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Preparation and characterization of novel chitosan/gelatin membranes using chitosan hydrogel

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ABSTRACT

Chitin and chitosan are novel biomaterials. The novel chitosan/gelatin membranes were prepared using the suspension of chitosan hydrogel mixed with gelatin. The prepared chitosan/gelatin membranes were characterized by scanning electron microscopy (SEM), X-ray diffraction (XRD), mechanical, swelling, and thermal studies. The morphology of these chitosan/gelatin membranes was found to be very smooth and homogeneous. The XRD studies showed that the chitosan/gelatin membranes have good compatibility and interaction between the chitosan and gelatin. The stress and elongation of chitosan/gelatin membranes on wet condition showed excellent when the mixture ratio of gelatin was 0.50. The prepared chitosan/gelatin membranes showed good swelling, mechanical and thermal properties. Cell adhesion studies were also carried out using human MG-63 osteoblast-like cells. The cells incubated with chitosan/gelatin membranes for 24 h were capable of forming cell adhesion. Thus the prepared chitosan/gelatin membranes are bioactive and are suitable for cell adhesion suggesting that these membranes can be used for tissue-engineering applications. Therefore, these novel chitosan/gelatin membranes are useful for biomedical applications.

1. Introduction

Chitosan is derived from chitin, a natural abundant substance found in the exoskeletons of insects, shells of crustaceans, and fungal cell walls. Chitosan has been studied as biomedical materials due to its wound healing effect, hemostasis, biocompatibility, biodegradability, antimicrobial activity, and so on (Hirano et al., 1990; Izume & Ohtakara, 1987; Mori et al., 1997; Okamoto et al., 1993; Tanigawa, Tanaka, Sashiwa, Saimoto, & Shigemasa, 1992; Tokura, Ueno, Miyazaki, & Nishi, 1997). For these reasons, chitosan is biomedically very valuable material. Chitosan is generally soluble in acids although it has crystalline structure and several hydrogen bonds (Ogawa, 1991; Okuyama, Nioguchi, Miyazawa, Yui, & Ogawa, 1997). A lot of studies have been reported about the chemical modification of chitosan which was regenerated into fiber, membrane and beads, because of its good solubility in acids (Jayakumar, Prabaharan, Reis, & Mano, 2005; Jayakumar, Reis, & Mano, 2006a, 2006b, 2007a, 2007b; Jayakumar, Nwe, Tokura, & Tamura, 2007c; Jayakumar, Nagahama, Furuike, & Tamura, 2008; Rinki, Dutta, & Dutta, 2007; Wang et al., 2006).

Gelatin is also biocompatible protein, and when it takes in living body, it shows low antigenicity 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 composite films prepared from chitosan and gelatin for biomedical applications have been reported (Arvanitoyannis et al., 1998; Kolodziejska, Piotrowska, Bulge, & Tylingo, 2006). However, most of these films were prepared by casting method using chitosan/gelatin solution in acetic acid. Recently, chitin hydrogel was prepared from calcium solvent systems (Jayakumar & Tamura, 2008; Nagahama, Higuchi, Jayakumar, Furuike, & Tamura, 2008a; Nagahama, et al., 2008b; Nagahama, Nwe, Jayakumar, Furuike, & Tamura, 2008c; Tamura, Nagahama, & Tokura, 2006). The chitin/gelatin membranes was also prepared by using chitin hydrogel reported by our group (Nagahama et al., 2008c, 2008d). In this paper, we are reporting the preparation of chitosan hydrogel and chitosan/gelatin membranes prepared from the chitosan hydrogel. The present paper clearly describes about the surface morphology, crystallinity, swelling, mechanical, thermal and cell attachment studies of chitosan/gelatin membranes.

2. Experimental

2.1. Materials

Chitosan (FM-80) was received from Koyo Chemical Co. Ltd. Gelatin and other chemicals were purchased from Wako Chemical

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Table 1
Preparation data of chitosan/gelatin membranes.

Sample		m-1	m-2	m-3	m-4
Chitosan	W_c (g)	0.30	0.50	0.70	1.00
Gelatin	W_g (g)	0.70	0.50	0.30	0
r	(1[a])	0.30	0.50	0.70	1.00

[a] The mixture ratio was calculated as follows; $r = W_c/(W_c + W_g)$.

