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Preparation of Chitinous Compound/Gelatin Composite and Their Biological Application

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Summary: Chitin, a natural abundant polysaccharide, have been investigated as prospected biochemical material due to its several biological advantages. It is insoluble in the most of the organic solvents due to its rigid crystalline structure. However, chitin regenerated hydrogel (RG) has been prepared by using the saturated calcium solvent system under mild conditions. And also, swelling hydrogel (SG) was prepared by using water. In this study, we prepared the suspension of chitinous hydrogel, and applied to fabricated the chitinous compound/gelatin composite sheets. Additionally, *N*-acetyl *D*-(+)-glucosamine was added into some composite sheets. We investigated the mechanical properties and growth of NIH/3T3 fibroblast cell for the prepared composite sheet.

Keywords: calcium solvent; chitin hydrogel; chitinous/gelatin composite sheet; flow curve; tensile strength

Introduction

Chitin is known to be biodegradable polymer in nature and in the animal body[1–3], and it is nontoxic when administrated into animal body within the range of dosages. Chitin can be easily degradated by lysozyme, which present in our body fluid. However, chitin is insoluble in general solvents due to its high crystallinity, which is based on the hydrogen bond through the acetamide group and hydrogen bonds[4–6]. Alhough several research work have been reported to dissolve chitin, the solvents caused to decrease the molecular weight of chitin during the dissolution procedure. In recent years, calcium chloride dihydrate saturated methanol was found to be the solvent for chitin under mild condition[7–8]. It has also been found that the chitin hydrogel could be prepared by using this solvent system[9].

Gelatin is 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[10]. The predominant property of gelatin would be the Sol-Gel transition under aqueous condition.

In this research, gelatin was blended with chitin gel and chitinous compoubd/gelatin composite sheets were prepared. Moreover, *N*-acetyl *D*-(+)-glucosamine (GlcNAc) was added and treated under heat treatment for the effect of cross-linking. The resultant chitinous compound/gelatin composite sheets were characterized and studied the biological activity of sheets by grown the fibroblast cells on the sheets.

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Experimental Part

Materials

α- and β- chitin were kindly supported by Koyo Chemical Co. Ltd. Chemicals. Calcium chloride dihydrate saturated methanol solvent (Ca solvent) have been prepared according to the reported method[9]. 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 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. The water used was double distilled water using the RFD 240NA water distillation apparatus (Advantec, Model RF 100170, serial No. 231042, Toyo seisakusho Kaisha, LTD., Japan). All other chemicals were an analytical grade purchased from Wako Chemical Co. (Japan) and were used without any further purification.

Preparation of Regenerated Hydrogel (RG)

20 g of α- and β-chitin powder was suspended in 1 L of Ca solvent and refluxed for 6 h with stirring, followed by filtration to remove 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. Arter 3 h, the precipitate was collected by centrifugation followed by several washing with distilled water and then homogenized by Wearing Blender. The homogenized gel was formed and then dialyzed against distilled water until no calcium ion was detected in outer solution.

Preparation of Swelling Hydrogel (SG)

10 g of β-chitin powder was suspended in 20 ml of distilled water and agitated by Warring Blender for 30 seconds at room temperature. The procedure was repeated several times by the stepwise addition of distilled water until the homogeneous gel was formed.

Preparation of Chitinous/Gelatin Composite Sheets

The hydrogel of RG and SG was mixed with 0.20 g as dry weight and suspended in distilled water. 0.1 g of gelatin was dissolved in distilled water at 60 °C and added to the chitinous solution suspended in distilled water. Then 20% (w/w) (according to chitin weight) of GlcNAc was added into the same suspension. Furthermore, the suspension was treated with autoclave at 120 °C for 2 h. The hydrogel solution was filtered though a saran and paper filter to remove the water. Resultant sheet was dried under pressure at room temperature for one day.

