Original Article

Enhancement of the Mechanical and Biological Properties of a Biomembrane for Tissue Engineering the Ocular Surface

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Abstract

Introduction: In this study, we have developed and optimised a novel gelatin-chitosan (GC) substrate for use as a cellular carrier for tissue-engineered conjunctival epithelium. Materials and Methods: The substrate was fabricated by casting and the mechanical properties of the substrate, including tensile strength and elongation, were measured. Using the MTT, cell proliferation assay with rabbit conjunctival fibroblasts, we optimised the G:C ratio to enhance cytocompatibility. Rabbit conjunctival epithelial cells were immunostained using monoclonal antibodies for keratin 4 and pancytokeratin to investigate the biological effects of the GC substrate on the proliferation and differentiation of epithelial cells. Results: We found that increasing the amount of gelatin resulted in an increase in elasticity (from 1:9 to 1:1 ratio), reaching a maximum (101.89% ± 7.13%) at a ratio of 1:1. The MTT assay showed that the proliferation of conjunctival fibroblasts significantly increased from 0.068 ± 0.017 to 0.177 ± 0.011 (P = 0.014) as the gelatin was increased from 20% (1:4) to 50% (1:1). Additional studies using tissue-cultured conjunctiva explants showed that these explants grew well on the substrate, forming a multilayered epithelium. Cell morphology on this substrate was similar to that of cells grown on culture dishes alone. Positive staining of keratin 4 and pancytokeratin indicated that the substrate supported normal differentiation of conjunctival epithelial cells. Conclusion: By enhancing the proportion of gelatin, both the mechanical and biological properties of the chitosan substrate were improved. The results also suggest that this GC biomembrane may be a useful candidate for reconstructive tissue engineering of the conjunctiva.

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Key words: Biomembrane, Chitosan, Conjunctival epithelia, Gelatin, Tissue engineering

Introduction

The most recent treatment for severe ocular surface disease is the transplantation of cultivated corneal and conjunctival epithelial stem cells, which makes use of human amniotic membrane (HAM) as a substrate and cell carrier.[1-10] Although the results are quite promising, this new procedure is still facing some challenges. One of the biggest problems is the difficulty in ensuring the biosafety of HAM in disease transmission, e.g., HIV, hepatitis B and C as well as from bacteria and fungus which will grow readily on HAM. Thus, procuring and storing HAM are serious issues. Another potential problem is the risk of immune-mediated graft rejection. In addition, as a natural product, HAM consistency cannot be controlled. From a surgical standpoint, the physical structure of HAM does not provide significant mechanical strength to act as a tectonic base for support of the sclera or cornea. Optically, when used on the cornea, it is not clear. To overcome the disadvantages of HAM, it would be desirable to develop a synthetic, optically clear membrane that can replace HAM, and provide structural integrity to an ocular surface wound defect. The materials used for this should be biocompatible

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and biodegradable and the membrane produced should be durable, suturable, gas-permeable, and allow the free diffusion of glucose, protein and ions.

Chitosan, a member of the family of glucosaminoglycans (GAGs), has been studied as a substrate and scaffold for tissue engineering of skin.[11] GAGs are major components of skin dermis and cornea, and play a critical role in the process of wound healing.[12-14] Chitosan has been found to have a beneficial role on wound healing in vitro and in vivo.[12] In addition, chitosan has already proven to be useful in ophthalmology, where it has been developed for contact lens fabrication and ocular bandage lenses. However, a pure chitosan substrate would be too stiff for application on the curved ocular surface. Therefore, gelatin, a soft, elastic natural material, can be introduced into the chitosan membrane to improve its chemical and physical properties. Integration of gelatin into chitosan will reduce the stiffness of a membrane and may also improve its biological properties. Gelatin, a biodegradable and biocompatible polymer, is a processed type I collagen so there are no immune properties remaining. In addition, collagen is of course the primary component of the extracellular matrix in the eye and skin. Gelatin, as a denatured collagen, may be expected to have useful biological properties on cell attachment, migration, proliferation and differentiation. A new gelatin-chitosan (GC) composite biomembrane developed here can form a stable network to provide a firm structure to prevent the gelatin from contracting. Thus, a chitosan and gelatin copolymer can make use of the merits of these 2 biomaterials for ocular surface tissue engineering.

