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Characterization of chitosan-chondroitin sulfate blended membranes and effects on the growth of corneal cells

Zi-Ang Yao[a,b*] and Hai-Ge Wu[a,b]

[a]*Bioengineering College of Dalian University, Dalian 116622, China*

[b]*Liaoning Key Laboratory of Bioorganic Chemistry, Dalian University, Dalian 116622, China*

[*]Corresponding author. Tel: (+86) 411 8740 3950; Fax: (+86) 411 8740 3910; E-mail: ziangyao@163.com

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ABSTRACT

In order to construct a suitable scaffold for corneal cell culture and transplantation in vitro, different chitosan-chondroitin sulfate blended membranes were prepared and the properties of blended membranes were studied. Corneal stroma cells and corneal endothelial cells were seeded onto the blended membrane surface and the effects of the blended membranes on corneal cell attachment and metabolism were investigated. The results showed that chitosan and chondroitin sulfate had good compatibility in blended membranes. Chondroitin sulfate improved the homogeneousness, crystallization, transparency, and tensile strength and decreased the water content of the blended membrane. Within the blending ratio of 1:0.1, chondroitin sulfate reduced the damage of chitosan membranes to cells and improved the biocompatibility between cells and membranes. Corneal cells grew and formed a confluent monolayer on chitosan-chondroitin sulfate blended membranes (CH-CS3). All results indicated that the blended membranes of chitosan and chondroitin sulfate could be used as a scaffold for corneal cell culture in vitro and have potential to be used as carriers for corneal endothelial cell transplantation.

Keywords: Chitosan; chondroitin sulfate; blended membrane; corneal endothelial cell; transplantation

Dr Ziang Yao is professor of Biomaterial and Marine Bioactive Product at Bioengineering College, Dalian University, China. He obtained bachelor (Marine Biology, 1995), Master (Marine Drugs, 1998) and PhD (Marine Biology, 2002) degree from Ocean University of Qingdao, China. Dr Yao was employed as associate professor at Dalian University in 2002. His major area of research interest is biomaterials for corneal and nervous tissue engineering.

Dr Haige Wu is associate professor of Biomaterial and Marine Bioactive Product at Bioengineering College, Dalian University, China. She obtained Bachelor (Marine Biology, 1998) and Master degree (Marine Drugs, 2001) from Ocean University of Qingdao, China. She received PhD degree in Biochemistry from Dalian Institute of Chemical Physics, Chinese Academy of Sciences in 2008. Her main area of research is biomaterials for corneal and nervous tissue engineering.

Introduction

The corneal endothelium is essential for the maintenance of normal corneal hydration, thickness and function [**1**]. A number of studies have shown that the proliferation of human endothelial cells is restricted in the body, and the recovery of impaired endothelium depends on the migration and reestablishment of endothelial cells around the impairment [**2-3**]. When the number of endothelial cells is under a critical level, the injury of the cornea could not be recovered by the migration of endothelial cells, and this would lead to corneal edema [**4**]. The most effective therapy for this disease is corneal transplantation, but now the therapy is restricted by the insufficiency of donor corneas. Because most corneal diseases are just due to the injury of endothelial cell monolayer, researchers try to replace the injured endothelial cells with cultured endothelial cells on scaffolds in vitro [**5,6**]. Several materials, such as collagen matrix [**7,8**], Descenet's membrane [**9,10**], amnion [**11,12**] and gelatin matrix [**13,14**], have been used as scaffolds to culture endothelial cells in vitro, and the complexes of cells and scaffold were

transplanted into animal eyes to replace the injured endothelial layers. These results indicated that the cells cultured on scaffolds could restore the transparency and function of the cornea in the short-term, but that failure was inevitable in the long-term due to some defects of the scaffolds [**15, 16**]. Therefore, it is important to construct a more suitable scaffold for corneal endothelial cell transplantation.

Chitosan, derived from chitin, is comprised of β-(1-4)-linked-2-amino-2-deoxy-D-glucopyranose (GlcN, D-unit) and 2-acetamido-2-deoxy-D-glucopyranose (GlcAc, A-unit). Chitosan molecules have positive charges and exhibit numerous other interesting physicochemical and biological properties, such as biological renewability, biodegradability, biocompatibility, non-antigenicity, non-toxicity and other bioactivities [**17, 18**]. More studies have shown that there are no inflammatory or allergic reactions following implantation, injection and topical application of chitosan in the body. Due to all these characteristics, chitosan has been regarded as one of the most promising materials for use in constructing tissue-engineered scaffolds. Numerous scaffolds made of chitosan have been widely applied in tissue engineering, such as bone tissue engineering [**19, 20**], skin tissue engineering [**21, 22**], cartilage tissue engineering [**23, 24**]. Chitosan concurrently drew more attention for its potential use in corneal cell transplantation [**25, 26**]. Our previous study showed that chitosan membranes had a hydrophilic surface, which could promote corneal cell adhesion and short-term proliferation, but a lot of corneal cells fell off from the chitosan membrane in long-term culture [**27, 28**]. In order to construct suitable membranes for corneal cell transplantation that have in vivo-like mechanical properties and can support long-term growth of corneal cells, we prepared blended membranes with chitosan and chondroitin sulfate. The properties of the blended membranes were characterized, and the biocompatibility of blended membranes with corneal cells was studied.