Co. (Japan) and used without any further purification. Gelatin and other chemicals were purchased from Wako Chemical Co. (Japan) and used without any further purification. Human immortalized osteoblast-like cells MG-63 were purchased from NCCS Pune.

2.2. Preparation of chitosan hydrogel

A mass of 1.0 g chitosan was suspended with Blender in 1.0 L of 2.0% (w/v) acetic acid solution. Then, 10.0% (w/v) sodium hydroxide was slowly added into chitosan solution till pH of the solution reached to 10–12. After that, the obtained hydrogel was dialyzed against distilled water until the outer solution was neutralized. After the dialysis, the chitosan hydrogel was separated by centrifugation. The water content of chitosan hydrogel was 97.3% (w/w).

2.3. Preparation of chitosan/gelatin solution

The gelatin was dissolved in 20 ml of distilled water at 50 °C. Then, the chitosan hydrogel was mixed with the gelatin solution and agitated at 50 °C in the different ratios. The mixing ratio of gelatin and hydrogel is shown in Table 1.

2.4. Preparation of chitosan/gelatin membranes

The chitosan/gelatin solution (different ratio) was filtered through a saran and paper filter to remove the broad water. Resultant chitosan/gelatin membranes pressed under 1T pressure and dried at room temperature for a day.

2.5. Swelling studies

The swelling studies of the chitosan/gelatin membranes were carried out by the following method. The membranes were cut into 2 × 2 cm length and measured the weight (W_0). Then, the chitosan/gelatin membranes were immersed in distilled water and 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 using the following equation:

Swelling ratio (R) = $(W_1 - W_0/W_0) \times 100$.

2.6. Dissolution behavior of gelatin

The dissolution behavior of gelatin from the chitosan/gelatin membrane (m-2, $r = 0.50$) was carried out by the following method. The membranes were cut into 2 × 2 cm length and measured the weight (W_0). Then, the chitosan/gelatin membranes were immersed in distilled water heated at 80 °C for 24 h. After the time, the samples were sufficiently dried and the weight (W_1) were measured. The dissolution ratio of the membranes was calculated using the following equation:

The dissolution ratio (R) = $(W_1/W_0) \times 100$.

2.7. Cell attachment studies

The chitosan/gelatin membranes of size 5 mm^2 were used for the cell study. Prior to cell culture work, the samples were surface sterilized by immersing in 70% alcohol for 30 min and kept under UV for 1 h. The samples were then pre-treated by immersing in Phosphate Buffer Saline–EDTA (PBS–EDTA) for 1 h and kept

