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Fabrication and characters of a corneal endothelial cells scaffold based on chitosan

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Abstract A novel chitosan-based membrane that made of hydroxyethyl chitosan, gelatin and chondroitin sulfate was used as a carrier of corneal endothelial cells. The characteristics of the blend membrane including transparency, equilibrium water content, ion and glucose permeability were determined. The results showed that the optical transparency of the membrane was as good as the natural human cornea. The water content of this scaffold was 81.32% which was remarkably close to the native cornea. The membrane had a good ion permeability and its glucose permeability was even higher than natural human cornea. The cultured rabbit corneal endothelial cells formed a monolayer on the membrane. The results demonstrated that the membrane was suitable for corneal endothelial cells to attach and grow on it. In addition, the membranes in vivo could be degraded steadily with less inflammation and showed a good histocompatibility. These results demonstrated that the hydroxyethyl chitosan-chondroitin sulfate-gelatin blend membrane can potentially be used as a carrier for corneal endothelial cell transplantation.

1 Introduction

The corneal endothelium, which exists as a monolayer, plays a critical role in preserving visual acuity by maintaining the hydration and thickness of the stroma. Only an intact endothelium with a sufficient cell density can allow the cornea to maintain clarity. During life-time, human experience a physiological reduction of corneal endothelial cell (CEC) density. The reduction rate is about 0.5% per year [1] and the loss cannot be compensated. Because CECs are arrested in the G1 phase of the cell cycle, they do not proliferate in vivo [2] but will undergo lateral cell expansion to replace lost or damaged cells. Therefore, corneal endothelial dysfunction occurs when the number of corneal endothelial cells becomes insufficient, with the subsequent occurrence of corneal stroma edema [3]. Up to now, the standard therapy for corneal endothelial dysfunction is the full-thickness corneal transplantation. However, this has several drawbacks, including limited availability of donor corneas, recurrent allograft rejection, and subsequent graft failure in certain cases. An alternative approach is that, instead of the full-thickness corneal, the in vitro cultured CECs can be used in transplantation [4]. To achieve this, a matrix that serves as a carrier for corneal endothelial cells is required.

Corneal replacements have been under speculative design for over 200 years. With the developments of the advanced cell culture methods and the tissue engineering, researchers have spurred numerous efforts to produce engineered corneal tissue since the early 1990s. Recently, scaffold design and fabrication are major areas of biomaterial research and they are important subjects for tissue engineering and regenerative medicine research.

Degradable scaffold plays an irreplaceable role in tissue regeneration and repair when a tissue engineering approach is applied [5]. Several decades ago, scientists tried to manipulate endothelial cell density by transplanting isolated cells using different methods. Strategies to culture corneal endothelial cells onto biodegradable membranes were also their goal. So far, the carriers used for corneal

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endothelial cells include allograft cornea, thin gelatin membrane, hydrogel membrane, collagen matrix, collagen sheet, biodegradable polymers, amniotic membrane, Descemet membrane, etc. Although these carriers had good biocompatibility, transplantation into the corneal stroma has caused a range of complications [6–14]. For example, the most common extra cellular matrix (ECM) component in mammalian tissues is collagen, which accounts for approximately 30% of all body proteins [15]. Extracted, purified collagen has been popular in tissue engineering due to its high biocompatibility. Although very robust in vivo, extracted collagen is rapidly degraded and lacks the mechanical toughness and elasticity, due to the dissociation of natural cross-links during isolation and purification process [16]. Though there has been significant progress toward the development of engineered corneal replacements, the results of these efforts has been somewhat disappointing. Currently, no clinically viable tissue-engineered corneas are approved for use in end-stage corneal disease.

The present study aims at searching for carrier materials that may have better cell attachment promoting properties and implantation applicability for corneal endothelial cells.

Chitosan (CTS) is a biomimetic, amino cationic polysaccharide derived by deacetylation of chitin, the main component of the exoskeleton of crustaceans [17, 18]. Chitosan has been widely applied in drug delivery, gene therapy and tissue engineering because of its biocompatibility and biodegradability [19–21]. In addition, the expression of ECM protein could be prompted by chitosan in human osteoblasts and chondrocytes. Chitosan could also enhance cell adhesion and proliferation, including the osteoblasts, fibroblast, keratinocyte and endothelium cells [22].

The biomembranes which were made of chitosan, hyaluronate, collagen, and chondroitin sulfate have been used in reconstruction of cornea [23]. However, when the chitosan membranes were implanted into cornea, severe host inflammation in signs of fibrous encapsulation and corneal neovascularization were observed. Particularly, the initially induced inflammation after implantation might endanger a successful engraftment [24, 25]. Therefore, the structure and composition of a carrier or scaffold for corneal cells must be modified to relieve the initial host tissue response after implantation.

