Available online at www.sciencedirect.com
ScienceDirect
Desalination 234 (2008) 166–174
DESALINATION
www.elsevier.com/locate/desal

Fabrication and characterization of chondroitin sulfate-modified chitosan membranes for biomedical applications

Nai-Yi Yuan[a], Ruei-Yi Tsai[a], Ming-Hwa Ho[b], Da-Ming Wang[a], Juin-Yih Lai[c], Hsyue-Jen Hsieh[a]*

[a]*Department of Chemical Engineering, National Taiwan University, Taipei 106, Taiwan*
Tel. +886(2)23633097; Fax +886(2)23623040; email: hjhsieh@ntu.edu.tw
[b]*Department of Chemical Engineering, National Taiwan University of Science and Technology, Taipei 106, Taiwan*
[c]*Research and Development Center for Membrane Technology, Chung Yuan University, ChungLi 320, Taiwan*

Received 31 July 2007; accepted revised 21 September 2007

Abstract

Chitosan (CH) is a biomaterial with antiseptic, bioactive, and biocompatible properties. To further enhance the performance of chitosan membranes, chondroitin sulfate (CS) was utilized to modify the porous chitosan membranes fabricated by the freeze-gelation method. The cross-linking of CS to CH was mediated by ionic interaction and covalent cross-linking using EDC/NHS coupling reagents. The pore structures, mechanical properties, and surface hydrophilicity of the porous chondroitin sulfate-modified chitosan (CH/CS) membranes could be altered by varying the weight ratio of CS to CH. The membranes had interconnected stratiform pore structure with surface pore size ranging from 10 to 40 μm. Among various CH/CS membranes the one with the weight ratio of 90/10 (CH to CS) possessed the highest mechanical strength (18.61 N/g), about 40% increase as compared with the unmodified CH membrane. The addition of CS improved the hydrophilicity of the membranes. Preliminary cell culture experiments revealed that the proliferation of the gingival fibroblasts on the CH/CS surface was slightly better than that on the CH surface. In summary, the CH/CS membranes (especially the 90/10 membrane), due to their higher mechanical strength, hydrophilicity, and better cell compatibility, are promising biomaterials for tissue engineering applications.

Keywords: Biomaterial; Chitosan; Chondroitin sulfate; Freeze-gelation; Porous membrane

*Corresponding author.

Presented at the Fourth Conference of Aseanian Membrane Society (AMS 4), 16–18 August 2007, Taipei, Taiwan.

doi:10.1016/j.desal.2007.09.083

1. Introduction

Biocompatibility and biodegradability are of the materials for tissue engineering applications [1]. Chitosan (CH) is well known for its numerous and interesting biological properties [2]. Chitosan, a copolymer of glucosamine and N-acetylglucosamine, is a biomaterial with antiseptic, bioactive, and biocompatible properties [3,4]. The protonation of the amino groups on the chitosan molecules lead to dissolution in organic acid at low pH value. The amino groups can be utilized for coupling chitosan with other biomolecules such as lectins [5].

Previous researches have discovered that the hydrophobic property of chitosan often leads to problems such as poor cell attachment, thus limiting its application as a biomaterial [6,7]. To further enhance the performance of chitosan membranes, chondroitin sulfate (CS) was utilized to modify the chitosan membranes for preparing composite membranes with better hydrophilicity and biological compatibility [8]. Chondroitin sulfate belongs to the GAG (glycosaminoglycan) family. It is an alternating copolymer of β-(1,4)-D-glucuronic acid and β-(1,3)-*N*-acetyl-D-galactosamine that is sulfated at the 4-position or at the 6-position for three isomers: chondroitin sulfate A (chondroitin 6-sulphate), chondroitin sulfate B (dermatan sulphate), chondroitin sulfate C (chondroitin 4-sulphate) [9]. According to the study of Denuziere et al., the carboxylic groups and sulfite groups of CS could form negatively charged functional groups in weak acid and react with positively charged molecules such as chitosan to produce polyelectrolyte complexes (PEC) [10].