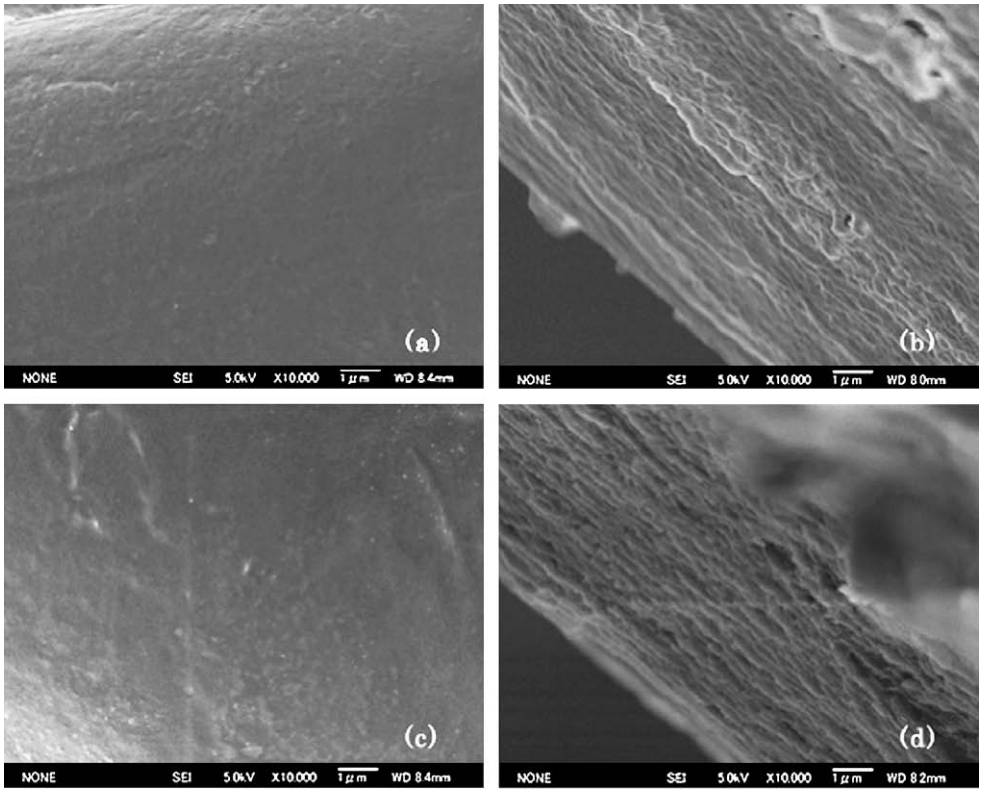

Fig. 1. SEM images of (a) Surface morphology of chitosan/gelatin membrane (m-2, $r = 0.50$), (b) Cross-section morphology of chitosan/gelatin membrane (m-2, $r = 0.50$) membrane, (c) Surface morphology of chitosan membrane (m-4, $r = 1.00$), (d) Cross-section morphology of chitosan membrane (m-4, $r = 1.00$).

immersed in 10% MEM 1 h. After the pre-treatment, the samples were carefully placed in 96 well plates and the cells were seeded at a density of 5000 viable cells/well. The morphology of the cells seeded on the membranes was investigated after 96 h of incubation with a scanning electron microscope. For preparing SEM analysis, the samples were placed in PBS–EDTA solution and rinsed quickly. The samples were subsequently fixed using 4% gluteraldehyde in PBS for 1 h and dehydrated through a graded series of alcohol (20%, 40%, 60%, 80% and 100%) for 10 min each and air-dried. The samples were sputter coated with platinum and the cell morphology was examined using SEM.

2.8. Measurements

The surface morphology of the chitosan/gelatin membranes were studied by scanning electron microscope (SEM, JEOL JSM-6700 microscope). X-ray diffraction (XRD) patterns were recorded using Rigaku RINT-2000. The X-rays were generated at 40 KV and 20 mA using nickel-filtered Cu Kα radiation. The 2θ (deg) of each peak in the range 0–40° on the equatorial peak and the meridian of these membranes was measured for 10 min. 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 on the dry or wet condition for the sample. The wet condition samples were prepared by immersed them into water for 2 min followed by removing the excess of water. The thermogravimetric (TG) and differential thermal analysis (DTA) was 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 chitosan/gelatin membranes

The chitosan/gelatin membranes were prepared using chitosan hydrogel with different amounts of gelatin (Table 1). Chitosan content (r) symbolized r was calculated as below; $r = W_c/(W_c + W_g)$, where W_c was weight of chitosan (g) and W_g was weight of gelatin (g). The chitosan hydrogel was added with gelatin before filtration. After that, the mixed chitosan/gelatin hydrogel was fabricated for the preparation of chitosan/gelatin membranes. The chitosan/gelatin membranes with gelatin were softer than the chitosan membranes without gelatin. These chitosan/gelatin membranes and chitosan membranes were not brittle.

3.2. Morphology studies

The SEM images of the chitosan/gelatin membrane (m-2) and chitosan membrane (m-4) were shown in Fig. 1. It was found that the surface morphology of the chitosan/gelatin membranes and chitosan membrane were relatively smooth surface. The cross-section morphologies of these membranes were not also rough. The large pores were not observed on the surface and cross-section of these membranes. This smooth morphology was due to the presence of chitosan hydrogel in the membranes. These results indicated that the gelatin and chitosan hydrogel were mixed well in the molecular level.

3.3. XRD studies

The XRD pattern of chitosan/gelatin membranes was shown in Fig. 2. All of these showed the main diffraction peaks around around $2\theta = 20°$ in the XRD pattern. The XRD results suggested that there were good compatibility and interaction between gelatin and chitosan molecules in the membranes. When gelatin component was added into chitosan membranes, the peak intensity ratio of chitosan membrane was reduced. The above results indicate the decreasing of crystallinity of chitosan. It was due to the incorporation of amorphous gelatin into chitosan hydrogel. A little decrease

