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Novel biodegradable chitin membranes for tissue engineering applications

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Abstract

Chitin is a natural biopolymer that has been investigated for its prospected biomedical applications due to its several biological advantages. However, the chitin is very difficult to dissolve in common solvents due to its rigid crystalline structure. But it is soluble in saturated calcium solvent system under mild conditions. By using the calcium solvent system, chitin regenerated hydrogel (RG) was prepared by using α-chitin. And we also prepared swelling hydrogel (SG) by using β-chitin with water. In addition, the SG was mixed with glutaraldehyde (GA) or *N*-acetyl D-(+)-glucosamine (GlcNAc) at 120 °C for 2 h. The chitin membranes were prepared by using RG and SG with or without GA or GlcNAc. The prepared chitin membranes were characterized by mechanical, swelling, enzymatic degradation, thermal, and growth of NIH/3T3 fibroblast cell studies. The stress and elongation of chitin membranes prepared from SG with GA or 10% (w/w) GlcNAc were increased due to the cross-linking effect. The chitin membranes prepared from SG showed higher swelling and degradation than the membranes prepared from RG. Moreover, the chitin membranes prepared from SG with GA or GlcNAc showed lower swelling and degradation than the untreated one. The thermal studies showed that the chitin membranes prepared from RG showed higher thermal stability than the other chitin membranes prepared from SG. And also, these membranes showed good growth of NIH/3T3 fibroblast cells although a little aggregation of cells was observed. So, these chitin membranes are promising biomaterials that can be useful for tissue engineering applications.

Keywords: Chitin hydrogel; Chitin membranes; Calcium solvent; Swelling ratio; Enzymatic degradation; NIH/3T3 fibroblast cell

1. Introduction

Chitin is known to be a biodegradable polymer in nature and in the animal body (Hirano et al., 1990; Sashiwa, Saimoto, Shigemasa, Ogawa, & Tokura, 1990) and to be of low toxicity when administered into animal body. For these reasons, chitin is useful for several biomedical applications (Okamoto et al., 1993; Nishimura et al., 1985). However, chitin is insoluble in general organic solvents due to its rigid crystalline structure, which is based on the hydrogen bond between the acetamide group, hydroxyl group, and carbonyl group (Austin, 1975; Delacruz et al., 1992; Kaifu, Nishi, & Tokura, 1981; Minke & Blackwell, 1978; Tamura, Sawada, Nagagama, Higuchi, & Tokura, 2006; Tamura et al., 2004). Chitin is also known to have a number of different crystalline structures dependent on its function in animal body. 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 works have been reported dissolve the chitin. But the used solvents caused to decrease the molecular weight of chitin during the dissolution procedure (Tokura, Nishi, & Noguchi, 1979). In recent years, the calcium solvent system was found to be a good solvent to dissolve the chitin in the mild conditions (Tamura et al., 2006; Tokura, Nishimura, Sakairi, & Nishi, 1996). It has also been found that the chitin hydrogel can also be prepared by using this solvent system (Jayakumar & Tamura, 2007: Nagahama, Higuchi, Jayakumar, Furuike, & Tamura, 2007; Tamura et al., 2006; Tamura, Nagahama, & Tokura, 2006).

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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 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). They also show some biological activities such as immunological, antibacterial, wound healing, drug delivery, and has been proposed for tissue engineering applications (Jayakumar et al., 2006, 2007; Jiang, Nair, & Laurencen, 2006; Rinki, Dutta, & Dutta, 2007; Verma, Verma, & Ray, 2005: Verma, Verma, Ray, & Ray, 2005; Wang et al., 2006). To improve the properties of the biopolymer, the chemical crosslinker is commonly used. In this study, the SG was mixed with GlcNAc and was modified by 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 be used for modification of polysaccharide, chitosan (Chung, Tsai, & Li, 2006; Tanaka, Huang, Chiu, Ishizaki, & Taguchi, 1993; Ueshima & Kawai, 2007). Due to its insoluble nature of chitin, its applications are limited. So that we modified the chitin into gel or membranes by using calcium solvent system for the purpose of biomedical applications. In this paper we are reporting that the chitin membranes were prepared from the different chitin hydrogels. And also, we are reporting in this paper about the morphology, swelling, enzymatic degradation, mechanical, thermal, and growth of NIH/3T3 fibroblast cell studies of these chitin membranes 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 of 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. Calcium chloride dihydrate, GlcNAc and the other chemicals, and lysozyme were purchased from Wako Pure Chemicals (Japan) and used without any further purification. The Ca solvent system has been prepared according to our previously reported method (Tamura et al., 2006).