However, due to the formation of PEC between CH and CS mediated by the strong ionic interaction, it was a difficult task to prepare homogeneous CH/CS composite membranes. To resolve the difficulty, we used a two-step cross-linking method followed by a freeze-gelation method to prepare CH/CS composite membranes with enhanced mechanical properties. The freeze-gelation method, based on thermally induced phase separation [11], is a novel method developed by Ho et al. [12]. The key steps of freeze-gelation are the freeze of a polymer solution, followed by the gelation of the polymer under the freezing condition to preserve the porous structure. In this work, we used the freeze-gelation method to prepare the porous CH/CS membranes with different weight ratios of CH to CS. The pore structures, mechanical properties, and surface hydrophilicity of these membranes were determined. The cell compatibility test of these membranes was also carried out.

2. Materials and methods

2.1. Materials

Chitosan (molecular weight = 300,000, degree of deacetylation = 90%) was purchased from Kiotek Co. (Taiwan). Chondroitin sulfate, *N*-(3-dimethylamino-propyl)-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) and other chemicals were purchased from Sigma (USA). Materials for cell culture including Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin (10,000 U/mL), phosphate-buffered saline (PBS), and trypsin-EDTA were purchased from GIBCO BRL (USA). Tissue culture flasks, 12-well plates and 96-well plates were obtained from Iwaki (Japan). Gingival fibroblasts (GF) were provided by Prof. L.T. Hou (National Taiwan University Hospital, Taiwan).

2.2. The preparation of porous CH/CS membranes

Chondroitin sulfate (CS) powders were dissolved in water, and then the solution was mixed with EDC (twice the moles of CS) and NHS (equal to the moles of CS) to react for 2 h. Afterwards chitosan (CH) powders were added and

stirred for 1 h for complete dispersion. The following weight ratios of CH/CS: 100/0, 95/5, 90/10, 87.5/12.5, 85/15, 80/20, 70/30 were selected to prepare various CH/CS composite membranes (Table 1). Then the acetic acid solution was added slowly under continuous stirring at 25°C and incubated for 24 h to generate a homogenous mixture containing 3 wt.% of chitosan and chondroitin sulfate. The mixture was then poured into a mould and frozen at –80°C for 12 h. The frozen mixture (membrane) was soaked in a NaOH/ethanol aqueous solution at −15°C for 6 h and then immersed in a 95% ethanol solution for 6 h [12]. The membrane was washed 3 times (30 min each time) at 25°C with phosphate-buffered saline (PBS) solution and then subjected to various analyses. In addition, the amounts of CS in the spent PBS were measured to calculate the amounts of CS cross-linked to the chitosan membranes [13]. The efficiency of the cross-linking of CS to CH was calculated as follows:

$$\text{Cross-linking efficiency (\%)} = \frac{CS_A - CS_R}{CS_A} \times 100\%$$

where CS_A and CS_R denote the amount of CS initially added and the amount of CS in the spent PBS (unbound CS), respectively.

Table 1
Abbreviations and compositions of the various CH/CS composite membranes or films

Abbreviations	Composition			
	CH (g)	CS (g)	EDC (g)	NHS (g)
100/0	3.000	0.000	0.000	0.000
95/5	2.850	0.150	0.114	0.045
90/10	2.700	0.300	0.228	0.090
87.5/12.5	2.625	0.375	0.285	0.113
85/15	2.550	0.450	0.342	0.135
80/20	2.400	0.600	0.456	0.180
70/30	2.100	0.900	0.683	0.270

2.3. Preparation of dense CH/CS films

For the preparation of dense CH/CS films, the 1 wt.% CH/CS solution (CH/CS = 90/10) was poured into dishes and dried in a vacuum oven for 24 h at 37°C. The dehydrated films were then immersed in a NaOH/70% ethanol solution for 6 h, and then rinsed with ethanol. The films were washed 3 times using a PBS solution (30 min each time). The films were dried again in the oven at 37°C for 24 h and then stored at 4°C.