Experimental

Materials

Chitosan (deacetylation degree 95%, average molecular weight 300 kDa) was obtained from Zhejiang Aoxing Biotechnology Co., Ltd. (Zhejing, China). The chondroitin sulfate was obtained from Sigma Corporation. New Zealand rabbits weighing between 2.0 and 2.5 kg were obtained from the Laboratory Animal Center of Qingdao Drug Inspection Institute (Qingdao, China). All other reagents used were of analytical grade.

Preparation of chitosan and chitosan-chondroitin sulfate blended membranes

Chitosan membranes, termed CH30, were obtained by drying 2% chitosan solution in 2% acetic acid on a glass plate at 40˚C in a drying oven for 48 h. Chitosan-chondroitin sulfate blended membranes were obtained by drying mixed solutions of chitosan and chondroitin sulfate on a glass plate at 40˚C in a drying oven for 48 h. The dried membranes were immersed in 10% sodium hydroxide solution for 30 min and then washed with distilled water until they were neutral. The concentration of chitosan solution was 2% in 2% acetic acid, and the concentration of chondroitin sulfate solution was 0.2% in water. The blending ratios of chitosan and chondroitin sulfate (v/v) were 1:0.02 (CH-CS1), 1:0.05 (CH-CS2), 1:0.1 (CH-CS3) and 1:0.2 (CH-CS4), chondroitin sulfate membranes were obtained by drying 2% chondroitin sulfate solution in distilled water on a plane plate at 40˚C in a drying oven for 48 h.

Corneal stroma cell culture

Corneal stroma cells were isolated from rabbit corneas according to previously used methods [29]. The corneal stroma cells were cultured in F12/DMEM medium supplemented with 20% new-born bovine serum, 100 units/ml penicillin, 100μg/ml streptomycin at 37^{o}C in 5% CO_2 atmosphere.

Corneal endothelial cell culture

Corneal endothelial cells were isolated from rabbit corneas according to previous methods [**29**] and cultured in the same medium and conditions as those of the corneal stroma cells.

Compatibility evaluation of chitosan and chondroitin sulfate in blended membranes

The surface structure of chitosan and blended membranes was observed with scanning electron microscopy (XZ-650 HITACHI). The crystallinity of chitosan and blended membranes was examined with a Rigaku D/max X-ray diffractometer (CuKα, 40kV, 100mA, 2θ=3-40, speed=6 degree/minute). The chemical bonds in chitosan and blended membranes were checked by Fourier transform infrared spectroscopy.

Evaluation of tensile strength

The tensile strength of dried chitosan and blended membranes (length of 10 cm and width of 1 cm) was determined with a tensile strength tester (landw SE060) at a pulling speed of 10 mm/min under a temperature of 25˚C.

Evaluation of optical properties

The membranes were fixed on silica glass (width of 1 cm), and then the silica glass with the membrane was inserted into a cuvette filled with 0.9% physiological saline solution. After 10 min, light transmittancy of chitosan and blended membranes was measured with a spectrophotometer (UV-1800) at room temperature at wavelengths between 345 nm and 800 nm.

Measurement of water content

Chitosan and blended membranes were dried at 70˚C in a drying oven over night, and then membranes were immersed in distilled water over night after being weighed (recorded as dry mass, mdry). The membranes were then taken out, absorbed the superficial liquid and weighed quickly (recorded as hydrate mass, mhydrate). The water

content of the membranes was defined as: [(mhydrate-mdry)/mhydrate] × 100%.

Measurement of permeability to small molecular compounds

Chitosan and blended membranes were fixed between the diffusion chamber (with 1% glucose, 0.05% tryptophan, 0.9% NaCl in distilled water) and receptor chamber (distilled water), and both chambers were mixed by an electromagnetic stirrer. The concentration of the tested compounds in the receptor chamber was checked after diffusion for 3, 6, 12, 24, 48, 96, 192 and 288 h using the 3,5-dinitrosalicylic acid method for glucose, ultraviolet absorption method for tryptophan and electric conductivity method for NaCl. The permeability values were calculated according to previous methods [**30**].

*Corneal stroma cell attachment tes*t

All membranes were immersed in 0.9% physiological saline solution and sterilized with a moist heat sterilization method for 20 min. The sterile chitosan and blended membranes were attached on the cell culture plate. After corneal stroma cells were seeded onto the surface of different membranes (5×10^5 cells/ml) for 30 min, 0.5 ml medium was added to each culture plate, and the cells were incubated for 6 h. Then, a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) test was carried out to quantify the viability of the cells that adhered to the chitosan and blended membranes. The percentage of cells adhering on the culture plate was regarded as 100%. The percentage of cells adhering on chitosan and blended membranes was calculated compared to the control.