In this study, we have investigated the use of gelatin to improve the mechanical and biological properties of a chitosan membrane and the optimisation of this novel GC copolymer. To achieve these goals, we have studied biomembrane preparation, the mechanical properties and cellular evaluation.

Materials and Methods

GC Biomembrane Fabrication and Mechanical Properties

The GC biomembrane was fabricated by casting the solution in a metal mould, evaporating the solvent in a vacuum oven and neutralising the biomembrane in an NaOH solution. The mechanical properties of the GC biomembrane were determined by an Instron tensile testing device (Instron model 5569) with a 10 Newton load cell. All tensile testing in this project was done at room temperature. The specimen was kept moist when it was mounted onto the pneumatic grippers for testing.

Evaluation of Biocompatibility of the GC Biomembrane

Rabbit conjunctival fibroblasts between passages 3-5 were grown on various GC biomembranes and in normal cell culture dishes, fed with DMEM culture medium supplemented by 10% FBS and 1% antibiotics. The cell culture medium was changed every 3 days. The cells were cultured at 37°C for 7 days prior to use for the proliferation study.

Cell proliferation of fibroblasts on the GC biomembrane was evaluated by determining the mitochondrial function of cells using the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT). The acidified isopropanol extracted solution containing blue formazan was analysed directly by a UV spectrophotometer (Tecan Genios pro). The absorbance at 570 nm was determined using 720 nm as reference.[15,16]

Expansion and Differentiation of Rabbit Conjunctival Epithelial Cells on the GC Biomembrane

Rabbit conjunctival epithelial tissue was minced into pieces of about 1 mm^2 and explanted onto the GC biomembrane or into a standard tissue culture dish. DMEM-F12 culture medium, 1:1, supplemented by 10% FBS and growth factors, was used for 10 days. From Day 10 onward, the culture medium was changed to 3:1 DMEM-F12, supplemented by 10% FBS and growth factors for cell differentiation.

Immunohistochemical Analysis

Rabbit conjunctival epithelial cells grown and differentiated on the GC biomembrane were harvested on Day 14. After culturing, the cells were rapidly frozen in OCT at -20°C, sectioned at 8 microns, exposed to 95% ethanol for 20 min, and after washing, incubated with primary antibody such as pancytokeratin (PCK) or cytokeratin 4 (K4) overnight, followed by washing and incubation with a second antibody labelled with fluorescein isothiocyanate (FITC) and counterstaining with propidium iodide (PI). The labelled cells were then examined by confocal laser (Olympus FV-500).

Results and Discussion

Mechanical Properties of GC Biomembranes

We used mechanical testing procedures to understand the interaction between the properties of the membrane, concentrations of the 2 primary components and what was considered to be necessary for surgical manipulation. Varying the gelatin concentration over a wide range from 0% to 80% had a first-order effect on the mechanical properties of the GC biomembrane. Membranes with gelatin concentrations greater than 80% were difficult to fabricate. Figure 1 shows that increasing the amount of gelatin resulted in an increase in elasticity (from 10% to 50%), which reached a maximum (mean ± SEM, 101.89% ± 7.13%, n = 6) at 50% (1:1 ratio). Increasing the gelatin component also led to a change of the tensile strength of the

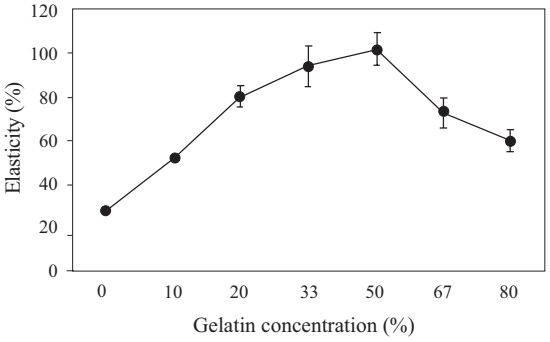

Fig. 1. Effect of gelatin concentration on elasticity of gelatin-chitosan biomembrane. All the specimens were kept in wet conditions as the tests were conducted. Data (n = 3 ± SEM) are presented as mean of elasticity reading in percentage.