Moreover, further biomedical applications of CTS are greatly limited because of its poor solubility in physiological solvents. Therefore, a water-soluble derivative of CTS becomes hot spot in this research area. To improve the water-solubility and biocompatibility of CTS, a derivative of CTS-Hydroxyethyl chitosan (HECTS) was prepared in our laboratory. It was synthesized by reaction of chitosan and ethylene oxide. The fabrication and characters of this material will be described more detail in a separated paper. The experimental results demonstrated that this material has a good solubility and good film-forming capability and can be applied to the reconstruction of tissue engineered cornea. Furthermore, gelatin (Gel) is derived from partial degradation of collagen and has low antigenicity. Chondroitin sulfate (CS) is a kind of mucopolysaccharide and present in a significant amount in human corneal stroma. Usually, CS is added in cornea preservation medium and has an outstanding effect on corneal endothelial cell cultures [26].

In the present study, we screened out a blend membrane based on HECTS. The properties of the blend membrane, the effects of the membrane on cell attachment and growth, the in vivo biodegradability and biocompatibility were investigated. The results showed the feasibility and suggests that HECTS is a suitable material as the carrier material in reconstruction of the tissue-engineered cornea.

2 Materials and methods

2.1 Materials and reagents

Wistar rats were purchased from Qingdao Laboratory Animal Center. New Zealand Rabbits were purchased from Agriculture Science Research Department of Shandong Province. HECTS (degree of deacetylation = 75%, Mw = 60 kDa) was prepared in our laboratory, CS (Qingdao Better Biotechnology Co. Ltd, China; Mw = 10 kDa) and biodegradable cross linker were prepared and purified in our lab. Gelatin(Gel), epithelium growth factor (EGF), 3-(4,5-dimethylthiazol-2yl)-2,5, diphenyl tetrazolium bromide (MTT) and basic fibroblast growth factor (bFGF) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Materials for cell culture, including Dulbecco's Modified Eagle Medium (DMEM)/F12 culture medium and fetal bovine serum (FBS) were purchased from Gibco Co. (Grand Island, N.Y., U.S.A). 3-0 non-absorbable surgical sutures were purchased from Shanghai Xincheng Medical Equipment Co. Ltd. (Shanghai, China). All other reagents used were reagent grade.

2.2 Preparation of HECTS blend membrane

2% HECTS solution was mixed with the 0.2% Cs aqueous solution and 2% Gel solution (weight ratio of HPCTS: Gel: Cs = 90:10:1) under agitation for 30 min at 35°C. Then, the solution was adjusted to pH 10. A suitable amount of potassium acetate and cross linker was added to the mixture. The mixture was poured into a flat-bottomed glass dish, and then dried at 35°C to form a thin membrane. The HECTS-Gel-Cs blend membrane was repeatedly washed with D-Hanks' balanced saline until the pH to reach a physiological range (Tables 1, 2).

Table 1 Optical transmittance of the membrane, each point represents the mean ± SD of three experiments

Wavelength (nm)	Mean ± SD
400	90.383 ± 1.321
500	93.333 ± 1.448
600	94.457 ± 1.520
700	95.960 ± 1.318
800	96.740 ± 1.271

Table 2 Analysis of the OD_{492} values on the 5th and 8th days for CECs. Each result represents the mean ± SD of three experimental data

Time	OD_{492} (Mean ± SD)	
	Control	Membrane-CEC
5 days	0.159 ± 0.012	0.229 ± 0.038*
8 days	0.215 ± 0.022	0.293 ± 0.067

Asterisk denotes significant differences of OD_{492} values compared to the control ($P < 0.05$) as determined by Student's *t*-test

Membranes for in vitro cell culture and in vivo implantation studies were prepared under sterile conditions. All regents were either autoclaved or sterilized by filtering with 0.22 μm filters.

2.3 Measurement of the properties of the blend membrane

2.3.1 Transparency measurement of the blend membrane

The transparency of the membrane pieces were examined by scanning them within the range of wavelengths (400–800 nm) using a TU-1800S UV–Visible Spectrophotometer (Beijing, China).