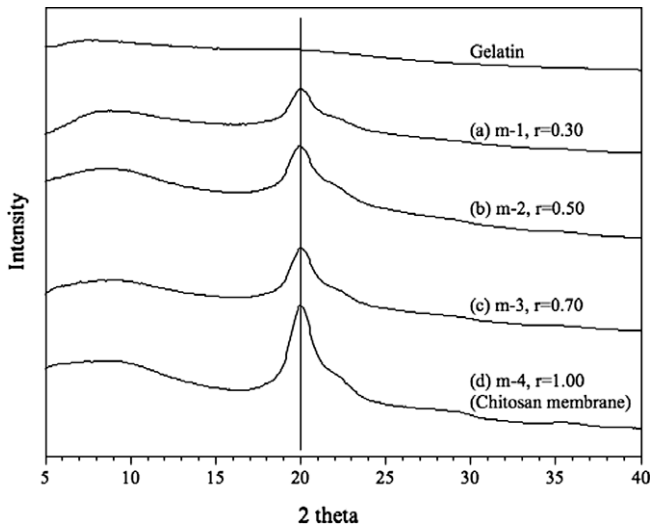

Fig. 2. XRD images of chitosan/gelatin membranes, (a) m-1 ($r = 0.3$), (b) m-2 ($r = 0.5$), (c) m-3 ($r = 0.7$) and (d) m-4 ($r = 1.0$).

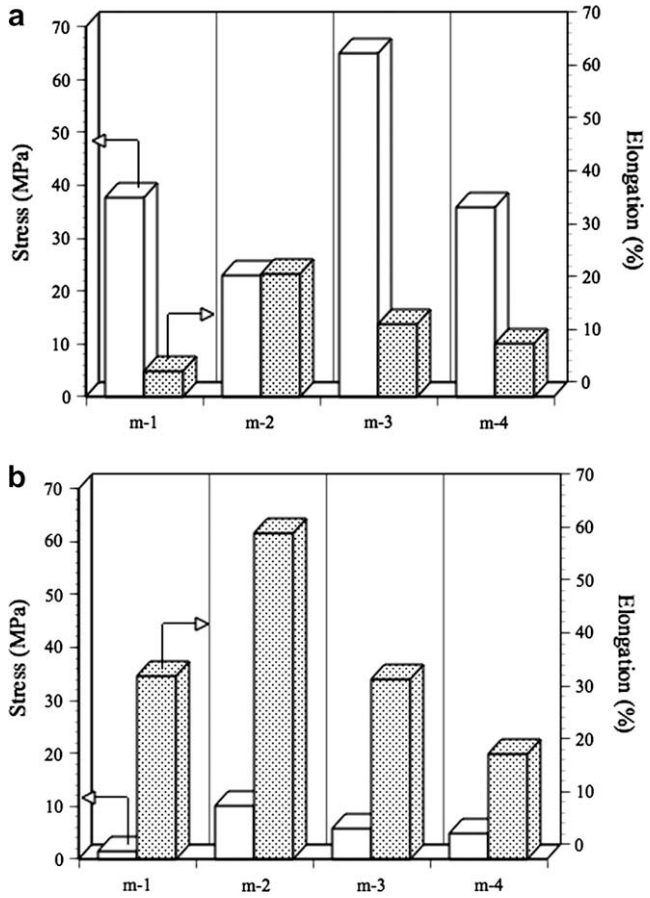

Fig. 3. Stress and elongation of chitosan/gelatin membranes on (a) dry condition and (b) wet condition.

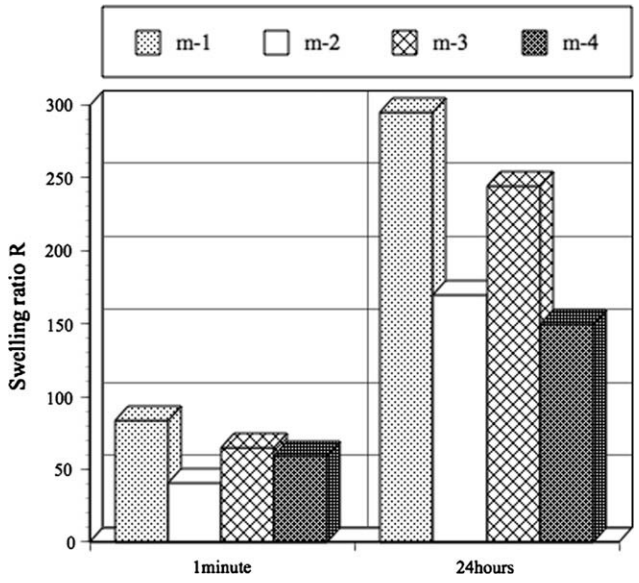

Fig. 4. The swelling studies of the chitosan/gelatin membranes. They were immersed in PBS (pH 7.2) at 37 °C after 1 min and 24 h.

in the crystallinity of chitosan/gelatin membranes is due to the hydrogen bonding between gelatin, which leads to their good compatibility (Cheng et al., 2003; Zhai, Zhao, Yoshii, & Kume, 2004).