2.2. Preparation of RG

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 washings with distilled water and then homogenized by Warring 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).

2.3. Preparation of SG

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 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).

2.4. Preparation of SG with GA or GlcNAc

The 0.25 g of SG was mixed as dry weight and suspended in water. GlcNAc of 10% or 30% (w/w), as per dry weight of chitin, was added into the chitin solution. Glutaraldehyde (GA) of 1.0% (w/w) was used for comparison with GlcNAc. These suspensions were treated in an autoclave at 120 °C for 2 h.

2.5. Preparation of chitin membranes

The chitin hydrogel (RG and SG) solution with or without GA or GlcNAc with heat treatment as 0.25 g of dry weight was filtered through a saran and paper filter to remove the excess water. Resultant chitin membranes were dried under 1t pressure at room temperature for a day.

2.6. Swelling studies

The swelling studies of the chitin membranes were carried out by the following method. The membranes were

cut into 2 cm × 2 cm length and the weight (W_0) measured. Then, the chitin membranes were immersed in phosphate-buffered saline (PBS, pH 7.2) and sodium acetate–hydrochloric acid buffer (AC-HA, pH 1.5) at 37 °C for 1 min and 24 h. After predetermined time, the samples were removed and the weight (W_1) was measured. The swelling rate was calculated as follow: swelling ratio (R) = (W_1/W_0).

2.7. Enzymatic degradation studies

The enzymatic degradation behavior of chitin membranes was studied by the following method. The membranes were cut into 2 cm × 2 cm length and the weight (W_0) measured. Then, the degradation buffer was prepared from PBS (pH adjusted to 5.2) with acetic acid and 0.01% (w/v) lysozyme added. 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 the weight (W_1) measured. The degradation rate was calculated from these weights as follows: degradation rate% (w/w) = ($W_0 - W_1$)/$W_0 \times 100$.

2.8. In vitro NIH/3T3 fibroblast cell studies

The different types of chitin 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 membranes and the cell attachment and viability studied. Each chitin membrane was inoculated with 150 μl of cell solution (6×10^4 cells/ml). The cells were allowed to attach under static condition at 37 °C in CO_2 incubator for 4 h. After cell attachment, chitin membranes were washed with 1× PBS to remove unattached cells and then added 5 ml of DMEM 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 microscope (Carl Zeiss Laser Scanning Microscope, Axiovert 200 M, LSM5PASCAL, Germany).

2.9. Measurements

The surface morphology of the chitin membranes was studied by a 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 was cut into the following shape, 5 mm wide and 10 mm long, and measured more than ten times at 3.0 mm/min rate. The thermogravimetric analysis (TGA) and differential thermal analysis (DTA) were performed by SII TG/DTA6200 (EXSTAR 6000) at a 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 membranes were prepared by using two different chitin hydrogels RG and SG with or without GA or GlcNAc. The preparation data of the chitin membranes are shown in Table 1. The chitin membranes except s-1 and s-2 were modified with GA or GlcNAc before the filtration. After that, the hydrogels were fabricated for the preparation of chitin membranes. In the case of SG it needed longer time to prepare the chitin membranes than RG because the SG was swollen highly by water. The chitin membranes treated with GA or GlcNAc showed slightly a brown colour. These chitin membranes were not brittle and kept in the form membranes.

3.2. Morphology studies

The SEM images of the chitin membranes are shown in Fig. 1. It was found that surface morphology of the chitin membranes was relatively smooth and homogeneous and they are not having any little pore on their surface. This may be due to the preparation of chitin membranes under mild conditions. The surface morphology of the membranes prepared with GA and GlcNAc also showed smooth morphology.

