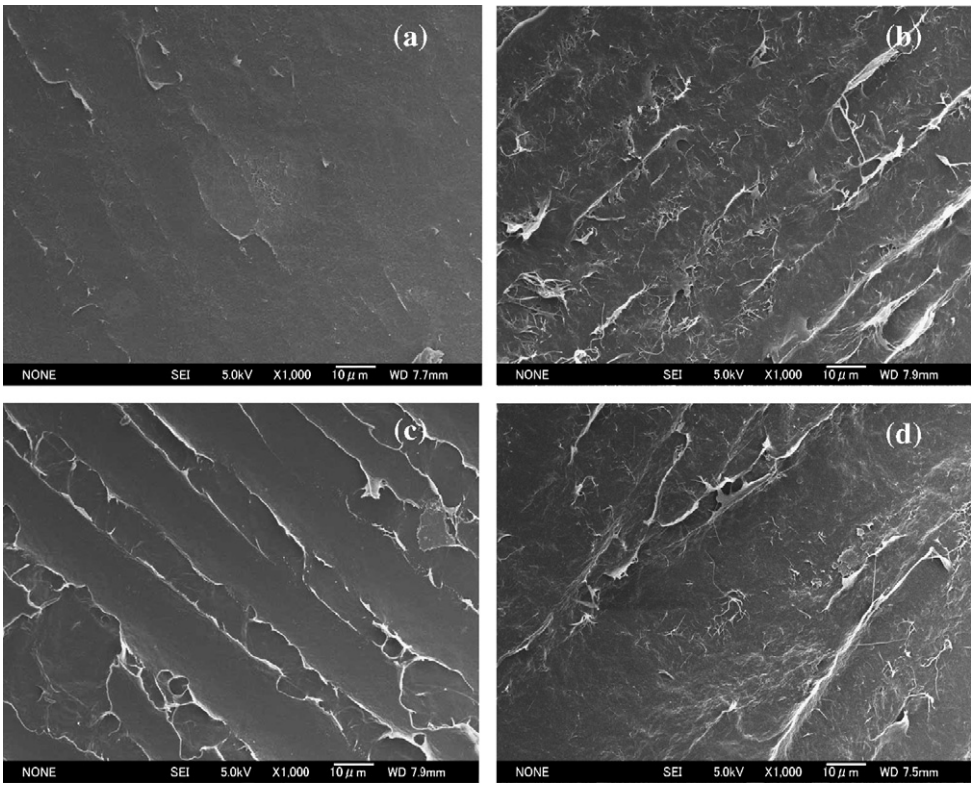

Fig. 1. SEM images of chitin membranes (a) s-1, (b) s-2, (c) s-3 and (d) s-4.

by using calcium solvent under mild conditions. In contrast, the chitin/gelatin membranes prepared from RG and SG with GlcNAc (s-2 and s-4) showed a little rough surface. It is due to the cross-linking of GlcNAc.

3.3. Tensile strength

Figs. 2 and 3 shows the tensile strength of the chitin/gelatin membranes. It was observed that the chitin/gelatin membrane with GlcNAc prepared from RG (s-2) was

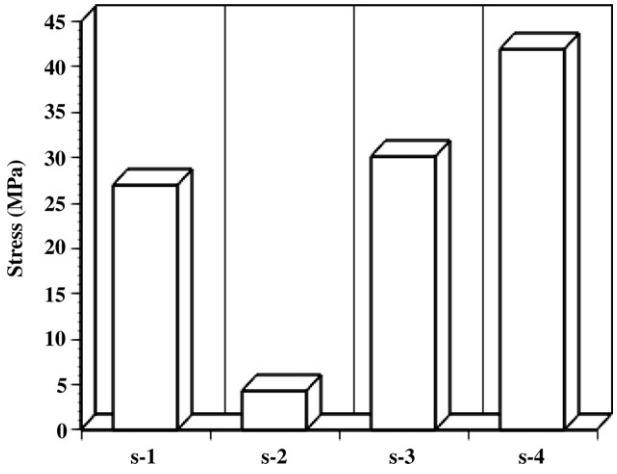

Fig. 2. Stress of prepared of chitin/gelatin membranes.