3.4. Tensile strength

Fig. 3 shows the tensile strength of chitosan/gelatin membranes in dry (Fig. 3a) and wet conditions (Fig. 3b). It was observed that the stress (m-3) and the elongation of chitosan/gelatin membranes (m-2) was higher than the other membranes. Especially, the membrane (m-2, $r = 0.50$) had a certain amount of high stress with strong strain. Moreover, the stress and elongation of membrane (m-2, $r = 0.50$) was higher than the other membranes on wet condition. It seems that the chitosan/gelatin membranes prepared from the same content of chitosan and gelatin were flexible and had high stress and elongation on wet condition. For the biomedical applications, the physical strength on wet condition is very important. These results indicated that these membranes are useful in biomedical applications.

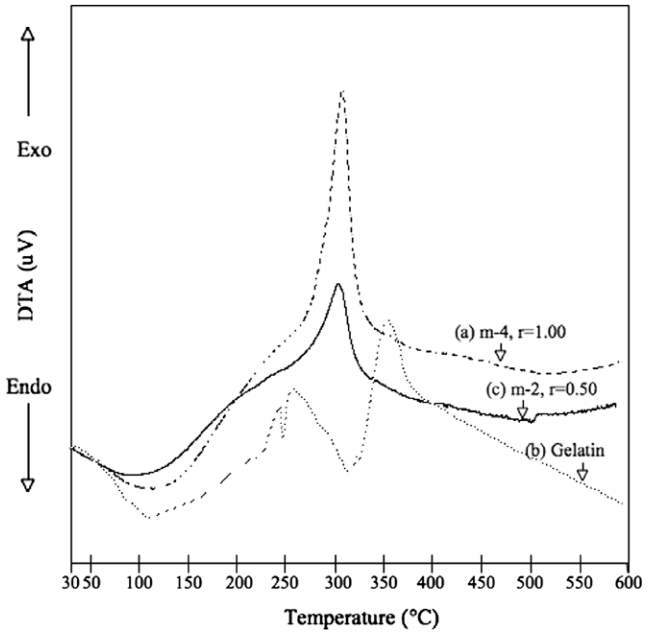

Fig. 6. DTA curve of (a) chitosan membrane (m-4), (b) gelatin and (c) chitosan/gelatin membrane (m-2).

3.5. Swelling studies

The swelling studies of the chitosan/gelatin membranes in PBS were shown in Fig. 4. The chitosan/gelatin membranes showed virtually constant degree of swelling ratio on 1 min and 24 h. The membranes (m-2, $r = 0.50$) showed lower swelling degree than other chitosan/gelatin membranes. It seemed that it had stronger hydrogen bonds among the other chitosan/gelatin membranes.

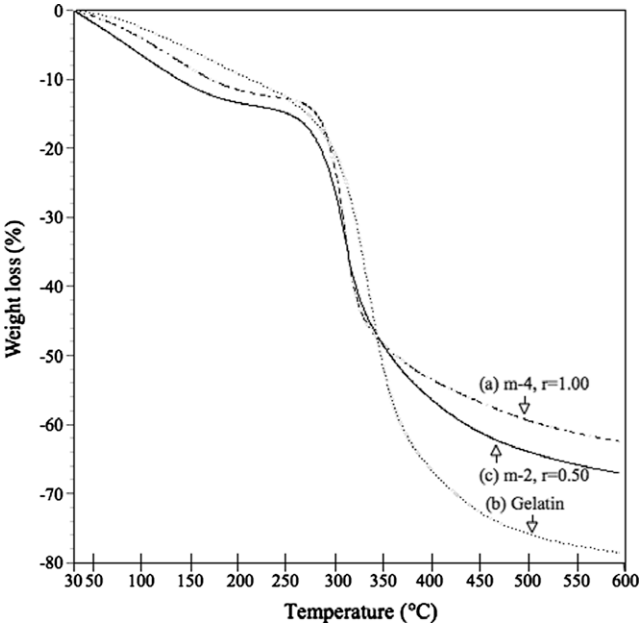

Fig. 5. TGA curve of (a) chitosan membrane (m-4), (b) gelatin and (c) chitosan/gelatin membrane (m-2).