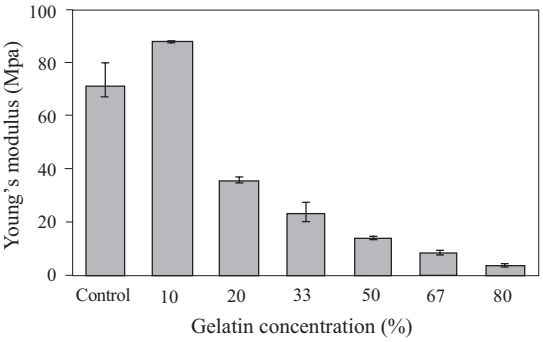

Fig. 3. Effect of gelatin concentration on Young's modulus of gelatin-chitosan biomembrane. All the specimens were kept in wet conditions as the tests were conducted. Data (n = 3 ± SEM) are presented as mean of Young's modulus in Mpa.

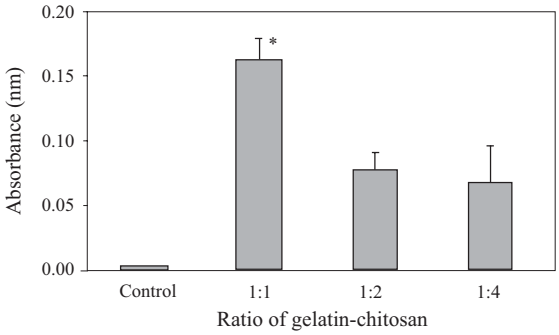

Fig. 5. Proliferation of rabbit conjunctival fibroblasts on gelatin-chitosan biomembrane. The rabbit conjunctival fibroblasts cultured on GC biomembrane with various concentration of gelatin for 7 days at 5% CO_2 and 37°C. Data (n = 3 ± SEM) are presented as mean of UV absorbance reading of optical density at 570 nm. P <0.05 (*) will be considered as significant difference.

GC biomembrane from 18.03 ± 1.18 Mpa for 10% gelatin to 1.28 ± 0.11 Mpa for 80% gelatin, as shown in Figure 2. Meanwhile, Young's modulus of this biomembrane changed from 88.10 ± 0.37 Mpa to 3.47 ± 0.27 Mpa by including additional gelatin (Fig. 3). In summary, increasing the amount of gelatin resulted in an increase in elasticity within a concentration range (from 1:9 to 1:1 ratio) and lowered the tensile strength of the membrane.

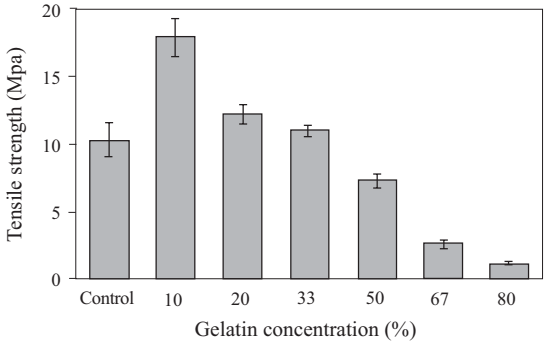

Fig. 2. Effect of gelatin concentration on tensile strength of gelatin-chitosan biomembrane. All the specimens were kept in wet conditions as the tests were conducted. Data (n = 3 ± SEM) are presented as mean of tensile strength reading in Mpa.

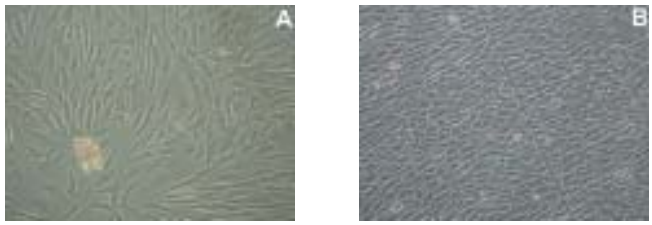

Fig. 4. Cultivation of rabbit conjunctival fibroblasts on the normal petri dish (A) and GC biomembrane (B) for 7 days at 5% CO_2 and 37°C. Magnification 100x.

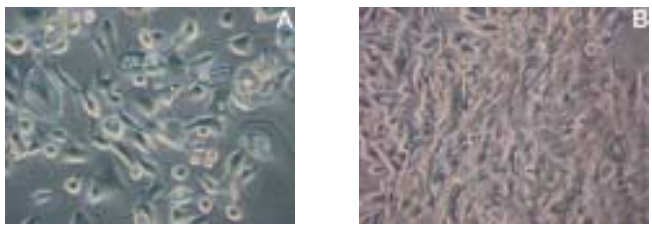

Fig. 6. Expansion of rabbit conjunctival epithelial cells on the normal petri dish (A) and the GC biomembrane (B) for 14 days. Magnification 200 x.