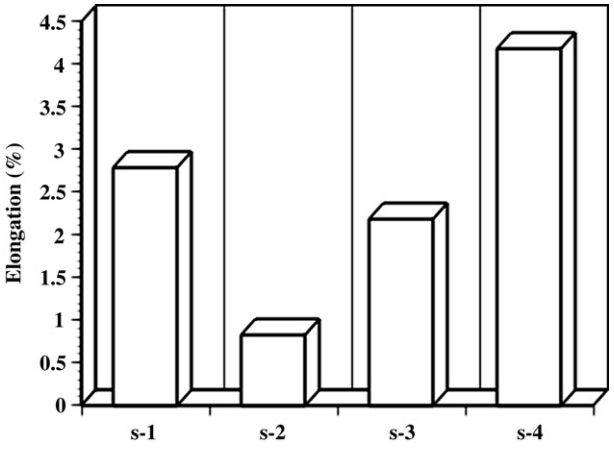

Fig. 3. Elongation of prepared chitin/gelatin membranes.

showed less stress and elongation than the chitin/gelatin membranes without GlcNAc (s-1). It is due to the excessive treatment of GlcNAc caused the cleavage in the sugar unit. The hydrogel of RG prepared by regeneration was seemed to be week due to the heat treatment with GlcNAc. And also, we observed that the chitin/gelatin membranes with GlcNAc prepared from SG (s-4) showed high stress and elongation than the chitin/gelatin membranes without GlcNAc (s-3). These results indicated that the chitin/gelatin membranes prepared from SG was very flexible and had high stress and elongation. And also, the cross-linking effect of GlcNAc was improved the tensile strength of the chitin/gelatin membranes prepared from SG.

3.4. Swelling studies

The swelling studies of the chitin/gelatin membranes with PBS were shown in Fig. 4. The swelling ratio of the chitin/gelatin membranes prepared from RG (s-1) and (s-2) was showed lower swelling than the chitin/gelatin membranes prepared from SG (s-3 and s-4). It was indicated that the β-chitin/gelatin membranes showed higher swelling than α-chitin/gelatin membranes. This phenomenon may indicate the tight adsorption of the water molecule to the β-chitin structure (Tamura, Sawada, et al., 2006; Tamura, Nagahama, et al., 2006). After 24 h, the chitin membrane prepared from SG with GlcNAc (s-4) was showed higher swelling than the chitin/gelatin membranes prepared from SG (s-3). It is due to the GlcNAc was cross-linked very well at the molecular level. So, that the cross-linking effect was influenced the swelling volume of the membranes.

3.5. Enzymatic degradation studies

The enzymatic degradation behavior of the chitin/gelatin membranes with lysozyme was shown in Fig. 5. These membranes pictures after 14 days were shown in Fig. 6. The chitin/gelatin membranes prepared from RG with or without GlcNAc (s-1 and s-2) were cracked their form after 14 days. In contrast, each of chitin/gelatin membranes prepared from SG with or without GlcNAc (s-3 and s-4) was kept the form as the sheet without highly embracement for 14 days. The degradation rate of the chitin membranes prepared from RG (s-1 and s-2) was showed higher degradation than the chitin/gelatin membranes prepared from SG. Moreover, the chitin/gelatin membranes prepared from SG with GlcNAc (s-4) were also showed lower degradation than the untreated one (s-3). So, the cross-linking effect was decreased the rate of degradation. These studies showed that all chitin/gelatin membranes were degraded by enzyme.