The test of Lactate Dehydrogenase (LDH) activities secreted by corneal stroma cells

After the corneal stroma cells were seeded on the surface of chitosan and blended membranes and cultured for 24 h, the activities of LDH in the medium were tested with an LDH assay kit (Nanjing University, China).

Corneal stroma cell proliferation test

Blended and chitosan membranes were attached on the bottom of the cell culture plate. Corneal stroma cells were subsequently seeded onto the surface of different membranes at a density of 5×10^4 cells/ml and cultured. Then, a MTT test was performed to test cell proliferation on the different membrane surfaces after culture periods of 1, 3, 8 and 16 days.

The measurement of protein secreted by corneal stroma cells

After the corneal stroma cells were cultured on the surfaces of different membranes for 24, 72 and 192 h, the protein content in the culture medium was tested by the Coomassie brilliant blue method. The medium was renewed 24 h before the test each time.

Morphological observation of corneal stroma cells

After cells were seeded on the surface of different membranes, cell morphology was observed under an invert phase contrast microscope. After 36 hours and 6 days of culture, all membranes with cells were fixed with 3% glutaraldehyde in a phosphate buffer (pH 7.4) for 2 hours and then washed with phosphate buffer for 30 minutes. The samples were osmicated in 1% OsO_4 for one hour and washed again. After dehydration in graded serious of ethanol, the samples were dried in a critical point dryer. After sputter coating samples with gold/palladium, the samples were observed with scanning electron microscopy (Hitachi XZ650).

Morphological observation of corneal endothelial cells

According to the above experimental results, CH-CS3 was selected as the optimal scaffold for corneal endothelial cell culture. Corneal endothelial cells were seeded on the surfaces of CH-CS3 and cultured for 72 h or 30 days. CH-CS3 scaffolds with cells were treated by the method described above and observed with scanning electron microscopy.

After 30 days of culture, a confluent endothelial cell monolayer formed. CH-CS3 with cells were fixed with 2.5% glutaraldehyde, washed with phosphate buffer, osmicated in 1% OsO_4 and washed again as described above. After dehydration in graded series of acetone, the samples were soaked and embedded with Epon-812 epoxide resin, longitudinally cut with an ultramicrotome, electron-stained with uranyl acetate-lead citrate and examined with a transmission electron microscope (H-600).

Statistical analysis

The experiments were performed in quintuplicate (n=5) unless otherwise specified. The data were analyzed using paired and unpaired t-tests with SPSS. $P \leq 0.05$ was considered statistically significant difference; $P \leq 0.01$ was selected as a statistically extremely significant difference.

Results and discussion

The compatibility of chitosan and chondroitin sulfate in blended membranes

Compatibility between different compositions is critical to the homogeneous structure of blended membranes, and the homogeneous structure is very important for the character and function of membranes. The compatibility of chitosan and chondroitin sulfate in blended membranes was evaluated by the differences in surface structure, crystallinity and chemical bonds of chitosan and blended membranes. The surface structures of chitosan membrane and the blended membrane CH-CS3 are shown in **Fig. 1** as typical examples. A fibriform structure was observed on the surface of chitosan membranes, but there was no such structure observed on the surface of blended membranes. Compared to that of the chitosan membrane, the surface of the blended membranes was more homogeneous, and there was no evident macroscopic phase separation phenomenon. It could therefore be concluded that chondroitin sulfate

could improve the homogeneous structure of the blended membranes.

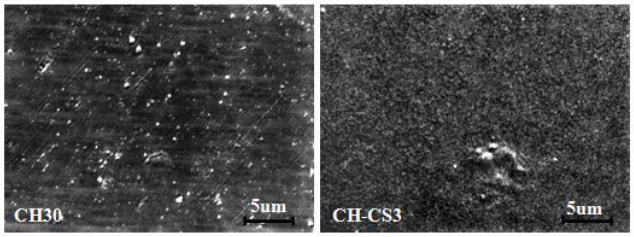

Fig. 1. SEM micrograph of surface structure of chitosan membrane (CH30) and chitosan-chondroitin sulfate blended membrane (CH-CS3). CH30: chitosan membrane with molecular weight of 300 KDa (×2000), CH-CS3: chitosan-chondroitin sulfate blended membrane (1:0.1) (×2000).

Chitosan is a kind of crystalline macromolecule with crystallization peaks at the degrees of 10.50, 15.00 and 20.50, whereas chondroitin sulfate is a macromolecule without crystallization. As shown in **Fig. 2** and **Table 1**, the crystallization degree of chitosan-chondroitin sulfate blended membranes was higher than that of chitosan membranes, and the highest crystallization degree was obtained at the blending ratio of 1:0.05 (CH-CS2). It could be concluded that chondroitin sulfate could increase the crystallization degree of the blended membranes, and a regular arrangement of chitosan in the membranes was favored.