2.4. Scanning electron microscopy (SEM)

The membranes were dehydrated step-wise with ethanol solution starting at 50% and continuing to 100% for approximately 15 min in each step. Afterwards, the samples were dried in a Tousimis PVT-3D critical point dryer (USA), sectioned, sputter-coated with gold in a Hitachi JEOL JFC-1100E and JEOL JEE-4X vacuum evaporator (USA), attached to sample stubs and then visualized using a Hitachi JSM-6300 scanning electron microscope (USA).

2.5. Differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA)

DSC analysis was conducted in a Mettler Toledo DSC821 differential scanning calorimeter (Switzerland) to examine the thermal properties of the prepared membranes. The samples of 5–8 mg were precisely weighted using an A&D HF-3000 electronic balance (Japan) in hermetic DSC aluminum sample pans. These sample pans were sealed. An empty sealed pan served as a reference standard. For DSC analysis, the sample and the reference standard were pre-equilibrated at 30°C, and then heated from 30 to 250°C at a heating rate of 5°C/min.

Thermogravimetric analysis (TGA) was performed in a Perkin Elmer Pyris 1 thermogravimetric analyzer (USA) to examine the thermal properties of the membranes. The samples of 10 mg were precisely weighted using an A&D

HF-3000 electronic balance (Japan). The sample and reference standard were pre-equilibrated at 30°C, and then heated from 30 to 500°C at a heating rate of 10°C/min.

2.6. Determination of membranes porosity

The porosity of a porous membrane was calculated as follows:

$$\text{Porosity } (\%) = \frac{V_m - V_c}{V_m} \times 100\% = \frac{l \times A - (W_m/\rho_c)}{l \times A} \times 100\%$$

where V_m and V_c are the volume of membrane and the volume occupied by the polymer (i.e. CH and CS), respectively. ρ_c is the intrinsic density of chitosan (1.342 g/cm^3 [14]). After measuring the area A, the mass W_m and the thickness l of the membrane, overall membrane porosity can be estimated using the above equation.

2.7. Determination of the mechanical properties

The mechanical properties of the porous membranes were determined using a Lloyd LRX tensile strength instrument (UK). The prepared membranes were swollen in PBS for 30 min and cut into a specific dog bone shape (6 cm long, 2 cm wide at the ends, and 1 cm wide in the middle). The thickness of each individual membrane was measured. The mechanical property analysis was performed at a stretching rate of 10 mm/min with a pre-load of 0.1 N to determine the tensile strength and elongation for each membrane.

2.8. Measurement of water uptake

The water uptake of the porous membrane was defined as the weight ratio of water to membrane in the swollen membrane. Since the membranes were vacuum-dried and stored in an electric desiccator with relative humidity maintained at 30%, only little water bound to the membranes as bound water and there was virtually no free water entrapped in the pores of membranes. Each membrane was soaked at room temperature in PBS and weighed repeatedly. The water uptake of a membrane was calculated as follows:

$$\text{Water uptake} = \frac{W_m - W_d}{W_d}$$

where W_m and W_d are the weights of swollen and dried membrane, respectively.

2.9. The FT-IR spectra analysis

Fourier-transformed infrared (FT-IR) spectra of membranes and powders were obtained in a Perkin-Elmer Spectrum One FT-IR spectrometer (USA). The range of the wave number was from 750 to 3750 cm^{-1}, and each sample was scanned 32 times.

2.10. Measurement of contact angle

The contact angles of the porous membrane surfaces were measured by the water-vapor contact angle measurement method [15]. The air bubble was released from a syringe (internal diameter of the needle: 0.65 mm) into a glass chamber containing water, so that the bubble floated 2 to 3 cm from the point of release to rest against a the membrane which was attached onto the polypropylene film (4.5 cm × 7.5 cm) fixed horizontally on a stage at the top of the water. The average bubble size was 2.5 ± 0.15 mm in diameter. Water contact angles were measured directly with a video camera mounted on a microscope to record the bubble image. The water contact angle of a membrane was calculated as follows:

$$\theta = 2\tan^{-1}(b/2h)$$

where θ is the contact angle of swollen membrane in water; h is the height of the bubble;

b is the interface length between bubble and membrane.