Measurements

X-ray diffraction (XRD) patterns were recorded using Rigaku R-AXIS IV. The X-rays were generated at 40 KV and 60 mA using nickel filtered CuKα radiation. The surface morphology of the samples were studied by scanning electron microscope (SEM, JEOL JSM-6700 microscope). Tensile strength and elongation 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 ten times at 3.0 mm/min rate. The flow curve of chitinous compounds were measured by Rheometer (Haake, Rheo Stress 600) and the setting of shear rate was from 0 to 50(1/s).

In Vitro Cultivation of Fibroblasts on Composite Sheets

The different types of chitinous sheet (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 sheets were used for growth of fibroblast NIH/3T3 cell. Cells were grown on chitin sheet and studied the cell attachment and viability. Each chitin sheet 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 sheets were washed with 1 x 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 x 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).

Results and Discussion

Preparation of Chitinous/Gelatin Composite sheets

The chitinous compound/gelatin composite sheets were prepared. Figure 1 shows the SEM images of α- and β-chitin regenerated hydrogel/gelatin sheet (α- and β-RG/GS) and β-chitin swollen hydrogel/gelatin sheet (β-SG/GS). The SEM of all samples were shown that the surface morphology of sheet was relatively smooth and homogeneous. The XRD studies was indicated that α-crystalline structure was suggested both chitin sheets prepared from α- and β- chitin RG. But, β-crystalline structure was suggested in the sheet prepared by β-chitin sheet from SG. It is due to the β-crystalline structure of RG was converted to α-crystalline though Ca solvent which was strongly influenced with hydrogen bond.

Mechanical Properties

The chitinous/gelatin composite sheet with GlcNAc were prepared as shown in Table 1. The hydrogel and sheets turned the color slightly smoky brown due to the addition of GlcNAc when they heated. Figure 2 shows the result of flow curve for β-SG/gelatin composite hydrogel with or without GlcNAc or heat treatment. Heat treated β- SG/gelatin composite hydrogel with GlcNAc showed stronger mechanical properties than other composite hydrogel. It seemed that some kind of the cross-linking effect was found in the result of flow curve. The tensile strength for β- SG/gelatin composite with or without GlcNAc (sample No.4 or 3) is shown in Figure 3. It seemed that those sheets prepared by the SG solution added gelatin and GlcNAc were physically- altered and improved. Meanwhile, the mixture of α-RG/gelatin without or with GlcNAc (sample No.1 and 2) was obtained as fragile gel and sheet when they were heated. The tensile strength of these samples were also very weak because these treatment condition was too strong and

Table 1.
The content of chitinous/gelatin composite sheet with or without GlcNAc.

No	The content of the sheet as dry weight (g)			
	α-RG	β-SG	Gelatin	GlcNAc
1	0.20		0.10	
2	0.20		0.10	0.04
3		0.20	0.10	
4		0.20	0.10	0.04

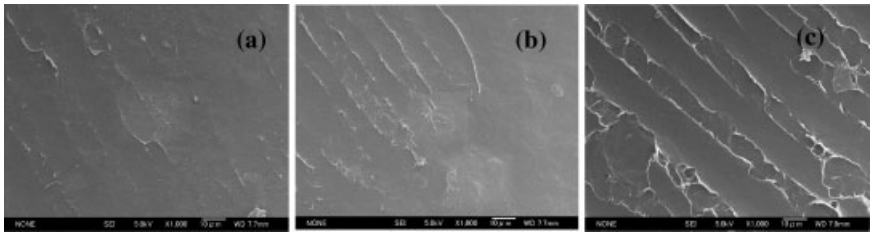

Figure 1.
SEM images of α and β-chitin regenerated hydrogel/gelatin sheet. These are SEM images on surface at 1000-fold magnification. The samples are described as follows. (a) α-RG/GS; (b) β-RG/GS; (c) β-SG/GS.