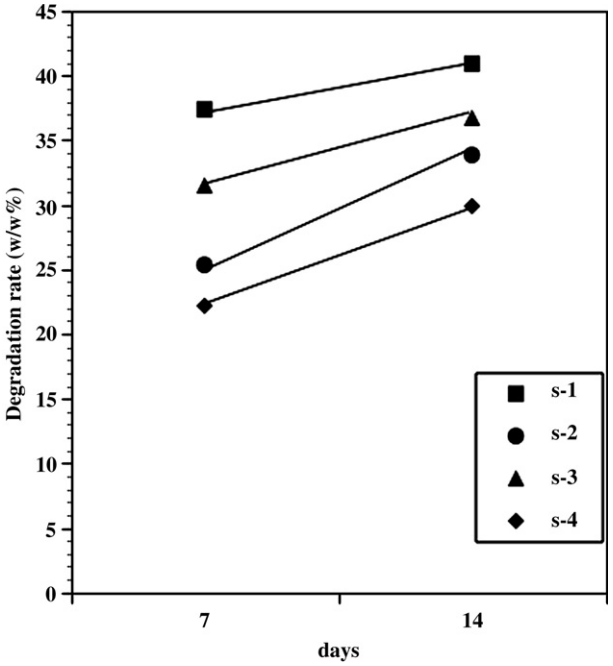

Fig. 5. The degradation behavior of chitin/gelatin membranes with lysozyme was investigated. The degradation buffer was prepared from PBS adjusted pH at 5.2 with acetic acid and added 0.01% (w/v) lysozyme.

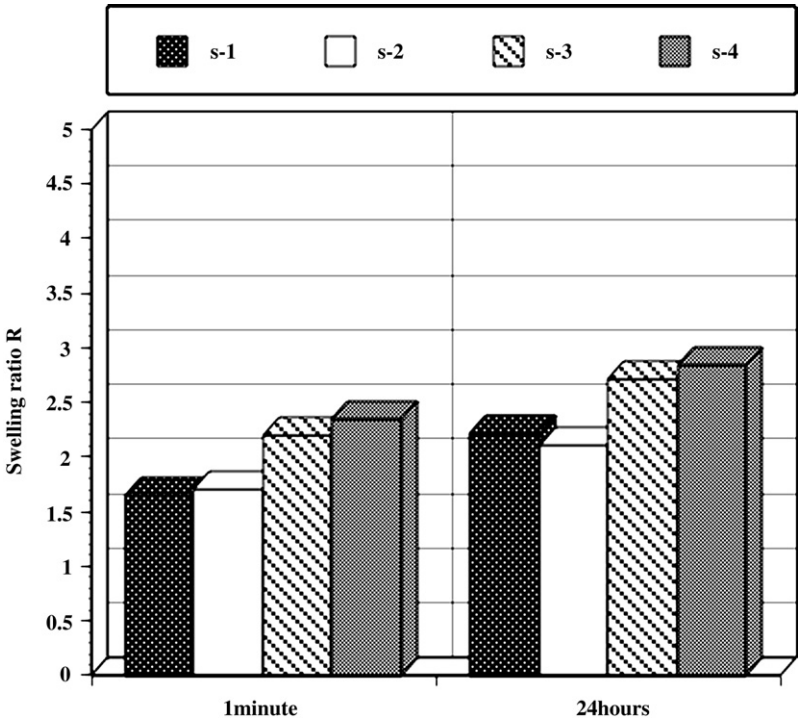

Fig. 4. The swelling studies of the chitin/gelatin membranes. They were immersed in PBS (pH 7.2) at 37 °C for 1 min and 24 h.