3.3. Tensile strength

Fig. 2 shows the tensile strength of the chitin membranes. It was observed that the stress and elongation of chitin membranes prepared from RG (s-1) were lower than those of the chitin membranes prepared from SG (s-2). It seems that the chitin membranes prepared from SG were very flexible and had high stress and elongation. Moreover, the stress and elongation of chitin membranes prepared from SG with 1.0% (w/w) GA (s-3) and SG with 10% (w/w) GlcNAc (s-4) were increased. These results

Table 1
Preparation data of chitin membranes

Sample No.	s-1	s-2	s-3	s-4	s-5
RG (g)	0.25				
SG (g)		0.25	0.25	0.25	0.25
GA % (w/w) (*1)			1.00		
GlcNAc % (w/w) (*1)				10.00	30.00

Each hydrogel except g-1 and g-2 was heated at 120 °C.
(*1), the concentration was calculated from the weight of chitin.

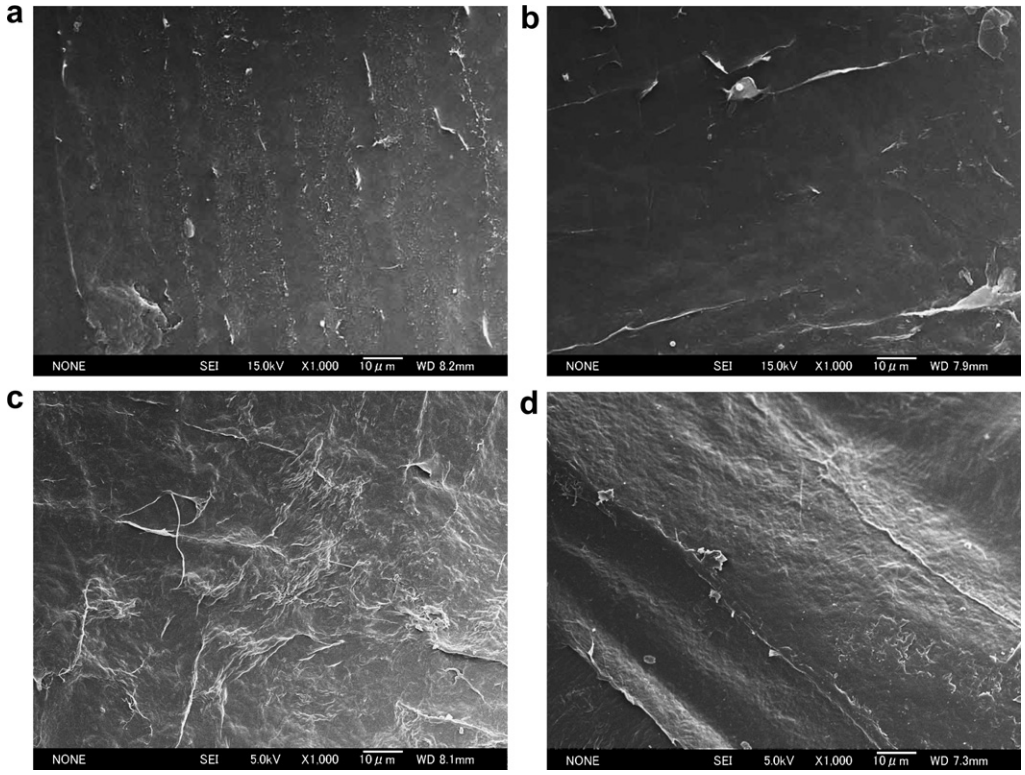

Fig. 1. SEM images of chitin membranes (a) s-1, (b) s-2, (c) s-3, and (c) s-4.