2.11. Cell compatibility test

For preliminary cell compatibility studies, the dense films were prepared in 12-well plates as described in Section 2.3. The films were sterilized in a 70% ethanol solution for 24 h. Gingival fibroblast (GF) cells cultured in MEM supplemented with 10% FBS were seeded onto various films at a density of 1.6×10^4 cells/well. After 1–6 days, cultured cells (on the films) were washed three times with PBS. Lysis buffer was introduced to the cells with a reaction time of 10 min. The resulting cell lysate was recovered for the protein concentration assay using a Pierce BCA protein assay kit (USA). The number of cells was indirectly expressed by the amount of cell proteins.

3. Results and discussion

3.1. Microstructure of porous CH/CS membranes

Fig. 1 shows the cross-section (a–h) and surface (i–l) of the CH/CS membranes with different weight ratios of CH/CS. The surface pore size ranged from 10 to 40 μm. The cross sectional micrographs revealed that interconnected stratiform pore structures were present in the interior region.

3.2. Thermal properties of the membranes

DSC and TGA were used to determine the changes in the membrane microenvironment that occurred during the fabrication process of the membrane. The DSC scans for the membranes are shown in Fig. 2. Endothermic peaks of the CH/CS membranes were around 90–110°C, which was close to the boiling point of water (100°C), and thereby these peaks indicated that the bound water of the CH/CS membranes was lost [16]. The TGA scans for the CH/CS membranes are shown in Fig. 3. There was a weight loss of 8–12% between 30 and 120°C. The results were consistent with that of DSC analysis.

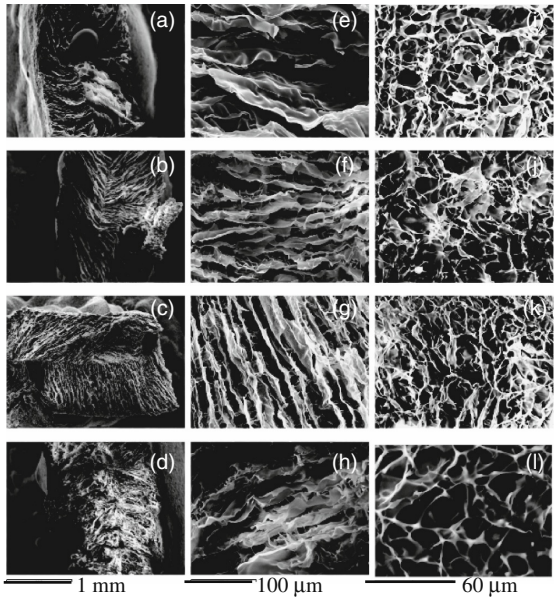

Fig. 1. The SEM images of the cross-section (a–h) and surface (i–l) of the CH/CS membranes. (a, e and i) for the 100/0 membrane; (b, f and j) for the 95/5 membrane; (c, g and k) for the 90/10 membrane; (d, h and l) for the 85/15 membrane.

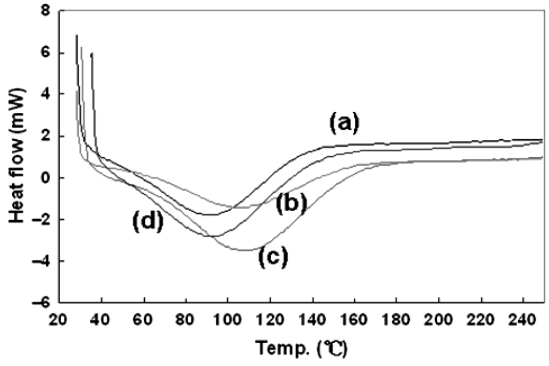

Fig. 2. The DSC curves of the CH/CS membranes: (a) 100/0, (b) 95/5, (c) 90/10 and (d) 85/15 membranes (heating rate 5°C/min).