2.3.2 Measurement of equilibrium water content

The equilibrium water content of membrane was defined as the weight ratio of water content to the swollen membrane. Membranes were soaked in 0.1 M phosphate buffered saline (pH 7.4) for 24 h at 37°C. Then the membranes were removed from the buffer solution, placed between two pieces of dried filter paper to remove excess solution, and then weighed (W_1). These samples were dried in a 105°C oven for 2 h, and weighed again to determine the dry weight (W_2). The percentage of equilibrium water content (%) was calculated with the following equation:

$$\text{Equilibrium water content } (\%) = [(W_1 - W_2)/\ W_1] \times 100\%$$

2.3.3 Ion and glucose permeability test

Ion and glucose permeabilities of the blend membranes were determined using a custom-made device. The blend membranes (1.5 cm in diameter) were placed into the apparatus without leaking. Sodium chloride solution (0.9 g NaCl, 100 ml, in triple distilled water) or glucose solution (1 g glucose, 100 ml, in triple distilled water) was at right side, and triple distilled water was at left side of the scaffold. Then the apparatus was placed into an incubator at 37°C and 100 rpm. The conductivity of the left side liquid was recorded to test the ion permeability until it reached its equilibration. The glucose concentration of the solutions in each chamber was periodically measured using DNS (3,5-Dinitrosalicylic acid) method [27]. The permeability coefficient (P [cm/s]) of glucose was calculated by the rate of glucose concentrations change with time using the following equation [28]:

$$P = [(dc/dt) \cdot V]/(A \cdot C_0 \cdot 60)$$

where dc/dt is the increase of permeated cumulative glucose amount versus time, V is the volume of the receiver compartment, A is the surface area of the membrane, C_0 is the initial glucose concentration, 60 is the conversion factor from minute into second.

2.4 Cyto-compatibility

2.4.1 Rabbit corneal endothelial cells culture

Rabbit corneal endothelial cells were cultured in DMEM/F12 (1:1) medium supplemented with 10% FBS. After reaching 80% confluence, the endothelial cells were rinsed twice with D-Hanks' balanced salt solution, incubated with a mixture of 0.25% trypsin and 0.02% EDTA at 37°C for 2 min, then neutralized with a culture medium containing 10% FBS. The cells at a density of 5.0×10^5 cells/ml were cultured in a CO_2 (5%) incubator at 37°C.

2.4.2 Corneal endothelial cells culture on scaffolds

Sterile blend membranes (11 mm in diameter) were put in the wells of 48-well tissue culture polystyrene (TCPS) plates. The P2 cells were seeded onto the membranes at a density of 1.0×10^5 cells/ml. And the same treated wells without membrane were the control group. They were cultured at 37°C/5%CO_2 with the medium changed every 3 days. Cell morphology and attachment were monitored by microscope. The growth of CECs was detected by MTT assay on the 5th and 8th days. All groups were performed by analyzing the ultimate results of the OD_{492} value. Experiments were done in triplicate.

2.4.3 SEM of CECs cultured on scaffolds

After cultured for 10 days, scaffolds were washed by D-Hanks' solution for three times before the SEM processing. Samples (HECTS/Gel/CS + Cells and HPCTS/Gel/CS) were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 1 h, dehydrated in ascending grades of alcohol, soaked in isoamyl acetate, CO_2 critical point drying, sputtered coating with gold and then examined using a scanning electron microscope (KYKY-2800B, KYKY Technology Development Ltd., China) at an accelerating voltage of 25 kV.

2.4.4 Fluorescence microscope imaging of CECs on scaffolds

CECs were labeled with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) before seeded on scaffolds. The labeled CECs were seeded onto the membranes as previously described in 2.4.2. Cell morphology and attachment were monitored using fluorescence microscope.

2.5 Evaluations of biocompatibility in vivo

The in vivo degradability of membranes was examined by implanting the membranes into the skeletal muscle of Wistar rats. Wistar rats weighing about 200 g were kept under a specific pathogen free condition throughout the experiment. The sterile membranes (6 mm in diameter) were implanted into the skeletal muscle of anesthetized rats. The rats implanted with 3-0 non-absorbable surgical sutures were set as controls. First, the sutures were twined cycle by cycle to form discs with 6 mm in diameter. And the following operation is homology to the experimental group. After that, three rats were sacrificed on 15, 30, 45, and 60 days after the implantation, respectively. Membranes and their surrounding tissue were removed, and subsequently fixed in 4% neutrally buffered formaldehyde, embedded in paraffin, stained with haematoxylin–eosin (HE) and then histologically analyzed.

2.6 Evaluations of histocompatibility in eyes

New Zealand rabbits were used as experimental animals. The sterile blend membranes (3.5 mm in diameter) were implanted into the anterior chamber of the right eye after the rabbit was anesthesia. On the 10th, 20th and 30th days, the eyes were imaged by camera, slit-lamp and after that three rabbits were sacrificed, respectively. The eyeballs were removed and analyzed for histology as described in Sect. 2.5.

2.7 Statistical analysis

Data were shown as means ± SD (standard deviation) of a representative point from three similar experiments. Statistical analysis of data was performed by one-way analysis of variance (ANOVA), and a value of $P < 0.05$ was considered significant (computed by SPSS version 13.0 Software).