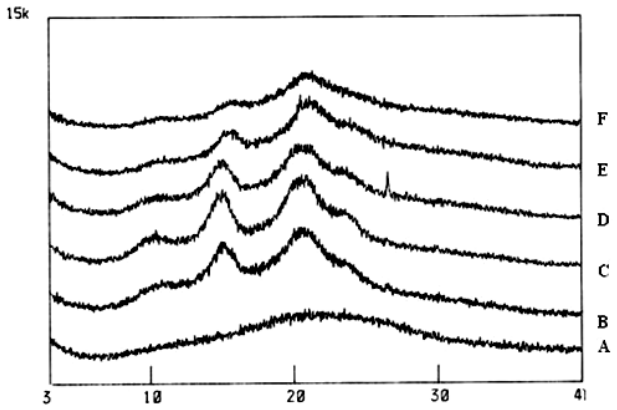

Fig. 2. X-ray diffraction pattern of chitosan and different chitosan-chondroitin sulfate blended membranes. A: chondroitin sulfate membrane, B: chitosan-chondroitin sulfate blended membrane (CH-CS1, 1:0.02), C: chitosan-chondroitin sulfate blended membrane (CH-CS2, 1:0.05), D: chitosan-chondroitin sulfate blended membrane (CH-CS3, 1:0.1), E: chitosan-chondroitin sulfate blended membrane (CH-CS4, 1:0. 2), F: chitosan membrane with molecular weight of 300 kDa.

IR spectrums of chitosan and blended membranes are shown in **Fig. 3**. Compared to that of chitosan membranes, there was no new absorption peak observed in the IR spectrum of blended membranes. In other words, there was no chemical reaction between chitosan and chondroitin sulfate in the blended membranes.

All the above results indicated that chitosan could be compatible with chondroitin sulfate in blended membranes, and chondroitin sulfate could make chitosan molecular arrays regularly, which caused the blended membranes to have a homogeneous structure and show high crystallinity.

Table 1. Crystallization of chitosan and chitosan-chondroitin sulfate blended membranes.

Membranes	Peak at 10.50 degree	Peak at 15.00 degree	Peak at 20.50 degree	Crystallinity
CH30	881	1491	2562	28.00%
CH-CS1	2104	3645	4208	31.30%
CH-CS2	2030	3728	4265	39.10%
CH-CS3	1650	3004	3675	34.70%
CH-CS4	1168	2298	3481	31.96%

Note: CH30: chitosan membrane with molecular weight of 300 KDa; CH-CS1, CH-CS2, CH-CS3 and CH-CS4 represent chitosan-chondroitin sulfate blended membranes with blending ratio of 1:0.02, 1:0.05,1:0.1and 1:0.2 respectively.

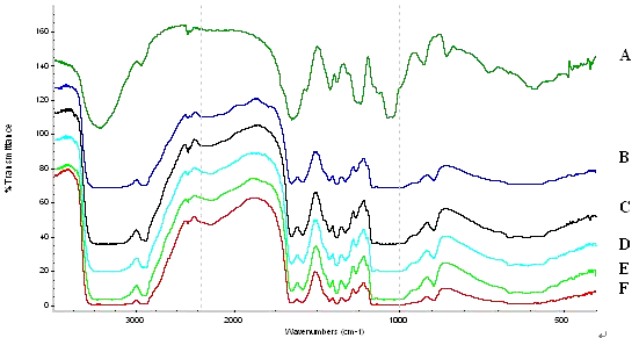

Fig. 3. IR spectrum of chitosan chitosan-chondroitin sulfate blended membranes. A: chondroitin sulfate membrane, B: chitosan membrane with molecular weight of 300 KDa, C: chitosan-chondroitin sulfate blended membrane (CH-CS1, 1:0.02), D: chitosan-chondroitin sulfate. blended membrane (CH-CS2, 1:0.05), E: chitosan-chondroitin sulfate blended membrane (CH-CS3, 1:0.1), F: chitosan-chondroitin sulfate blended membrane (CH-CS4, 1:0.2).

Table 2. Mechanical property and water content of chitosan and chitosan-chondroitin sulfate blended membranes.

Membranes	Tensile strength (MPa)	Water content (%)
CH30	20.42±1.23	87.78±3.45
CH-CS1	25.51±1.54	78.40±3.06
CH-CS2	17.79±1.09	59.24±2.78
CH-CS3	17.16±1.12	69.65±2.89
CH-CS4	24.74±1.67	73.98±3.16

Note: CH30: chitosan membrane with molecular weight of 300 kDa; CH-CS1, CH-CS2, CH-CS3 and CH-CS4 represent chitosan-chondroitin sulfate blended membranes with blending ratio of 1:0.02, 1:0.05,1:0.1and 1:0.2, respectively.