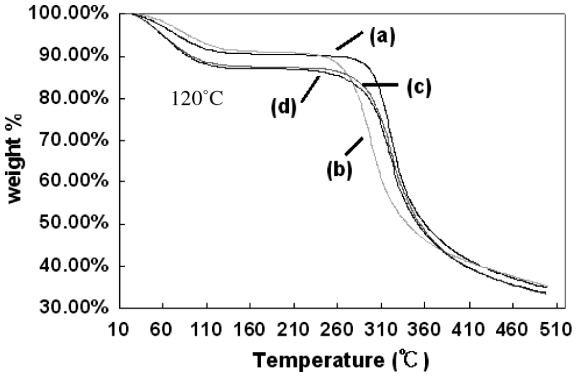

Fig. 3. The TGA curves for the CH/CS membranes: (a) 100/0, (b) 95/5, (c) 90/10 and (d) 85/15 membranes (heating rate 10°C/min).

3.3. Porosity, water uptake and cross-linking efficiency of the membranes

The porosity, water uptake and cross-linking efficiency of the CH/CS membranes were defined and measured as described in the materials and methods. The results are shown in Table 2. The porosities of the membranes were all about 96%, demonstrating that the membranes prepared by the freeze-gelation method were highly porous. As for the water uptake, the 87.5/12.5 group had the maximum water uptake, reaching 23.66 ± 0.46. In the 70/30 group, due to very strong ionic interaction between CH and CS, precipitation occurred and thereby porous membranes could not be fabricated. The cross-linking efficiency of the CH/CS membranes was increased when the amount of CS was increased and the efficiencies were all above 90%, demonstrating that two-step cross-linking method may result in very high cross-linking efficiency.

3.4. Mechanical properties of the membranes

The tensile strength and elongation of the CH/CS membranes are shown in Fig. 4. The tensile strength of chitosan (100/0) membrane was lower than that of CH/CS membranes (except 80/20 ones). Among various CS/CH membranes the 90/10 membrane possessed the highest tensile strength (18.6 N/g), about 40% increase as compared with the unmodified CH (100/0) membrane. The addition of small amounts of chondroitin sulfate that can be cross-linked to chitosan may increase the tensile strength of the CH/CS membranes such as 95/5, 90/10 and 87.5/12.5 membranes. However, because chitosan is a crystallizable material [17,18], the addition of large amounts of chondroitin sulfate may decrease the crystallinity of chitosan and thus reduce the strength the membranes such as 85/15 and 80/20 membranes. The elongation property of the membranes also followed similar trend. Among various CS/CH membranes the 95/5

Table 2
The porosity, water uptake and cross-linking efficiency of the CH/CS membranes

Sample	Porosity	Water uptake	Cross-linking efficiency
100/0	96.52 ± 0.11	20.73 ± 0.63	–
95/5	96.53 ± 0.04	20.71 ± 0.27	90.55 ± 0.50
90/10	96.61 ± 0.07	21.22 ± 0.43	94.60 ± 0.27
87.5/12.5	96.94 ± 0.06	23.66 ± 0.46	97.82 ± 0.17
85/15	96.40 ± 0.07	19.95 ± 0.42	97.26 ± 0.07
80/20	96.25 ± 0.28	18.86 ± 1.47	97.50 ± 0.15
70/30[a]	–	–	99.27 ± 0.054

[a]Precipitation occurred.

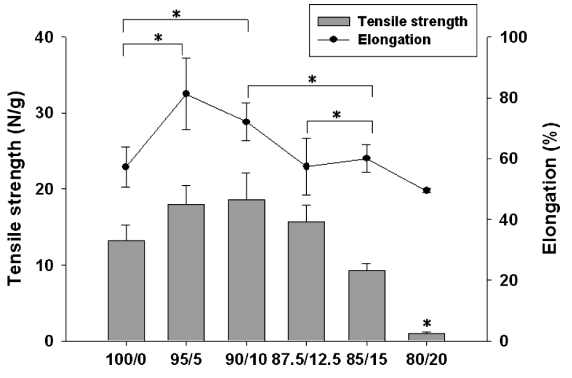

Fig. 4. The mechanical properties (tensile strength and elongation) of the porous CH/CS membranes ($n \geq 5$, mean $\pm$ S.D.). The tensile strength is defined as the load to fracture (maximum load) divided by the weight of membrane. The elongation is defined as the strain at maximum load. The fabrication conditions for the membranes: 3 wt.% CH/CS solution (see Table 1), frozen at –80°C, gelled in NaOH/ethanol solution at –15°C, and rinsed with 95% ethanol solution (*$p < 0.05$: significantly different from each other by the student's *t*-test).

membrane possessed the highest elongation (72%), about 25% increase as compared with the unmodified CH (100/0) membrane.