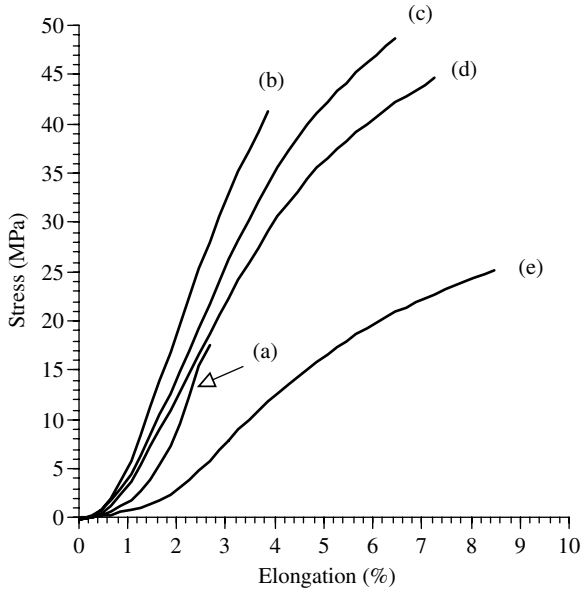

Fig. 2. The tensile strength of chitin membranes (a) s-1, (b) s-2, (c) s-3, (d) s-4, and (e) s-5.

indicated that the cross-linking effect improved the tensile strength of the chitin membranes. In contrast, the stress of chitin membranes prepared from SG with 30% (w/w) GlcNAc (s-5) was decreased much more than that of the chitin membranes prepared from SG (s-2), 1.0% (w/w) GA (s-3), and 10% (w/w) GlcNAc (s-4). It is due to the excessive amounts of GlcNAc that caused the cleavage in the sugar unit.

3.4. Swelling studies

The swelling studies of the chitin membranes with PBS are shown in Fig. 3. The swelling ratio of the chitin membranes prepared from RG (s-1) showed lower swelling than that of the other chitin membranes prepared from SG (s-2, s-3, s-4, and s-5). It indicated that the β-chitin membranes showed higher swelling than α-chitin membranes. This phenomenon may indicate the tight adsorption of the water molecule to the β-chitin structure (Tamura et al., 2006). After 24 h, the chitin membrane prepared from SG with 1% (w/w) GA (s-3) showed lower swelling than the chitin membranes prepared from SG (s-2, s-4, and s-5). It is due to the GA that crosslinked very well at the molecular level. So, the cross-linking effect influenced the swelling volume of the membranes.

The swelling ratio of the chitin membranes with AC-HA is also shown in Fig. 4. The swelling ratio of the chitin membrane prepared from RG (s-1) showed lower swelling than the other chitin membranes prepared from SG. But, the chitin membrane SG (s-2) showed higher swelling than the other chitin membranes prepared from SG with GA or GlcNAc. It seems that the cross-linking effect influenced the swelling property. The swelling ratio with AC-HA was also higher than with PBS. These results demonstrated that the swelling was higher in the acidic conditions.

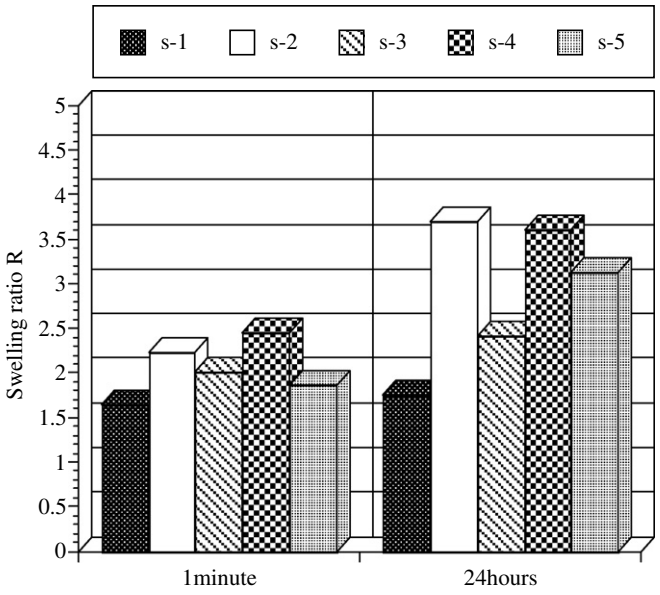

Fig. 3. The swelling studies of the chitin membranes. They were immersed in PBS (pH 7.2) at 37 °C for 1 min and 24 h.