Tensile strength

The tensile strength of chitosan and blended membranes is shown in **Table 2**. The tensile strength of dry blended membranes changed with the changing ratio of chondroitin sulfate in an interesting way. The tensile strengths of blended membranes CH-CS1 and CH-CS4 were much higher than those of chitosan membranes, but the tensile strengths of blended membranes CH-CS2 and CH-CS3 were lower than those of chitosan membranes. These varying tendencies are contrary to those associated with crystallinity, perhaps due to the rearrangement of chitosan molecules by blending of chondroitin sulfate.

Optical properties

As shown in **Fig. 4**, chitosan and blended membranes were optically transparent at all wavelengths of visible light, and the maximal transparency was observed at 700 nm. The transparency of all blended membranes was higher than that of the chitosan membrane, and the maximum transparency was obtained when the blending ratio of chitosan and chondroitin sulfate was 1:0.2 (CH-CS4). This result indicated that chondroitin sulfate could improve the transparency of the blended membranes, perhaps due to the regular rearrangement of chitosan molecules and the homogeneous structure of blended membranes.

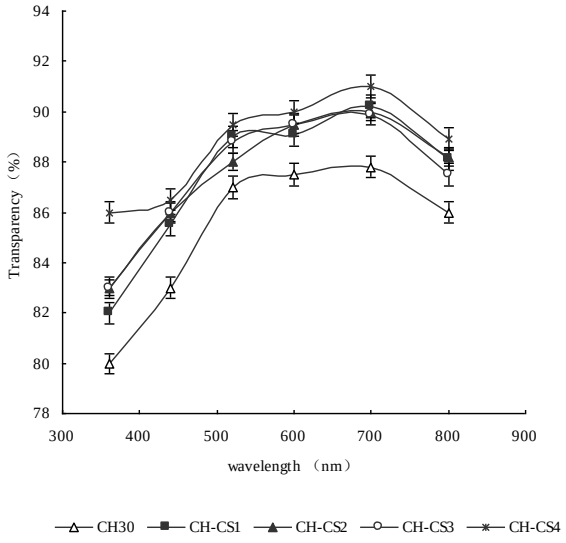

Fig. 4. Transmittancy of chitosan and chitosan-chondroitin sulfate blend membranes. CH30: chitosan membrane with molecular weight of 300 KDa; CH-CS1, CH-CS2, CH-CS3 and CH-CS4 represent chitosan-chondroitin sulfate blended membranes with blending ratio of 1:0.02, 1:0.05, 1:0.1 and 1:0.2, respectively.

Water content

The water contents of chitosan and blended membranes are shown in **Table 2**. Water contents of blended membranes were lower than those of chitosan membranes, and the lowest water content was observed in CH-CS2. This was in accordance with the results of crystallization degree detection. These results could perhaps be explained as being due to the stronger interaction between the two molecules and the more regular array of the molecules at this blending ratio. When the blending ratio of chondroitin sulfate was more than 1:0.05, the chondroitin sulfate molecule played a primary role in the characteristics of the blended membranes and caused the trends in the characteristics to reverse directions.

Permeability to small molecular compounds

The permeability of chitosan and blended membranes to the small molecular compounds (glucose, tryptophan, NaCl) is shown in **Table 3**. The effect of chondroitin sulfate on the permeability of the blended membranes was relative to the membrane structure, molecular size and the interaction between molecules and membranes. Chondroitin sulfate increased the permeability of blended membranes to glucose and tryptophan, but for NaCl, the highest diffusivity was obtained when the blending ratio was 1:0.05.

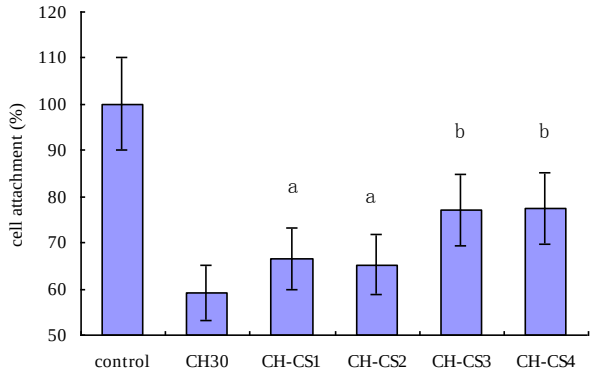

Fig. 5. Attachment of corneal stroma cells on chitosan and chitosan-chondroitin sulfate blended membranes (t-test). control: culture plate; CH30: chitosan membrane with molecular weight of 300 KDa; CH-CS1, CH-CS2, CH-CS3 and CH-CS4 represent chitosan-chondroitin sulfate blended membranes with blending ratio of 1:0.02, 1:0.05,1:0.1and 1:0.2 respectively . a: $P<0.05$, b: $P<0.01$, compared with chitosan membranes.