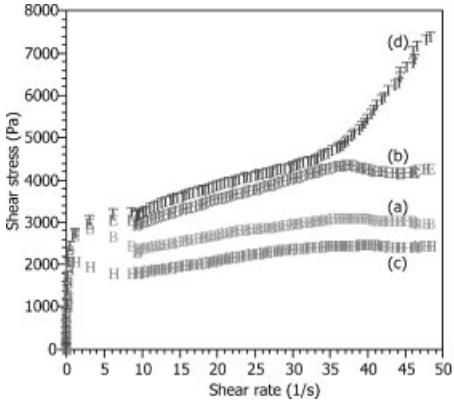

Figure 2.
Flow curve of β-SG/gelatin hydrogel with an added GlcNAc. The water content of hydrogel was 99.0% (w/w). The sample of hydrogel was described as follows (a) Unheated β-SG/gelatin composite hydrogel without GlcNAc (Unheated sample No.3); (b) Heated β-SG/gelatin composite hydrogel without GlcNAc (Heated sample No.3); (c) Unheated β-SG/gelatin composite hydrogel with GlcNAc (Unheated sample No.4); (d) Heated β-SG/gelatin composite hydrogel with GlcNAc (Heated sample No.4).

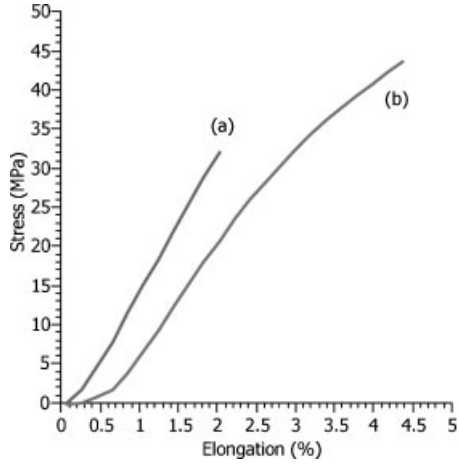

Figure 3.
The tensile strength of β-SG/gelatin composite sheet prepared from with heat treatment. The condition of β-SG was described as follows (a) Heated β-SG/gelatin composite sheet without GlcNAc (sample No.3) (b) Heated β-SG/gelatin composite sheet with GlcNAc (sample No.4).

occured cleavage in the molecule. The improvement of tensile strength for α-RG/gelatin composite with GlcNAc is in progress and it will be reported in future article.

Cell Culture Study

Figure 4 shows the growth of NIH/3T3 fibroblast cell on the sheets. The life cells FDA stained cells were observed on the sheets with polygonal morphology. Fibroblast cells were separated and proliferated on the surface of the sheels. However some aggregation of cells were observed on some part of the compositive sheets. Therefore, it is necessary to improve the properties of composite sheets. The improve composite sheets might be attractive for tissue engineering application.

Conclusions

The chitinous/gelatin composite sheets were prepared from chitin hydrogel. These

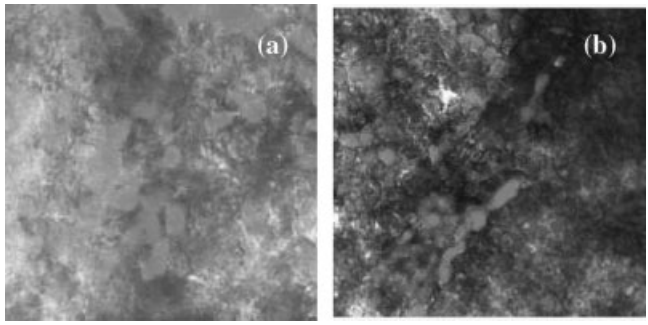

Figure 4.
The growth of fibroblast cell on chitinous sheet. The sample was described as follows, (a) α-RG/GS with GlcNAc (sample No.2); (b) β-SG/GS with GlcNAc (sample No.4).

sheets were characterized by XRD, SEM and tensile strength. The XRD studies were indicated in that α-crystalline structure seems to be the most stable crystalline structure. The addition of GlcNAc into chitinous sheets influenced the mechanical properties of the sheets. These chitinous/gelatin composite sheet showed growth of fibroblast NIH/3T3 cells. Therefore, these composite sheets are very useful for biomaterial applications.

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