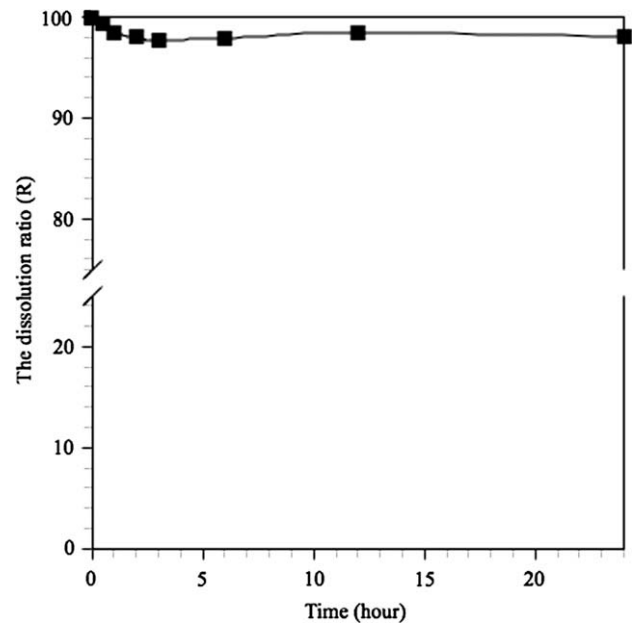

Fig. 7. The dissolution behavior of chitosan/gelatin membrane (m-2, $r = 0.50$). They were immersed in distilled water heated at 80 °C for 24 h.

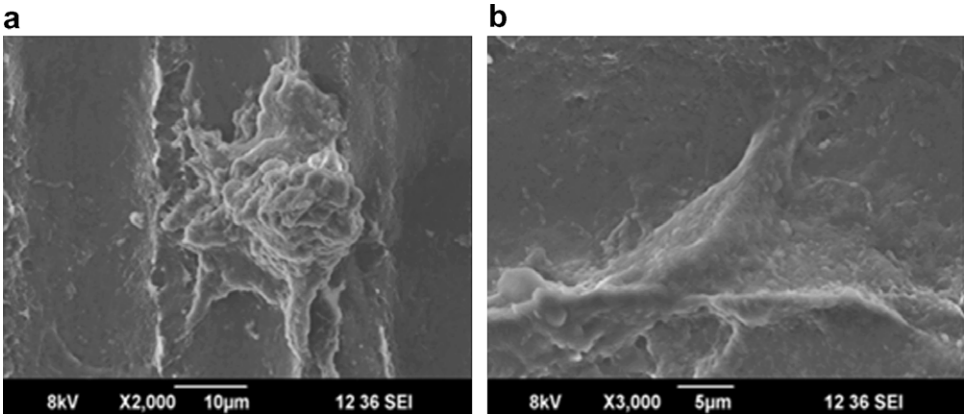

Fig. 8. MG-63 Cell attachments of chitosan/gelatin membranes of (a) 24 h and (b) 96 h after seeding the cells.

The chitosan/gelatin membranes (m-2) were immersed in PBS for 24 h. After 24 h, the shape of the membrane was flexible without brittle in nature. This phenomenon indicated that the chitosan/gelatin membranes (m-2, $r = 0.50$) especially could maintain stable swelling ratio.

3.6. Thermal studies

Fig. 5 shows the TGA curves of the prepared chitosan/gelatin membranes. The chitosan/gelatin membrane (m-2) showed less thermal stability than the chitosan membrane (m-4). The chitosan/gelatin membrane (m-2) showed the second degradation at 252 °C, while the chitosan membrane (m-4) was showed at 270 °C. It is due to the incorporation of amorphous gelatin into chitosan. In contrast, the chitosan/gelatin membrane (m-2) showed the higher thermal stability than gelatin powder. The gelatin degraded faster than the chitosan/gelatin membranes. Fig. 6 shows the DTA curves of the prepared chitosan/gelatin membranes. The DTA peaks of chitosan/gelatin membrane (m-2) were similar to chitosan membrane (m-4) peaks while those of gelatin powder were not similar to chitosan/gelatin membrane. These results also caused from the difference in crystal structure and hydrogen bonding network between gelatin and chitosan. It also indicated that the prepared membranes with chitosan hydrogel and gelatin were mixed at the molecular level.