3 Results

3.1 Properties of the blend membrane

3.1.1 Optical transmittance

Transparency of implanted membrane is critical to the quality of implantation. We measured the light transmission through the membrane in the light wavelength range (400–800 nm) as the indicator of the membrane transparency and the results is shown in Table 1. Over 90% optical transmittance was observed in all tested visible wavelengths. The results indicate that light transmission meets the quality criteria of corneal carrier.

Previous work [18] demonstrates in spectral regions (at 450, 500, 550, 600 and 650 nm), light transmission values of human corneas in eye bank increased from 50 to 75%. According to this data, the scaffold is obviously more transparent than human cornea.

3.1.2 The equilibrium water content

The membrane prepared in this study had a fitting equilibrium water content of 81.32%. As it is known that the water content of human cornea is between 72 and 82%. Therefore, the water content of this scaffold is in the normal range of the native cornea.

3.1.3 The permeability

The permeable rate changed with time of membranes for NaCl and glucose was shown in Fig. 1. NaCl had permeated to the left side after 4 h, and the conductivity rose rapidly during 48 h. On the 1st day, the conductivity was 2720 μs/cm and reached 5190 μs/cm after 48 h. From 48 to 240 h, the permeable rate rose steadily and the conductivity reached 9950 μs/cm. After that, the increase of the permeability slowed down and both sides almost reached their equilibrations on the 13th day. The result indicates the scaffold is permeable for NaCl and the iron could pass the membrane persistently and steadily.

The rate of glucose concentration changed with time was shown in Fig. 1. Within 48 h, glucose went across the

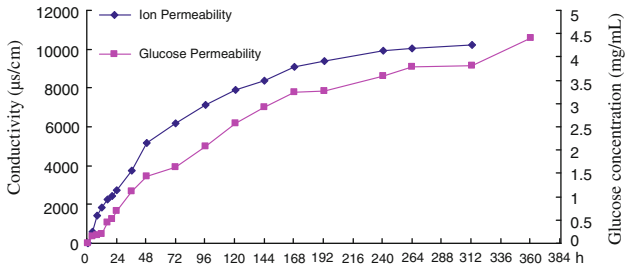

Fig. 1 The ion and glucose permeability of the membrane changed with time. The iron could pass the membrane persistently and steadily. Both sides almost reached their equilibrations on the 13th day, and the glucose contents of the two sides were almost equal at 360 h

membrane at a remarkable rate. After that, the rate increase slowed down but the glucose concentration increased steadily. On the 15th day, the glucose contents of the two sides were almost equal. At this time, the glucose permeability coefficient of the blend membrane was 1.93×10^{-5}cm/s. These results demonstrate the membrane is glucose permeable.

According to the previous studies [29], the permeability of native cornea is between 10^{-6} and 10^{-7}cm/s. Therefore, the permeability of the membrane is comparable to or even better than the native cornea.

3.2 Morphology and activity of the cells on the membrane

3.2.1 Morphology and growth of CECs cultured on scaffolds

To investigate the cyto-compatibility of the scaffold, the rabbit corneal endothelial cells were cultured on the membrane. Figure 2A–C) showed the micrographs of rabbit corneal endothelial cells spread on the membrane surface. As a control, the same experiment was conducted on 48-well plate (Fig. 2a–c).

The situations of cell adhesion and growth were observed on both control and experimental groups (Fig. 2a, A). The results showed that the cells maintained normal morphology in both control and experimental conditions after the cells were cultured for 3 days. However, compared to the control, the cells cultured on the blend membranes grew faster. On day 6, for the experiment group (Fig. 2B), a large number of CECs reached confluence with a cobblestone appearance and cohesive organization. Upon longer culture time, there was a trend that the cells could proliferate faster on blend membrane than the control (Fig. 2c, C,). At the same time, the activity of CECs was detected by MTT assay (Table 2). On the 5th and 8th days, the OD_{492} values of experiment groups were higher than those in the control group. Therefore, all results suggest that the blend membrane has a good cyto-compatibility and it can enhance the growth rate of the cells.

3.2.2 Observation of CECs cultured on scaffolds by SEM

The surfaces of scaffolds were important to evaluate the scaffolds' potential to sustain growth of the cells. The scaffolds had rough surfaces to support the cell (Fig. 3A1). We could observe the lamellipodia of the spread CECs on the membrane (Fig. 3A2). As it is known that lamellipodia symbolizes the attachment of cells. So it showed that the CECs could attach and live on the scaffold well. In other words, the scaffold is suitable for activated cells growing on it.