Corneal stroma cell attachment rate

The attachment rate on the cell culture plate was used as the control, and the results of corneal stroma cells attaching on the chitosan and blended membranes are shown in **Fig. 5**. The attachment rates of corneal stroma cells on the blended membranes (66.68% for CH-CS1, 65.18% for CH-CS2, 77.11% for CH-CS3 and 77.41% for CH-CS4) were lower than those of the control but were higher than those of cells on chitosan membranes (59.26%). The attachment rate increased with the content of chondroitin sulfate increasing in the blended membranes within the blending ratio of 1:0.2. It could therefore be concluded that chondroitin sulfate can promote corneal stroma cell attachment on the blended membranes.

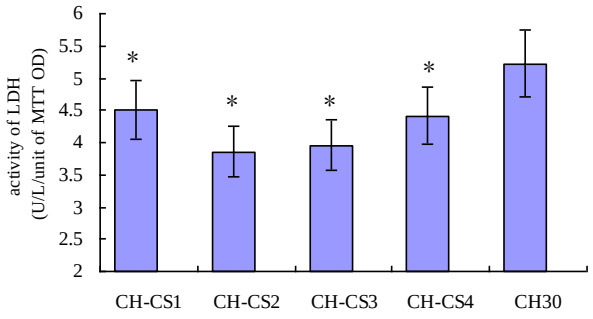

Fig. 6. LDH activities in the medium after culturing corneal stroma cells on chitosan and chitosan-chondroitin sulfate blended membranes for 24 h. CH30: chitosan membrane with molecular weight of 300 KDa; CH-CS1, CH-CS2, CH-CS3 and CH-CS4 represent chitosan-chondroitin sulfate blended membranes with blending ratio of 1:0.02, 1:0.05,1:0.1 and 1:0.2 respectively, * $P<0.01$, compared with CH30 (t-test).

The activities of Lactate Dehydrogenase (LDH) secreted by corneal stroma cells

The activities of LDH in the culture medium of corneal stroma cells cultured on chitosan and blended membranes are shown in **Fig. 6**. Compared to the cells on the blended membranes, cells on the chitosan membrane secreted more LDH. It could be presumed that chondroitin sulfate could improve the compatibility of corneal stroma cells with chitosan and reduce the injury of chitosan membranes to corneal stroma cells. When the blending ratio was less than 1:0.1, the activities of LDH decreased with the content of chondroitin sulfate increasing in the blended membranes. However, the activities of LDH increased when the blending ratio exceeded 1:0.1. Thus, the content of chondroitin sulfate should be controlled at a proper ratio. The blended membranes with too much chondroitin sulfate were also harmful to cells.

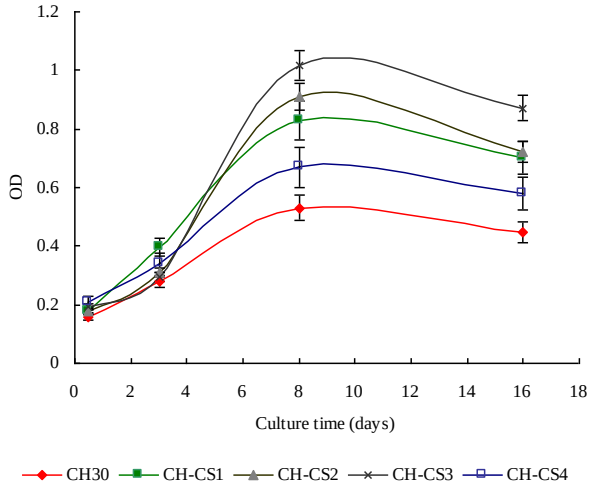

Fig. 7. Proliferation curves of corneal stroma cells cultured on chitosan and chitosan-chondroitin sulfate blended membranes. control: culture plate; CH30: chitosan membrane with molecular weight of 300 kDa; CH-CS1, CH-CS2, CH-CS3 and CH-CS4 represent chitosan-chondroitin sulfate blended membranes with blending ratio of 1:0.02, 1:0.05,1:0.1and 1:0.2, respectively .

Corneal stroma cell proliferation

Fig. 7 shows the growth curve of corneal stroma cells on chitosan and blended membranes. Reproductive activity of corneal stroma cells on blended membranes was better than that of the cells on chitosan membranes, and the reproductive activity of cells increased with the content of chondroitin sulfate increasing in the blended membranes. The best cellular reproductive activity was observed on CH-CS3 scaffolds. Reproductive activities decreased when the chondroitin sulfate blending ratio exceeded 1:0.1, in accordance with the LDH activities.

The protein secreted by corneal stroma cells

The effect of different membranes on the protein secreted by corneal stroma cells is shown in **Fig. 8**. In the initial growing stage on the membranes, the cells metabolized vigorously and the protein was synthesized quickly. As the culture time increased, the protein secreted by the cells diminished, perhaps due to the growth rate becoming slower as the number of cells increased. The metabolic activities of cells on CH-CS1 and CH-CS2 were more vigorous than those of cells on the chitosan membrane, but the protein synthesis speed by the cells on CH-CS4 was lower than that of cells on chitosan membranes. This indicated that the blended membrane could accelerate cell growth, but too much chondroitin sulfate blended membrane could also slow cell growth. This was in accordance with the results of cell adhesion, cell proliferation and LDH activity experiments.