3.5. Contact angles of the membranes

Hydrophilicity is an important property for biomaterials. The water contact angles of the CH/CS membrane surfaces as well as the chitosan control are shown in Fig. 5. The results indicated that the contact angles decreased when more chondroitin sulfate was mixed with chitosan to form porous membranes. In other words, the addition of chondroitin sulfate enhanced the hydrophilicity of the membranes.

It looked like that the data in Fig. 5 were not consistent with the result from the Table 2, because for the content of CS over 12.5% the water uptake started to decrease (Table 2), even though the hydrophilicity continued to increase (Fig. 5). To explain this discrepancy, it should be mentioned that the water uptake of a porous

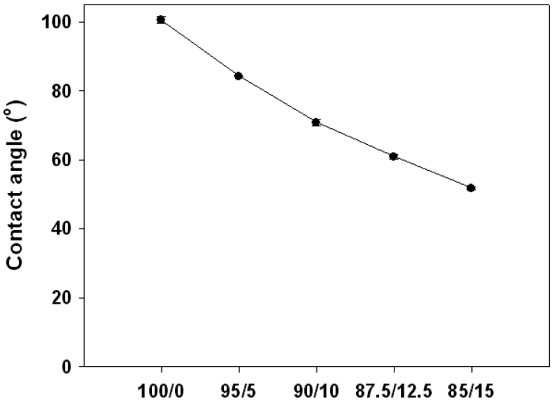

Fig. 5. Water contact angles of the CH/CS membranes ($n = 4$, mean $\pm$ S.D.).

membrane is affected by many factors including: 3-D pore structure, porosity, mechanical strength, and hydrophilicity of the membrane material. Thus hydrophilicity is only one of the factors that have to be considered. Even though the hydrophilicity was enhanced with increasing of CS contents (Fig. 5), the tensile strength of the membranes started to decrease when the content of CS reached over 12.5 (Fig. 4), making the 3-D pore structure of the membrane probable partially collapsed during the measurement of the water uptake due to lower mechanical strength, thus resulting in lower value of water uptake in Table 2. Therefore, the hydrophilicity shown in Fig. 5 only partially contributed to the water uptake. Other factors have to be considered. That could be why there was discrepancy of the data between Table 2 and Fig. 5.

3.6. FT-IR analysis

FT-IR spectra of chitosan powder, chitosan membrane, chondroitin sulfate powder, and CH/CS membranes were shown in Fig. 6. The peak at 2950 cm^{-1} in the CH/CS membranes but not in chitosan corresponded to the protonated carboxylic acid (COOH). The O=C–NH adsorption peak at 3311 cm^{-1} was stronger

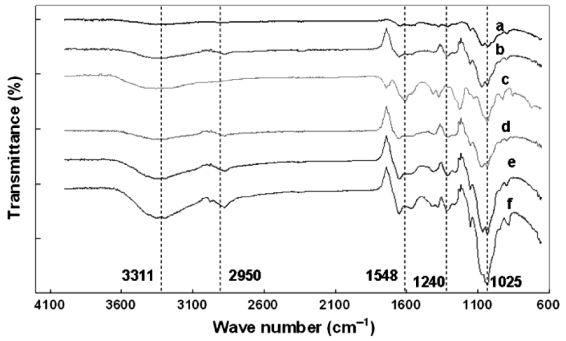

Fig. 6. The FT-IR spectra of CH, CS and CH/CS membranes: (a) CH powder, (b) 100/0 membrane, (c) CS powder, (d) 95/5 membrane, (e) 90/10 membrane and (f) 85/15 membrane.

in the CH/CS membranes than that in chitosan and chondroitin sulfate powders. The peak at 1240 cm^{-1} corresponding to the R-OSO_2-OR decreased in the CH/CS membranes. The above evidence indicated that the EDC/NHS-mediated coupling reaction between chitosan and chondroitin sulfate might occur. Moreover, the electrostatic interaction between SO_3^-/COO^- groups (on CS) and NH_3^+ groups (on CH) might drive CS and CH to form polyelectrolyte complexes (PEC).