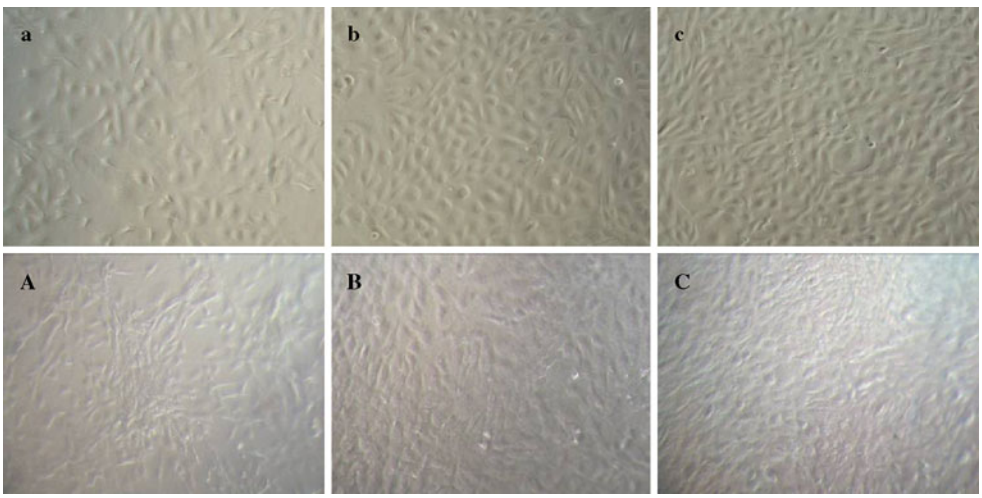

Fig. 2 The micrographs of rabbit corneal endothelial cells spread on the TCPS (a–c) and the membrane (A–C) after they were cultured for 3d (a, A), 6d (b, B) and 8d (c, C) (100×). Cells could maintain normal morphology in both groups, and the cells cultured on the blend membranes reached confluence and cohesive organization

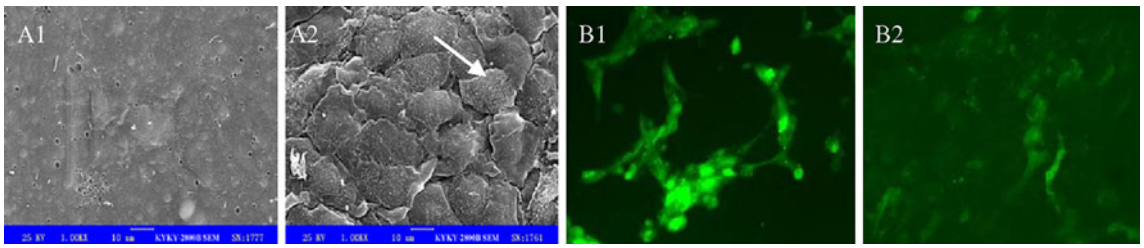

Fig. 3 Observation by SEM(1.00 K×)and fluorescence microscope (100×). The surface of the blend membrane (A1) and CECs cultured on the membrane (A2) were observed by SEM on the 10th day. The lamellipodia of the spread CECs was marked with arrow; Cells connected with each other to form a mesh and had strong green fluorescence on the 3rd day (B1), and cells multiplied to link into pieces and the fluorescence becomes dimmer on the 8th day (B2)

3.2.3 Fluorescence microscope imaging of CECs on scaffolds

CECs labeled with CFSE were seeded on scaffolds. On the 3rd day, the cells on scaffolds had strong green fluorescence (Fig. 3B1). The cells could connect with each other to form a mesh and attach on the membrane closely. With the fluorescent protein adduct formed by CFSE and cytoskeleton allocated equally to two second generation cells during proliferation. The green fluorescence weakened on the 8th day (Fig. 3B2). But the cell profile can still be found easily and cells linked into pieces. This result indicates that the CECs cultured on the membrane can attach and proliferate well on it.

3.3 Biodegradability in vivo

All mice were alive and no observable signs of infection and distress were noticed with the blend membranes implanted for 60 days. No sign of inflammation was found when the muscles of the experimental groups were examined by post-mortem visual examination. And the inflammation of the tissues just could be observed by making histological sections. On day 7, a large number of inflammatory cells were observed both in control and experimental groups (Fig. 4a, A). During the following days, the inflammatory reactions were lightened with the membranes gradually degraded. And the inflammatory cells of the experimental group faded more quickly than the control (Fig. 4b–d, B–D). On day 60 postoperatively, the control group had a light inflammatory response around the implants and their surrounding area (Fig. 4e). In contrast, the membranes were totally degraded and the tissues around the embedded point were almost the same as the normal muscles (Fig. 4E). And there weren't any fibrous encapsulation surrounding the implanted area during the whole degradation process.