3.7. Dissolution behavior of chitosan/gelatin membranes

The dissolution behavior of chitosan/gelatin membrane (m-2, $r = 0.50$) was shown in Fig. 7. The chitosan/gelatin membrane showed little variation of dissolution ratio after heating for 24 h with water. In contrast, gelatin cast film as a reference was completely dissolved within 10 min in the water (data not shown). After 24 h, there was no apparent color found in the hot water treated with the chitosan/gelatin membrane by the ninhydrin test (data not shown). These results indicated that the dissolution of chitosan/gelatin membrane is little influenced by hot water. It also supported that hydrogen bonding network between gelatin and chitosan was caused and led to their good compatibility.

3.8. Cell attachment studies

Fig. 8 shows the cell attachment on chitosan/gelatin membranes. Osteoblastic cells (MG63) were seeded on chitosan/gelatin membranes. After 24 h, there was cell adhesion on chitosan/geletin membranes (Fig. 8a). After 96 h of incubation it was found that cells adhered and completely spread on the surface of the membrane (Fig. 8b). They had many pseudopodia and formed a complete layer on the surface of the membranes, so that the membrane surface was not at all visible. This preliminary experiment suggests that chitosan/gelatin membranes have excellent biocompatibility in terms of osteoblastic cell culture. Further investigation such as cellular proliferation and differentiation assays are underway.

4. Conclusions

Novel chitosan/gelatin membranes were prepared using chitosan hydrogel mixed with gelatin. The surface morphology of these chitosan/gelatin membranes were found to be very smooth. The XRD studies of the chitosan/gelatin membranes showed that the incorporation of gelatin decreased the crystallinity of chitosan. The maximum stress and elongation of chitosan/gelatin membranes on wet condition was found when the mixture ratio of gelatin was 0.50. These chitosan/gelatin membranes showed good swelling, mechanical and thermal properties. The little dissolution of gelatin from the membrane was observed because of their good compability. Cell adhesion studies were carried out using human MG-63 osteoblast-like cells. The cells incubated with chitosan/gelatin membranes for 24 h were capable of forming cell adhesion. So, the prepared chitosan/gelatin membranes are bioactive and may be suitable for cell adhesion/attachment suggesting that these membranes can be used for tissue-engineering applications.

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References

Achet, D., & He, X. W. (1995). Determination of the renaturation level in gelatin films. *Polymer, 36*, 787–791.

Arvanitoyannis, I. S., Nakayama, A., & Aiba, S. (1998). Chitosan and gelatin based edible films: state diagrams, mechanical and permeation properties. *Carbohydrate Polymers, 37*, 371–382.

Cheng, M., Deng, J., Yang, F., Gong, Y., Zhao, N., & Zhang, X. (2003). Study on physical properties and nerve cell affinity of composite films from chitosan and gelatin solutions. *Biomaterials, 24*, 2871–2880.

Hirano, S., Itakura, C., Seino, H., Akiyama, Y., Nonaka, I., Kanbara, N., et al. (1990). Chitosan as an ingredient for domestic animal feeds. *Journal of Agricultural and Food Chemistry, 38*, 1214–1217.

Izume, M., & Ohtakara, A. (1987). Preparation of D-glucosamine oligosaccharides by the enzymatic hydrolysis of chitosan. *Agricultural Biological Chemistry, 51*, 1189–1191.

Jayakumar, R., Nagahama, H., Furuike, T., & Tamura, H. (2008). Synthesis of phosphorylated chitosan by novel method and its characterization. *International Journal of Biological Macromolecules, 42*, 335–339.

Jayakumar, R., Prabaharan, M., Reis, R. L., & Mano, J. F. (2005). Graft copolymerized chitosan–Present status and applications. *Carbohydrate Polymers, 62*, 142–158.

Jayakumar, R., Reis, R. L., & Mano, J. F. (2006a). Chemistry and applications of phosphorylated chitin and chitosan. *E-Polymers*, 035.

Jayakumar, R., Reis, R. L., & Mano, J. F. (2006b). Phosphorous containing chitosan beads for controlled oral drug delivery. *Journal of Bioactive and Compatible Polymers, 21*, 327–340.