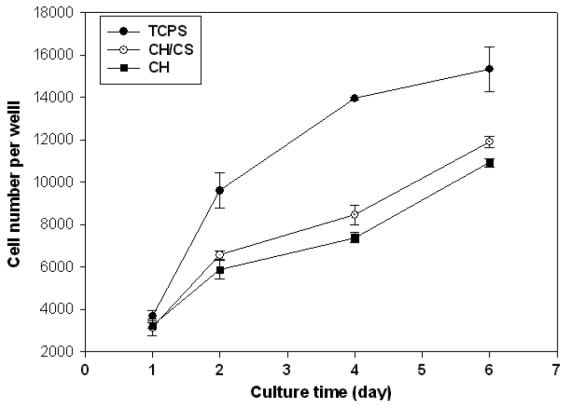

Fig. 7. The proliferation of GF cells on the surfaces of TCPS, CH/CS (90/10) and CH (100/0) (n = 4, mean ± S.D.).

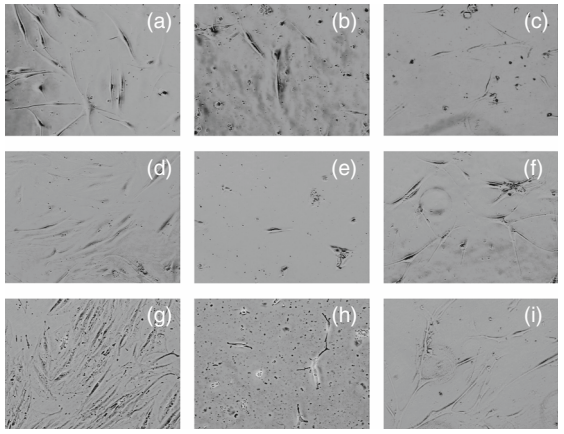

Fig. 8. Microscopic images of GF cells cultured on the surfaces of TCPS, CH (100/0) and CH/CS (90/10) for different times. (a, d, g): TCPS; (b, e, h): 100/0 surface; (c, f, i): 90/10 surface. (a, b, c): 1 day; (d, e, f): 7 days; (g, h, i): 14 days (100×).

3.7. Cell compatibility of the membranes

The preliminary cell compatibility experiments were carried out on the TCPS and CH/CS surfaces. As shown in Fig. 7, the proliferation of the gingival fibroblast (GF) cells on the CS/CH surface was slightly better than that on the CH surface, and the TCPS was used as the control surface. In Fig. 8 the morphology of GF cells on the CH/CS surface was similar to that on the TCPS surface, suggesting that the CH/CS surface was more suitable than the CH surface for GF cells to proliferate.

4. Conclusions

By using the freeze-gelation method, we successfully prepared the porous CH/CS composite membranes. The pore structure, mechanical properties, and water uptake of these membranes could be altered by varying the weight ratio of CH to CS. The two-step crosslinking method developed in this study increased the crosslinking efficiency to more than 90%. The addition of small amounts of CS increased the mechanical strength of the membranes. Among these membranes, the

90/10 membrane possessed higher tensile strength, appropriate hydrophilicity, and better biocompatibility. Therefore, it is a promising biomaterial for a variety of biomedical applications.

Acknowledgements

The authors thank Prof. L.T. Hou (National Taiwan University Hospital, Taiwan) for supplying GF cells for cell compatibility test. This study was financially supported by the Center-of-Excellence Program on Membrane Technology, the Ministry of Education, Taiwan and the National Science Council, Taiwan (grant number: NSC94-2214-E-002-011).

References

[1] S. Cohen, M.C. Baño, L.G. Cima, H.R. Allcock, J.P. Vacanti, C.A. Vacanti and R. Langer, Design of synthetic polymeric structures for cell transplantation and tissue engineering, Clin. Mater., 13 (1993) 3–10.