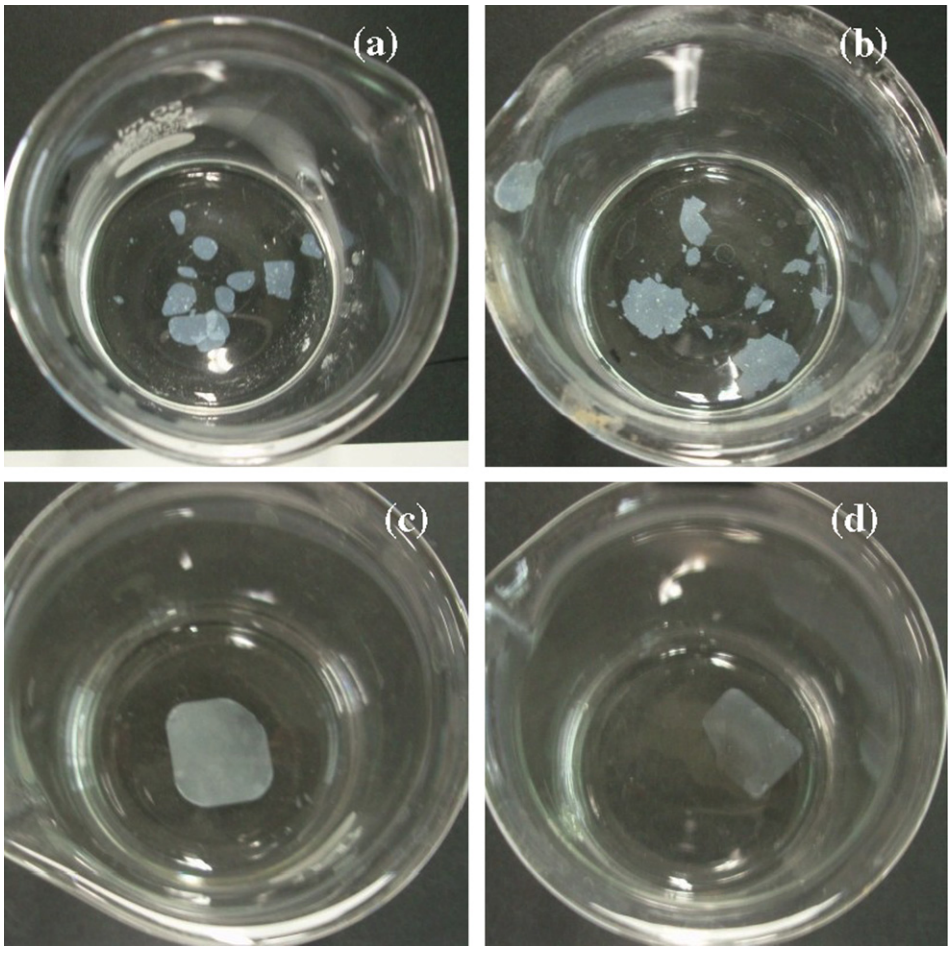

Fig. 6. The pictures of degraded chitin/gelatin membranes after 14 days (a) s-1, (b) s-2, (c) s-3 and (d) s-4.

3.6. Thermal studies

Fig. 7 shows the TGA curve of the prepared chitin/gelatin membranes. The chitin/gelatin membrane prepared from SG (s-1) and SG with GlcNAc (s-2) showed less thermal stability than other chitin/gelatin membranes prepared from RG (s-3) and with GlcNAc (s-4). The chitin/gelatin membrane prepared from SG was showed the second degradation at 250 °C, while the chitin/gelatin membranes prepared from RG was showed at 265 °C. The chitin/gelatin membranes prepared from SG degraded very faster than the chitin/gelatin membranes prepared from RG. It is due to be caused the difference in crystal structure and hydrogen bonding network of chitin hydrogel between RG and SG (Nagahama et al., in press-b).