3.4 Histocompatibility in anterior chamber of eyes

Postoperatively, there were no observable infections or diseases in the anterior chamber of rabbit eyes implanted with membranes. During the 1st week, the corneas of the operated eyes had complete clarity and there were no signs of turbidity. The irises, alone, were slightly engorged. The intact membrane in the anterior chamber could be seen clearly (Fig. 5A1). The eyeballs were stained with HE. This observation showed that there was a light

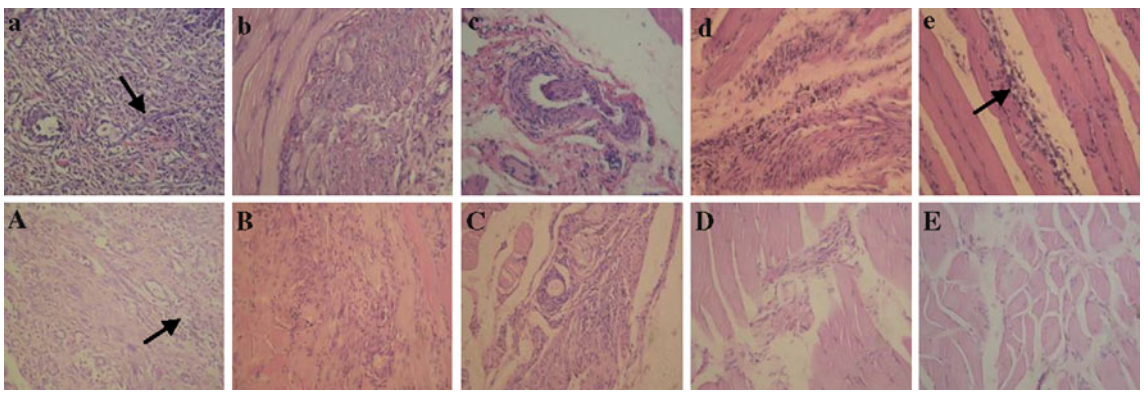

Fig. 4 Photomicrographs of the muscle histological sections of the control (a–e) and experimental (A–E) groups on the 7th, 15th, 30th, 45th, and 60th day (200×). Note the inflammatory cells (*arrows*) surrounding the tissues in close vicinity to the implant. The inflammatory cells surrounding the tissues implanted with the blend membrane were almost disappeared on the 45th day

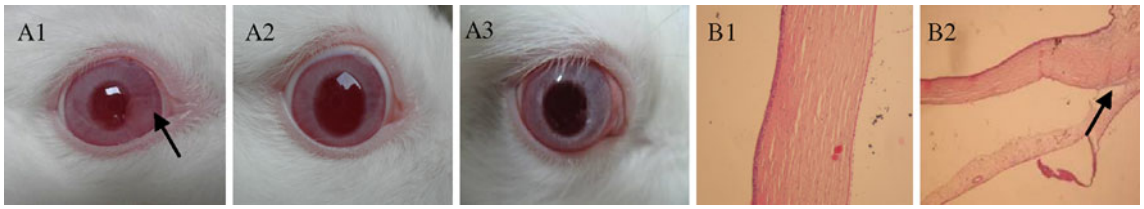

Fig. 5 Eyeball images after implantation with membrane into the anterior chamber (A1–A3) and HE staining images of experimental groups on day 7 (B1, B2) (100×). The intact membrane in the anterior chamber could be seen clearly during 1 week (A1), the corneas maintained normal structure and curvature (B1), and an *arrow* noted a small quantity of inflammatory cells at the contact point between the cornea and iris (B2). The membrane almost degraded totally during 3 weeks (A2) and the eyes were almost the same as the normal (A3)

inflammatory response in the anterior chamber. The corneas were not influenced and maintained normal structure and curvature (Fig. 5B1). Only a small quantity of inflammatory cells at the contact point between the cornea and iris were observed (Fig. 5B2). During 3 weeks, the engorgement had almost disappeared and the cornea still retained clarity (Fig. 5A2). The eyes of experimental groups were almost the same as the normal (Fig. 5A3). This means that the membrane has a good histocompatibility with the anterior chamber of eyes and is suitable for being the scaffold of CECs.

4 Discussion

In this study, a novel chitosan based blend membrane was developed using solvent casting/particulate leaching technique. The potassium acetate is used to function a porogenic agent. The presence of KAc resulted in the formation of holes between pore walls in the carrier. Salt fusion treatment caused an increase in the compressive modulus of solvent cast scaffolds, possibly due to the formation of thick annular struts adjacent to holes in pore walls [1]. For the materials with different molecular weights, the amount of potassium acetate and the amount of crosslinker are different. It is depended on the physical, chemical and biological properties of the membrane.

In this study, Gel, Cs and HECTS were used for the membrane synthesis. The three materials form a homopolymer. Among the three materials, HECTS is able to form ionic complexes with the negatively charged Cs and Gel and the ionic cross-linking could immobilize Cs and Gel. For the membrane synthesis, we have tested the three components at different ratios. Eventually, a ratio of 90:10:1 was chosen. At this ratio, the transparency and attachment for corneal endothelial cells of the synthesized membrane are optimal.