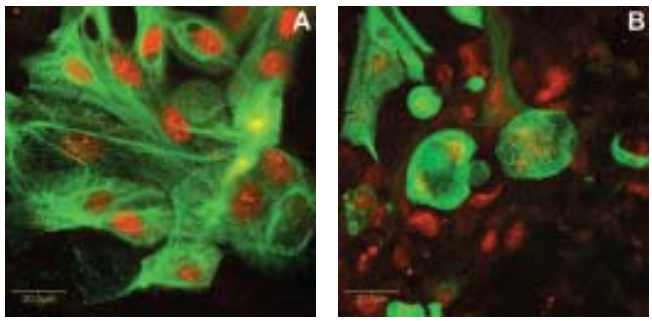

Fig. 7. Confocal laser microscopy of PCK and K4 in rabbit conjunctiva epithelial cells grown on GC biomembrane. Bar represents 20 microns.

Biological Evaluation of GC Biomembrane

1. Biocompatibility of GC biomembrane

Growth of rabbit conjunctival fibroblasts on the GC biomembrane was carried out to determine the cellular compatibility of GC biomembranes. In Figure 4, rabbit conjunctival fibroblasts seeded onto the plastic bottom of

petri dishes (A) or on top of GC biomembranes (B) in the presence of DMEM containing 10% FBS showed that cells could grow to confluence by Day 7. These images also showed that cells grew readily on the GC biomembrane, attached and spread in spindle-shaped morphology, which is similar to the response of explants on the plastic surface of cell culture petri dishes.

In addition to the cellular compatibility, the effect of the GC membrane on cell proliferation is critical. Our results showed that the proliferation of rabbit conjunctival fibroblasts on different ratios of G:C formulated in the biomembranes varied (Fig. 5). After 1 week in culture, conjunctival fibroblast proliferation increased significantly, as shown by an increase in the blue dye from 0.068 ± 0.017 to 0.177 ± 0.011 ($P = 0.014$, n = 3; mean ± SEM) when the gelatin component increased from 20% (1:4) to 50% (1:1).

2. Expansion of Rabbit Conjunctival Epithelial Cells on GC Biomembrane

Growth of rabbit conjunctival epithelial cells and immunostaining with monoclonal antibodies for K4 and PCK was used to investigate the biological effects of the GC biomembranes on the proliferation and differentiation of epithelial cells. Figure 6 shows the expansion of rabbit conjunctival epithelial cells on the plastic surface of the cell culture dish (A) and after placement on a GC biomembrane (B). We found that rabbit conjunctival epithelial cells were able to migrate out of the explants beginning on Day 3 to form cell colonies on both substrates. The epithelial cells took 10 days to grow to confluence. The cell behaviour and morphology were similar to that observed from explants grown on culture dishes without the GC biomembrane.

After changing the medium to a differentiating medium and maintainance for 3 days, we found that the K4 primary antibody, a biomarker for conjunctival epithelial cells, positively stained the conjunctival epithelial cells cultured on the GC biomembrane. This result illustrated that the epithelial cells could differentiate into mature forms on GC biomembrane in vitro. Additional studies on conjunctival epithelial cells were carried out using confocal laser microscopy (Fig. 7). The rabbit conjunctival epithelial cells grown on the GC biomembrane were labelled by PCK (A) and K4 (B), respectively. We found that epithelial cells were labelled by both PCK and K4 antibodies. The positive staining indicated that the cells were epithelial in origin and maintained the epithelial phenotype on the GC biomembrane, after culture for 14 days. This suggests that the GC biomembrane supported the normal biological activity and function of rabbit conjunctival epithelial cells in vitro.

Conclusion

In conclusion, by changing the proportion of gelatin, the mechanical properties of GC biomembrane varied according to the ratio of the GC components. A 1:1 biomembrane (50%) possessed the best elasticity and a 1:9 biomembrane (10%) was the strongest. Also, the ratio of gelatin and chitosan could significantly affect the biological properties of the GC biomembrane. Both epithelial cells and fibroblasts grew to confluence, illustrating good cytocompatibility of the GC biomembrane and good cell viability. The study also suggests that the GC biomembrane may be a useful potential candidate, as a cellular carrier and/or scaffold, for reconstructive tissue engineering of the conjunctiva.

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