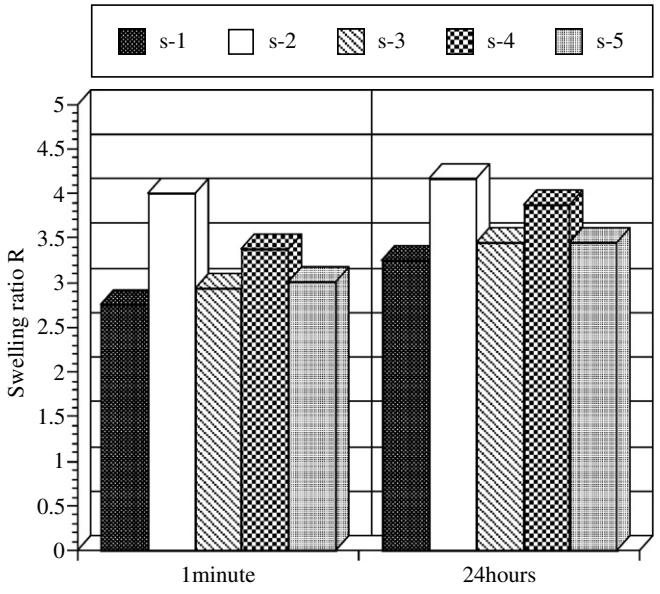

Fig. 4. The swelling studies of the chitin membranes. They were immersed in sodium acetate–hydrochloric acid buffer (AC-HA, pH 1.5) at 37 °C for 1 min and 24 h.

3.5. Enzymatic degradation studies

The enzymatic degradation behavior of the chitin membranes with lysozyme is shown in Fig. 5. Each chitin membrane kept the form as the sheet without high embracement for 14 days. The degradation rate of the chitin membranes prepared from RG (about 4% (w/w), (s-1)) showed lower degradation than the other chitin membranes. The other chitin membranes prepared from SG with GA (s-3) or GlcNAc (s-4 and s-5) also showed lower degradation than the untreated one (s-2). So, the cross-linking effect decreased

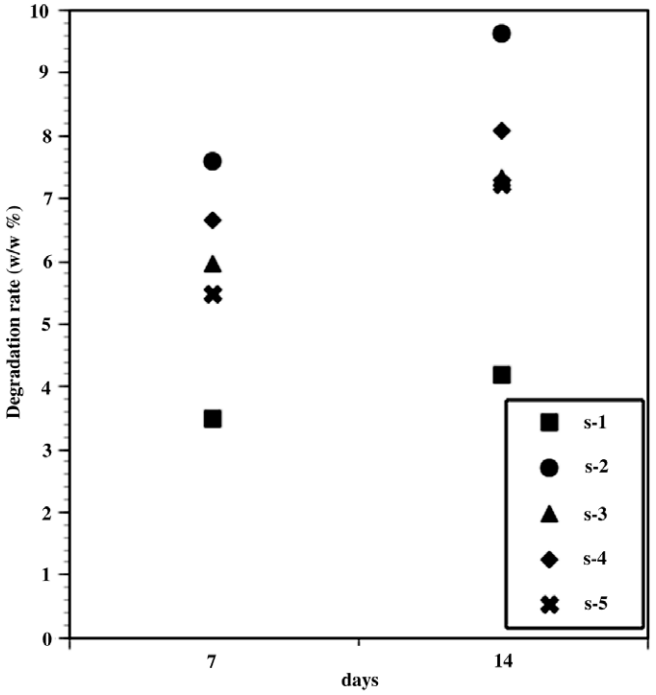

Fig. 5. The degradation behavior of chitin membranes with lysozyme was investigated. The degradation buffer was prepared from PBS adjusted pH at 5.2 with acetic acid and added 0.01% (w/v) lysozyme.

the rate of degradation. Moreover, the chitin membranes prepared from SG showed higher swelling and degradation than the chitin membranes prepared from RG. These results indicated that the α-chitin has higher rigid crystalline structure than β-chitin (Tamura et al., 2006). These studies showed that all the chitin membranes were