For successful tissue engineering scaffolds of cornea, some characteristics, such as vascularization, permeability to glucose and other small molecules had been suggested to be critical important [30]. The good permeability of the membrane can make sure the nutrition in anterior chamber transfer to the stroma and epithelium [31]. The water content of an artificial cornea was affected by several parameters, including the hydrophilicity, stiffness and pore structure. The membranes prepared in this study had moderate water content that closed to the native cornea. The results suggest that the synthesized membrane provides a similar circumstance as native cornea.

In this study, the blend membrane was designed to meet the requirements of cell growth and proliferation. Gelatin has been widely used in medicine for its excellent biocompatibility and biodegradability. Besides, HECTS derived from natural polymers mimicke many features of the extracellular matrix (ECM) and thus have the potential to direct cell migration, growth and organization during tissue regeneration. Our results demonstrated that CECs cultured on the membranes maintained good activity and multiplied properly.

The biodegradability and histocompatibility in vivo of the membrane were assessed in a rat model via the intramuscular implantation and in a rabbit model via the anterior chamber implantation. It has been reported that when a biomaterial is implanted, the local tissue reacts initially to the injury, and then to the presence of the material. Inflammation is the most common reaction to all injury forms. However, the abnormal wound healing can result in the damage of the host tissue [32]. In this study, we found out that the inflammatory reactions in the muscle of experimental groups were less than medical suture groups (Fig. 4). Additionally, the membranes in muscle were not surrounded and encapsulated by fibrous connective tissue. In the same way, the membranes in the anterior chamber could be degraded steadily with less inflammation. Besides, the corneas were not influenced and maintained normal structure and curvature, and corneal neovascularization was not observed (Fig. 5). In our previous studies, we have investigated a water-soluble derivative of CTS-Hydroxypropyl chitosan (HPCTS) [1]. In that study, we found that the HPCTS-based blend membrane was suitable for adhesion and growth of corneal epithelial cells, but it produced the encapsulation during degradation in the

muscle. And in the following studies, when the membranes were implanted into the anterior chamber, severe host inflammation in signs of fibrous encapsulation and corneal neovascularization were observed. All in all, the HECTS based blend membrane has better biodegradability and histocompatibility. And these characteristics are the biggest advantages compared to any other scaffold based on chitosan in our previous studies.

As this experiment is still in the exploration stage, many details will be supplemented and urgently improved in future studies. The transplantation for the scaffold carrying cells should be put in practice and the final function of this cell-carrier constructs in vivo need to be further assessed using animal models.

5 Conclusions

In this study, the blend membrane was successfully prepared with Gel, Cs and HECTS. The blend membrane was characterized by its good transparency, ion and glucose permeability, cyto-compatibility for CECs, degradability and biocompatibility. It demonstrates the potential use of this membrane as a new biomaterial for tissue engineering. This study also provides an opportunity for the future development of chitosan-based scaffolds for corneal tissue regeneration.