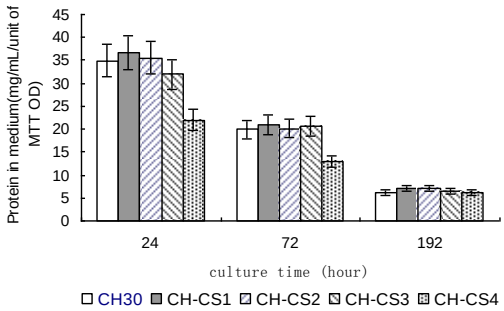

Fig. 8. Protein synthesized by corneal stroma cells after cultured on chitosan and chitosan-chondroitin sulfate blended membranes for 24, 72 and 192 h. CH30: chitosan membrane with molecular weight of 300 kDa; CH-CS1, CH-CS2, CH-CS3 and CH-CS4 represent chitosan-chondroitin sulfate blended membranes with blending ratio of 1:0.02, 1:0.05, 1:0.1 and 1:0.2, respectively.

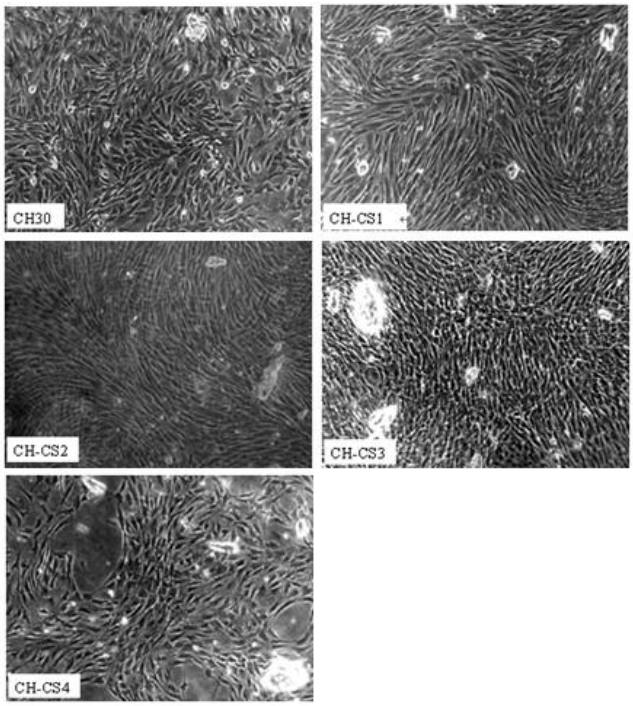

Fig. 9. Status of corneal stroma cells after cultured on chitosan and chitosan-chondroitin sulfate blended membranes for 6 days (×20).CH30: chitosan membrane with molecular weight of 300 KDa; CH-CS1, CH-CS2, CH-CS3 and CH-CS4 represent chitosan-chondroitin sulfate blended membranes with blending ratio of 1:0.02, 1:0.05,1:0.1 and 1:0.2, respectively.

Corneal stroma cell morphology

The growth status of cells on chitosan and blended membranes was observed under an inverted microscope. Cells could attach on different membranes, and they were full, polygonal or rhombic. During the culture process, the cells exhibited a fibriform shape, long and narrow. **Fig. 9** shows the morphology of corneal stroma cells after six days seeded on different membranes. It was clear that cells on CH-CS1, CH-CS2 and CH-CS3 scaffolds had formed confluent monolayers, and that the growth pattern was vorticose, but cells on chitosan membranes and CH-CS4 scaffolds could not form confluent monolayers as the cell density was lower. In the SEM photograph shown in **Fig. 10**, it is clear that when the cell density was lower, corneal stroma cells formed many pseudopods on the surface of the membranes, the compatibility between cell and membrane was good, and the interface between pseudopod and membrane was clear. After six days of the cells being seeded on the membranes, the cells formed confluent monolayers and many root-like pseudopods stretched out from the cell surfaces.

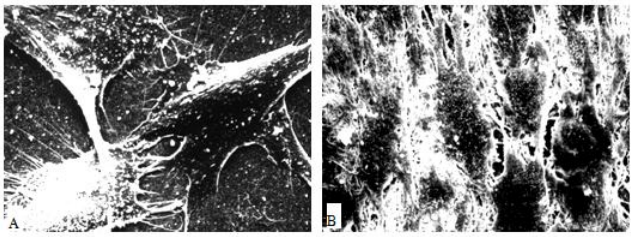

Fig. 10. Status of corneal stroma cells cultured on CH-CS3 examined by scanning electron microscopy. CH-CS3: chitosan-chondroitin sulfate blended membrane with blending ratio of 1:0.1; A : cultured for 36 h (×1000), B: cultured for 6 days (×350).