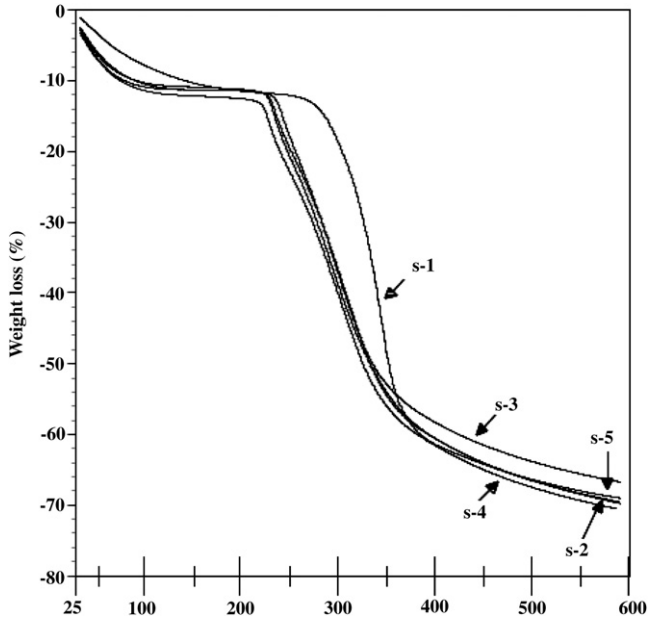

Fig. 6. TGA curve of the prepared chitin membranes.

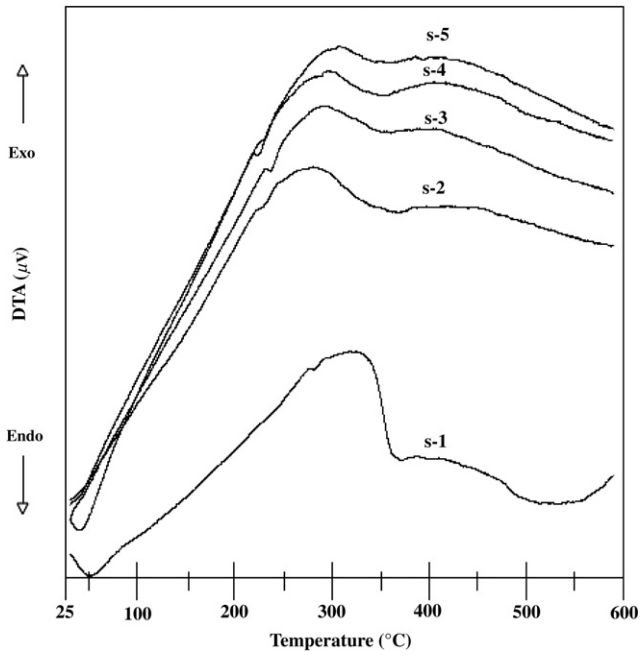

Fig. 7. DTA curve of the prepared chitin membranes.

degraded by enzyme. So, these novel chitin membranes are useful in the biomedical field.

3.6. Thermal studies

Fig. 6 shows the TGA curve of the prepared chitin membranes. The chitin membrane prepared from RG (s-1) showed higher thermal stability than all other chitin membranes prepared from SG (s-2, s-3, s-4, and s-5). The chitin membrane of RG (s-1) showed the second degradation at 257 °C, while the other chitin membranes prepared from SG showed at 220 °C. It indicated that the membranes prepared from RG showed higher thermal stability than the SG membranes. It was caused by difference in crystal structure and hydrogen bonding network of RG and SG, because the chitin membrane of RG was prepared from α-chitin (Tamura et al., 2006).

Fig. 7 shows the DTA curve of the prepared chitin membranes. Here also we found the difference between the chitin membrane prepared from RG (s-1) and other membranes prepared from SG (s-2, s-3, s-4, and s-5). DTA curve showed that the chitin membrane of RG (s-1) showed exothermic peak at 318 °C, while the other chitin membranes s-2, s-3, s-4, and s-5 from SG showed corresponding exothermic peaks at 279, 286, 299, and 308 °C, respectively. These DTA results also indicated that the chitin membranes prepared from RG showed higher thermal stability than the membranes prepared from SG. In this