[2] R.A.A. Muzzarelli, V. Baldassare, F. Conti, P. Ferrara, G. Biagini, G. Gazzanelli and V. Vasi, Biological activity of chitosan: ultra structural study, Biomaterial, 9 (1988) 247–252.

[3] M. Goozen, Applications of Chitin and Chitosan, Technomic Publishing, Lancaster, PA, 1997.

[4] J. Rhoades and S. Roller, Antimicrobial actions of degraded and native chitosan against spoilage organisms in laboratory media and foods, Appl. Environ. Microbiol., 66 (2000) 80–86.

[5] Y.C. Wang, S.H. Kao and H.J. Hsieh, A chemical surface modification of chitosan by glycoconjugates to enhance the cell-biomaterial interaction, Biomacromolecules, 4 (2003) 224–231.

[6] L. Ma, C.Y. Gao, Z.W. Mao, J. Zhou and J.C. Shen, Enhanced biological stability of collagen porous scaffolds by using amino acids as novel cross-linking bridges, Biomaterials, 25 (2004) 2997–3004.

[7] L. Ma, C.Y. Gao, Z.W. Mao, J. Zhou and J.C. Shen, Collagen/chitosan porous scaffolds with improved biostability for skin tissue engineering, Biomaterials, 24 (2003) 4833–4841.

[8] J.S. Pieper, T. Hafmans, J.H. Veerkamp and T.H. Kuppevelt, Development of tailor-made collagen-glycosamino glycan matrices: EDC/NHS crosslinking, and ultrastructural aspects, Biomaterials, 21 (2000) 581–593.

[9] J. Zaia, J.E. McClellan and C.E. Costello, Tandem mass spectrometric determination of the 4S/6S sulfation sequence in chondroitin sulfate oligosaccharides, Anal. Chem., 73 (2001) 6030–6039.

[10] A. Denuziere, D. Ferrier, O. Damour and A. Domard, Chitosan-chondroitin sulfate and chitosan-hyaluronate polyelectrolyte complexes: biological properties, Biomaterials, 19 (1998) 1275–1285.

[11] Y.S. Nam and T.G. Park, Porous biodegradable polymeric scaffolds prepared by thermally induced phase separation, J. Biomed. Mater. Res., 47 (1999) 8–17.

[12] M.H. Ho, P.Y. Kuo, H.J. Hsieh, T.Y. Hsien, L.T. Hou, J.Y. Lai, et al., Preparation of porous scaffolds by using freeze-extraction and freeze-gelation method, Biomaterials, 25 (2004) 129–138.

[13] Y.L. Chen, H.C. Chen, H.P. Lee, H.Y. Chan and Y.C. Hu, Rational development of GAG-augmented chitosan membranes by fractional factorial design methodology, Biomaterials, 27 (2006) 2222–2232.

[14] R.C. Thomson, A.G. Mikos, E. Beahm, J.C. Lemon, W.C. Satterfield, T.B. Aufdemorte and M.J. Miller, Guided tissue fabrication from periosteum using preformed biodegradable polymer scaffolds, Biomaterials, 20 (1999) 2007–2018.

[15] M. Fletcher and K.C. Marshall, Bubble contact angle method for evaluating substratum interfacial characteristics and its relevance to bacterial attachment, Appl. Environ. Microbiol., 44 (1982) 184–192.

[16] M.N. Khalid, F. Agnely, N. Yagoubi, J.L. Grossiord and G. Couarraze, Water state characterization, swelling behavior, thermal and mechanical properties of chitosan based networks, Eur. J. Pharm. Sci., 15 (2002) 425–432.

[17] T. Yui, K. Imada, K. Okuyama, Y. Obata, K. Suzuki and K. Ogawa. Molecular and crystal-structure of the anhydrous form of chitosan, Macromolecules, 27 (1997) 7601–7605.

[18] R.J. Samuels. Solid state characterization of the structure of chitosan films, J. Polym. Sci., Polym. Phys. Ed., 19 (1981) 1081.