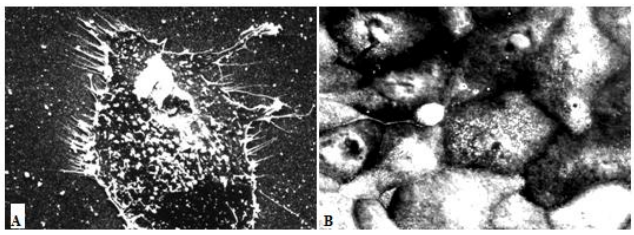

Fig. 11. Status of corneal endothelial cells on CH-CS3 examined by scanning electron microscopic. CH-CS3: chitosan-chondroitin sulfate blended membrane with blending ratio of 1:0.1; A: cultured for 72 h (×1000), B: cultured for 30 days (×400).

Corneal endothelial cell morphology

All above results indicated that CH-CS3 (blending ratio of chitosan and chondroitin sulfate was 1:0.1) displayed better characteristics, so CH-CS3 was used as a scaffold to culture corneal endothelial cells in this study. The growing status of corneal endothelial cells on CH-CS3 was observed under a scanning electron microscope, and the representative result is shown in **Fig. 11**. After 72 h of culture, the cell shapes were deplanate and abundant microvilli were observed on the cellular membranes. At the interface of cells and blending membranes, many pseudopods were observed, which grew into the membrane (**Fig. 11A**). After 30 days of culture, a confluent monolayer was formed and the cell shape became elliptical or polygonal, with some cells even shaped hexagonally as they are in vivo. The cells arranged compactly, and no vacuoles were observed at the junction of cells. Many microvilli were observed on the cellular membrane (**Fig. 11B**) just as is the case of normal corneal endothelial cells in vivo. These results indicated that corneal endothelial cells could grow well on CH-CS3 and had similar structure as normal cells in vivo.

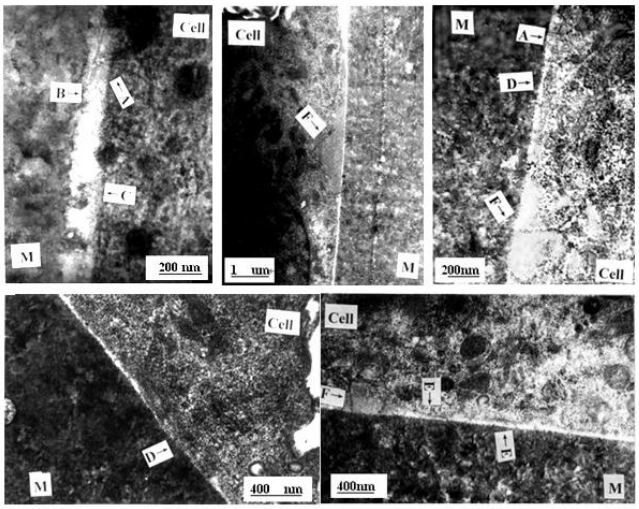

Fig. 12. Attachment of corneal endothelial cells on CH-CS3 examined with a transmission electron microscope. M: CH-CS3 blended membrane, Cell: corneal endothelial cells on CH-CS3 blended membrane, A: Microfilament stretching cross plasma membrane, B: adhesive substance secreted into the gap between cell and CH-CS3 membrane by cell, C: adhesive substance outside of the cellular membrane, D: junction between cell and CH-CS3 membrane, E: hemidesmosomes structure, F: protein structure secreted on CH-CS3 membrane by cell.

The interaction of endothelial cells and CH-CS3 was observed under a transmission electron microscope. As shown in **Fig. 12**, the structure of the cellular membrane where cells attached to the blended membrane was blurry, perhaps due to the vigorous secretary activity of the cellular membrane. Some discontinuous space (<50 nm) was observed between the cells and blended membrane, and the space was filled with an adhesive substance secreted by cells (**Fig. 12B**), which enhanced the attachment of the endothelial cells on CH-CS3. Some cellular membranes attached to CH-CS3 so tightly that no space was observed, and the outline between cellular membrane and CH-CS3 was undistinguishable (**Fig. 12D**). Many discontinuous

distribution structures were observed on the cellular membranes, which were similar to hemidesmosomes (**Fig. 12E**). Outside the cellular membrane, some substance secreted by the cells connected the cellular membrane and CH-CS3 tightly, and some of this substance even permeated into CH-CS3 (**Fig. 12C**). In addition, some microfilament bundles were observed, which stretched out from the cells and extended into intercellular substances or CH-CS3 (**Fig. 12A**). These results showed that the corneal endothelial cells on CH-CS3 could attach to the blended membranes in multiple ways and had good biocompatibility with the blended membranes.

Conclusion

Ultimately, all results indicated that chondroitin sulfate was very compatible with chitosan in the blended membranes and that a proper blending ratio of chitosan and chondroitin sulfate (≤1:0.1) could improve the biocompatibility between cells and membranes and accelerate cell attachment and growth on blended membranes. Therefore, the chitosan-chondroitin sulfate blended membrane has potential applications as a biocompatible scaffold for corneal tissue engineering.

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