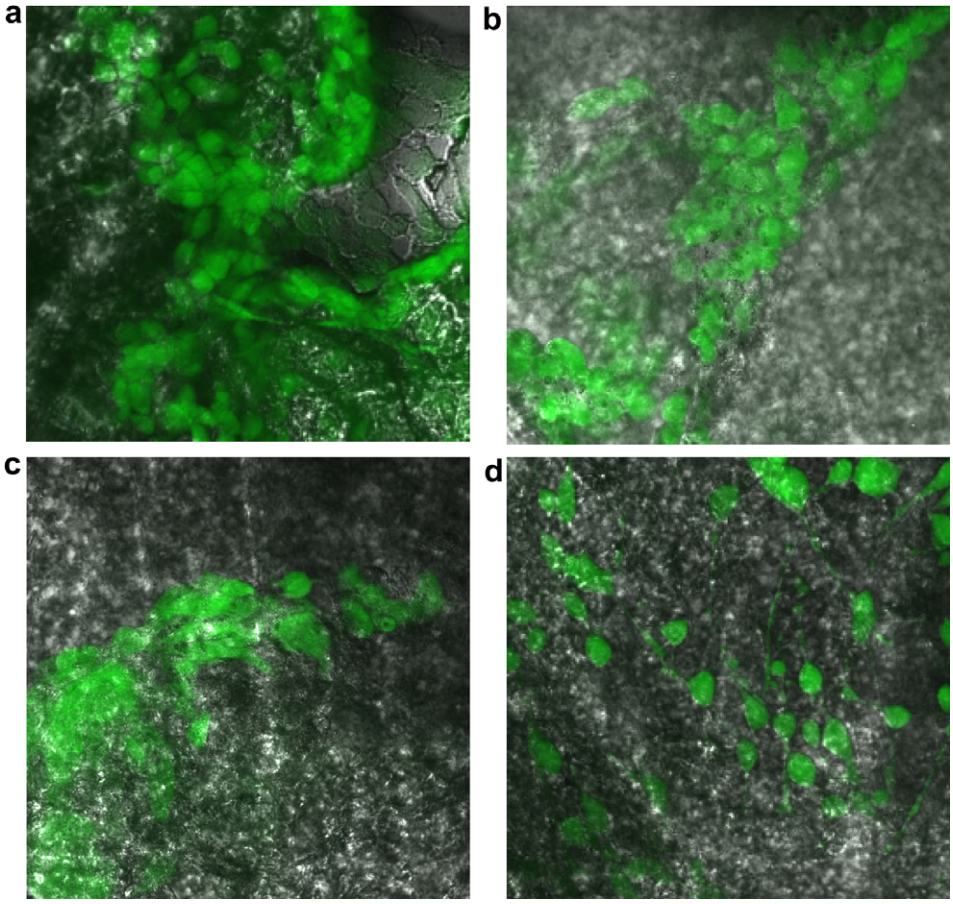

Fig. 8. The growth of NIH/3T3 fibroblast cell on the chitin membranes, (a) s-1, (b) s-2, (c) s-3, and (d) s-4.

case, the exothermic peaks of chitin membrane sheets prepared from SG with GA and GlcNAc (s-3, s-4, and s-5) were slightly shifted to higher temperature than the SG chitin membrane (s-2). It is due to the cross-linking effect making the difference in the shifting of the exothermic peaks.

3.7. In vitro NIH/3T3 fibroblast cell studies

Fig. 8 shows the growth of fibroblast cells on chitin membranes. The life cells FDA stained cells were observed on the membranes with polygonal morphology. Fibroblast cells were totally well separated and proliferated on the surface of each membrane. However, an aggregation of cells was also observed on some part of the chitin membranes. Therefore it is needed to improve the properties of chitin membranes. The improved chitin membranes must be attractive for tissue engineering applications.

4. Conclusions

Novel α- and β-chitin membranes were prepared by using RG and SG with or without GA or GlcNAc. The morphology of these chitin membranes was found to be very smooth and homogeneous. The stress and elongation of chitin membranes prepared from SG with GA and 10% (w/w) GlcNAc increased from untreated one due to the cross-linking effect. The chitin membranes prepared from SG showed higher swelling and degradation than the chitin membranes prepared from RG. Moreover, the chitin membranes prepared from SG with GA or GlcNAc showed lower swelling and degradation than untreated one. These chitin membranes showed biodegradation, swelling, mechanical, and NIH/3T3 fibroblast cell growth properties. So these novel biodegradable chitin membranes are promising biomaterials in the tissue engineering field.

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