Jayakumar, R., Reis, R. L., & Mano, J. F. (2007a). Synthesis and characterization of pH-sensitive thiol-containing chitosan beads for controlled drug delivery applications. *Drug Delivery, 14*, 9–17.

Jayakumar, R., Reis, R. L., & Mano, J. F. (2007b). Synthesis and characterization of N-methylenephenyl phosphonic chitosan. *Journal of Macromolecular Science, Pure and Applied Chemistry, A44*, 271–275.

Jayakumar, R., Nwe, N., Tokura, S., & Tamura, H. (2007c). Sulfated chitin and chitosan as novel biomaterials. *International Journal of Biological Macromolecules, 40*, 175–181.

Jayakumar, R., & Tamura, H. (2008). Synthesis, characterization and thermal properties of chitin-*g*-poly(ε-caprolactone) copolymers by using chitin gel. *International Journal of Biological Macromolecules, 43*, 32–36.

Kolodziejska, I., Piotrowska, B., Bulge, M., & Tylingo, R. (2006). Effect of transglutaminase and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide on the solubility of fish gelatin–chitosan films. *Carbohydrate Polymers, 65*, 404–409.

Mori, T., Okumura, M., Matsuura, M., Ueno, K., Tokura, S., Okamoto, Y., et al. (1997). Effects of chitin and its derivatives on the proliferation and cytokine production of fibroblasts in vivo. *Biomaterials, 18*, 947–951.

Nagahama, H., Higuchi, T., Jayakumar, R., Furuike, T., & Tamura, H. (2008a). XRD studies of β-chitin from squid pen with calcium solvent. *International Journal of Biological Macromolecules, 42*, 309–313.

Nagahama, H., Kashiki, T., Nwe, N., Jayakumar, R., Furuike, T., & Tamura, H. (2008b). Preparation of biodegradable chitin/gelatin membranes with GlcNAc for tissue engineering applications. *Carbohydrate Polymers, 73*, 456–463.

Nagahama, H., Nwe, N., Jayakumar, R., Furuike, T., & Tamura, H. (2008c). Preparation of chitinous compound/gelatin composite and their biological applications. *Macromolecular Symposia, 264*, 8–12.

Nagahama, H., Nwe, N., Jayakumar, R., Koiwa, S., Furuike, T., & Tamura, H. (2008d). Novel biodegradable chitin membranes for tissue engineering applications. *Carbohydrate Polymers, 73*, 295–302.

Ogawa, K. (1991). Effect of heating an aqueous suspension of chitosan on the crystallinity and polymorphs. *Agricultural Biological Chemistry, 55*, 2375–2377.

Okamoto, Y., Minami, S., Matsuhashi, A., Sashiwa, H., Saimoto, H., Shigemasa, Y., et al. (1993). Polymeric *N*-acetyl-D-glucosamine (Chitin) induces histionic activation in dogs. *Journal of Vertinary Medical Science, 55*, 739–742.

Okuyama, K., Nioguchi, K., Miyazawa, T., Yui, T., & Ogawa, K. (1997). Molecular and crystal structure of hydrated chitosan. *Macromolecules, 30*, 5849–5855.

Rinki, K., Dutta, J., & Dutta, P. K. (2007). Chitosan based scaffolds for tissue engineering applications. *Asian Chitin Journal, 3*, 69–70.

Tamura, H., Nagahama, H., & Tokura, S. (2006). Preparation of chitin hydrogel under mild conditions. *Cellulose, 13*, 357–364.

Tanigawa, T., Tanaka, Y., Sashiwa, H., Saimoto, H., & Shigemasa, Y. (1992). In C. J. Brine, P. A. Sanford, & J. P. Zikakis (Eds.), *Advances in chitin and chitosan* (pp. 206). London: Elsevier.

Tokura, S., Ueno, K., Miyazaki, S., & Nishi, N. (1997). Molecular weight dependent antimicrobial activity by chitosan. *Macromolecular Symposia, 120*, 1–9.

Wang, A. J., Cao, W. L., Gong, K., Ao, Q., Jun, K. L., He, C. Z., et al. (2006). Development of porous chitosan tubular scaffolds for tissue engineering applications. *Asian Chitin Journal, 2*, 53–60.

Zhai, M., Zhao, L., Yoshii, F., & Kume, T. (2004). Study on antibacterial starch/chitosan blend film formed under the action of irradiation. *Carbohydrate Polymers, 57*, 83–88.