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References

1. Gao XS, Liu WS, Han BQ, Wei XJ, Yang CZ. Preparation and properties of a chitosan-based carrier of corneal endothelial cells. J Mater Sci Mater Med. 2008;19:3611–9.
2. Ide T, Nishida K, Yamato M, Sumide T, Utsumi M, Nozaki T, et al. Structural characterization of bioengineered human corneal endothelial cell sheets fabricated on temperature responsive culture dishes. Biomaterials. 2006;27:607–14.
3. Yang J, Yamato M, Shimizu T, Sekine H, Ohashia K, Kanzaki M, et al. Reconstruction of functional tissues with cell sheet engineering. Biomaterials. 2007;28:5033–43.
4. Dobbins KRB, Price FWJ, Whitson WE. Trends in the indications for penetrating keratoplasty in the Midwestern United States. Cornea. 2000;19:813–6.
5. Liu W, Cao YL. Application of scaffold materials in tissue reconstruction in immunocompetent mammals: our experience and future requirements. Biomaterials. 2007;28:5078–86.
6. Aboalchamat B, Engelmanna K, Böhnke M, Eggli P, Bednarz J. Morphological and functional analysis of immortalized human corneal endothelial cells after transplantation. Exp Eye Res. 1999; 69(5):547–53.
7. Lloyd AW, Faragher RGA, Denyer SP. Ocular biomaterials and implants. Biomaterials. 2001;22:769–85.
8. Mohay J, Lange TM, Soltau JB, Wood TO, McLaughlin BJ. Transplantation of corneal endothelial cells using a cell carrier device. Cornea. 1994;13(2):173–82.
9. Orwin EJ, Hubel A. In vitro culture characteristics of corneal epithelial, endothelial and keratocyte cells in a native collagen matrix. Tissue Eng. 2000;6(4):307–19.
10. Mimura T, Yamagami S, Yokoo S, Usui T, Tanaka K, Hattori S, et al. Cultured human corneal endothelial cell transplantation with a collagen sheet in a rabbit model. Invest Ophthalmol Vis Sci. 2004;45:2992–7.
11. Hadlock T, Singh S, Vacanti JP, Mclaughlin BJ. Ocular cell monolayers cultured on biodegradable substrates. Tissue Eng. 1999;5(3):187–96.
12. Ma DHK, Chen JK, Zhang F, Lin KY, Yao JY, Yu JS. Regulation of corneal angiogenesis in limbal stem cell deficiency. Prog Retin Eye Res. 2006;25:563–90.
13. Engelmanna K, Drexler D, Böhnke M. Transplantation of adult human or porcine corneal endothelial cells onto human recipients in vitro. Cornea. 1999;18(2):199–206.
14. Ishino Y, Sano Y, Nakamura T, Connon CJ, Rigby H, Fullwood NJ, et al. Amniotic membrane as a carrier for cultivated human corneal endothelial cell transplantation. Invest Ophthalmol Vis Sci. 2004;45:800–6.
15. Nimni ME, Cheung D, Strates B, Kodama M, Sheikh K. Chemically modified collagen: a natural biomaterial for tissue replacement. J Biomed Mater Res. 1987;21:741–71.
16. Friess W. Collagen-biomaterial for drug delivery. Eur J Pharm Biopharm. 1998;45:113–36.
17. Berger J, Reist M, Mayer JM, Felt O, Peppas NA, Gurny R. Structure and interactions in covalently and ionically crosslinked chitosan hydrogels for biomedical applications. Eur J Pharm Biopharm. 2004;57:19–34.
18. Rafat M, Li FF, Fagerholm P, Lagali NS, Watsky MA, Griffith M, et al. PEG-stabilized carbodiimide crosslinked collagen–chitosan hydrogels for corneal tissue engineering. Biomaterials. 2008;29:3960–72.
19. Shirosaki Y, Okayama T, Tsuru K, Hayakawa S, Osaka A. Synthesis and cytocompatibility of porous chitosan–silicate hybrids for tissue engineering scaffold application. J Chem Eng. 2008;137:122–8.
20. Mori T, Okumura M, Matsuura M, Ueno K, Tokura S, Minami YS, et al. Effects of chitin and its derivatives on the proliferation and cytokine production of fibroblasts in vitro. Biomaterials. 1997;18:947–51.
21. Pangburn SH, Trescony PV, Heller J. Lysozyme degradation of partially deacetylated chitin, its films and hydrogels. Biomaterials. 1982;3:105–8.
22. Suh JKF, Matthew HWT. Application of chitosan-based polysaccharide biomaterials in cartilage tissue engineering: a review. Biomaterials. 2000;21(24):2589–98.
23. Chen JS, Li QH, Xu JT, Huang YX, Ding Y, Deng HW, et al. Study on biocompatibility of complexes of collagen–chitosan–sodium hyaluronate and cornea. Artif Organs. 2005;29:104–13.
24. Sung HJ, Meredith C, Johnson C, Galis ZS. The effect of scaffold degradation rate on three-dimensional cell growth and angiogenesis. Biomaterials. 2004;25:5735–42.
25. Laschke MW, Haufel JM, Thorlacius H, Menger MD. New experimental approach to study host tissue response to surgical mesh materials in vivo. J Biomed Mater Res A. 2005;74: 696–704.
26. Wang SL, Liu WS, Han BQ, Yang LL. Study on a hydroxypropyl chitosan–gelatin based scaffold for corneal stroma tissue engineering. Appl Surf Sci. 2009;255:8701–5.
27. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem. 1959;31:426–8.

28. Telse R, Sigrid K. In vitro permeation studies comparing bovine nasal mucosa, porcine cornea and artificial membrane: androstenedione in microemulsions and their components. Eur J Pharm Biopharm. 2004;58:137–43.
29. McCarey BE, Schmidt FH. Modeling glucose distribution in the cornea. Curr Eye Res. 1990;9:1025–39.
30. Chirila TV. An overview of the development of artificial corneas with porous skirts and the use of PHEMA for such an application. Biomaterials. 2001;22:3311–7.
31. Li CX, Siedlecki CA. Effects of surface wettability and contact time on protein adhesion to biomaterial surfaces. Biomaterials. 2007;28:3273–83.
32. Vijayasekaran S, Fitton JH, Hicks CR, Chirila TV, Crawford GJ, Constable IJ. Cell viability and inflammatory response in hydrogel sponges implanted in the rabbit cornea. Biomaterials. 1998;19:2255–67.