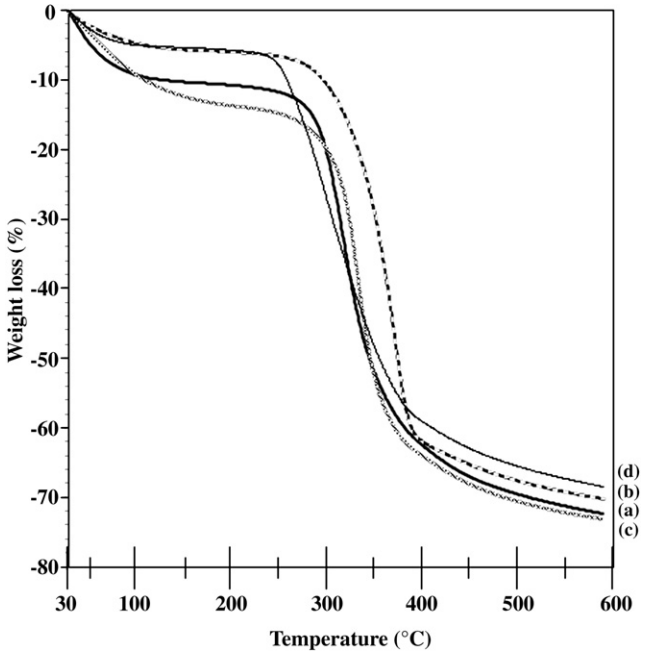

Fig. 7. TGA curve of the prepared chitin/gelatin membranes (a) s-1, (b) s-2, (c) s-3 and (d) s-4.

3.7. In vitro NIH/3T3 fibroblast cell studies

Fig. 8 shows the growth of fibroblast cells on chitin/gelatin membranes with GlcNAc and heat treatment. The life cells FDA stained cells were clearly observed on the membranes with polygonal morphology. Fibroblast cells were totaly good separated and proliferated on the surface of

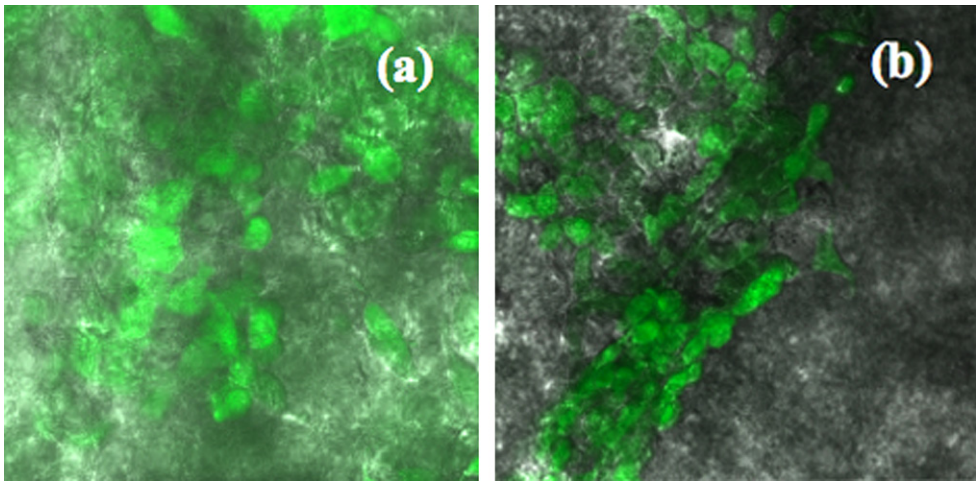

Fig. 8. The growth of NIH/3T3 fibroblast cell on the chitin/gelatin membranes (a) s-2, (b) s-4.

the each membrane. However, a little aggregation of cells was also observed on surface of the chitin/gelatin membranes with GlcNAc. Therefore it is needed to improve the properties of chitin/gelatin membranes. The improved chitin/gelatin membranes must be useful for tissue engineering applications.

4. Conclusions

Novel α- and β-chitin/gelatin membranes were prepared by using RG and SG with GlcNAc. The surface morphology of these chitin/gelatin membranes with GlcNAc was found to be little rough morphology. The chitin/gelatin membranes prepared from SG showed higher swelling than the chitin/gelatin membranes prepared from RG. In contrast, the chitin/gelatin membranes prepared from RG showed higher degradation than the chitin/gelatin membranes prepared from SG. But, the chitin/gelatin membranes with GlcNAc were showed the same tendency of swelling and degradation. These chitin/gelatin membranes with GlcNAc are showing good biodegradation, swelling, mechanical and NIH/3T3 fibroblast cell growth properties. So these biodegradable chitin/gelatin membranes with GlcNAc are useful in the tissue engineering field.

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