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A novel collagen film with micro-rough surface structure for corneal epithelial repair fabricated by freeze drying technique

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ABSTRACT

Corneal epithelial defect is a common disease and keratoplasty is a common treatment method. A collagen film with micro-rough surface was fabricated through a simple freeze drying technique in this study. Compared with the air-dried collagen film (AD-Col), this freeze-dried collagen film (FD-Col) has a more suitable water uptake capability (about 85.5%) and toughness performance. Both of the two films have good optical properties and the luminousness of them is higher than 80%. Besides, the adhesion and proliferation rate of human corneal epithelial cells on the micro-rough surface of FD-Col film is higher than that on the smooth surface of AD-Col film. The results indicate that this FD-Col film may have potential applications for corneal epithelial repair.

1. Introduction

Corneal epithelial defects or lesions are one of the most common cases of ophthalmic diseases and keratoplasty is a usual treatment method [1,2]. Unfortunately, the availability of donor tissue fails to meet the demand in many countries and regions [3]. To address this situation, various attempts have been made to reconstruct human corneas using a tissue engineering approach [4–9]. Collagen, as the main component of corneal tissue and the main load bearing component in connective tissues, has been extensively studied as cornea tissue engineering scaffold materials [10–12].

A suitable surface roughness of the film materials is conducive to cell adhesion, growth and proliferation [13]. Currently, many collagen membrane materials used for tissue repair have flat surfaces [14,15]. However, the adhesion and proliferation rate of corneal epithelial cells on the smooth surfaces of those collagen membranes is usually slower than that on the rough surface of acellular corneal stroma [16,17]. The aim of the this study is to analyze the viability of using freeze drying technique to fabricate a collagen film with micro-rough surface structure to provide implants for use during severe donor cornea shortages.

2. Material and methods

2.1. Materials

TypeIcollagen (HM Biotech Ltd., Guangzhou, PR China) was extracted from bovine tendon. 1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) were supplied by GL Biochem Ltd. (Shanghai, PR China). All cell-culture related reagents were purchased from Sigma Chemical (St. Louis, MO, USA).

2.2. Preparation of films

The preparation of collagen film was reported previously [18]. In brief, collagen was dissolved in 0.01 mol L^{-1} HCl (7.5 mg mL^{-1}). EDC and NHS were added to the collagen solution and thoroughly mixed at 4 °C to form a solution with mass ratio of EDC:NHS:Col = 0.5:0.5:6, crosslinking was carried out by stirring the solution for 4 h. The crosslinked collagen solution was dispensed into a specific mould, which has the same curvature of contact lens. Then, the collagen solution was air dried to form a cornea shape film (AD-Col). After that, the films were rinsed three times with deionized water and followed by an immersion in ultrapure water to absorb water for 2 h. The wet samples were frozen at −20°C and then placed

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into a freeze dryer (VIRTIS Genesis, USA) for lyophilization (FD-Col). The prepared AD-Col and FD-Col films were sterilized with γ-irradiation before use.

2.3. Scanning electron microscopy

Surface morphology of the AD-Col and FD-Col films were examined by scanning electron microscopy (SEM). Prior to imaging, the collagen films were fixed by conductive tapes after which the specimens were coated with gold at 2–5 nm thickness in a sputter coater (Hitachi, Tokyo, Japan) before examination. Observations were performed with a ZEISS EVO 18, Oberkochen scanning electron microscope (Germany) at 10 kV.

2.4. Analysis of swelling properties

Water absorption of the AD-Col and FD-Col films was measured by swelling them in PBS (pH = 7.4) at 35 °C. Wet weight of the samples was, after gently blotting the film surface with filler paper to remove the absorbed water, weighed immediately.

$$\text{water absorption} = \frac{(W_t - W_0)}{W_t} \times 100\%; \quad (1)$$

where, W_t represents the wet weight of the films and W_0 is the initial dry weight of the samples. The values are expressed as the mean ± standard error (n = 10).

2.5. Transmittance

After equilibrium swelling was reached, the samples were removed from the PBS and blotted quickly with filter paper to remove the excess water on the surface. Transmittance of the wet AD-Col and FD-Col films in the visual wavelength range (300–800 nm) was measured using a UV3802 ultraviolet–visible spectrophotometer (Shanghai UNICO, China).

2.6. Assessment of mechanical properties

The Mechanical tests were performed according to previously published methods [19]. The mechanical characteristics of the AD-Col and FD-Col films were measured using a uniaxial load testing equipment (Model no. 5567, Instron Corporation, Issaquah, WA, USA) at a rate of 1 mm min^{-1}. After equilibrium swelling was reached, the samples (1.0 cm of width × 2.0 mm of length × 0.15 ± 0.01 mm of thickness) were clamped for axial tensile testing (n = 6). The stress was monitored as a function of strain, and the elastic modulus was obtained by analyzing the linear region of the resulting stress–strain curve.

2.7. Human corneal epithelial cells experiment

2.7.1. In vitro corneal epithelial cell culture

Human corneal epithelial cells (HCECs) were obtained from State Key Lab. of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, China. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL) with high glucose, supplemented with 15% fetal bovine serum (Sijiqing, China), 5 μg mL − 1 insulin, 100 U mL^{-1} penicillin, 5 μg mL^{-1} human transferrin (Sigma), 2 mM L-glutamine, 100 μg mL^{-1} streptomycin (HyClone) and 10 ng mL^{-1} human epidermal growth factor (EGF; Gibco BRL). HCECs were incubated in a humidified atmosphere containing 5% carbon dioxide at 37°C. The cell supernatant was replaced every other day to maintain an adequate supply of cell nutrients.

2.7.2. The response of HCECs to the film

Before cell experiments, the films were washed three times in phosphate buffered saline under aseptic conditions, and then sterilized by ultraviolet radiation for 2 h, and washed three times in PBS again at last. After sterilization, the film with the thickness of about 0.205 mm was transferred to a 6-well tissue culture plate (Corning, UK). The seeded cell's density was 500 cells cm^{-2}. Then, the cell seeded film was incubated in a humidified atmosphere (5% CO_2, 37 °C). The culture medium was replaced every 2 days. The response of HCECs to the samples and the morphology of HCECs were examined. Before observed by an inverted fluorescence microscope (Olympus IX-70, Japan), the film's surface was washed with PBS.

2.7.3. The proliferation of HCECs to the film

After the samples were transferred to a 96-well tissue culture plates (Corning, UK). HCECs suspension was seeded onto the FD-Col films (experimental group) and AD-Col films (control group), respectively. The seeded cell's density was 5000 cells cm^{-2}. After 1–5 days, the proliferation activity of the HCECs on the films was determined by methylthiazol tetrazolium (MTT) assay (n = 10).

2.8. Statistical analysis

All data were shown as mean ± standard deviation. Experiments were analyzed using analysis of variance (ANOVA) to determine the significant differences among the groups. Statistical significance was defined as $P < 0.05$.

3. Results and discussion

3.1. Observation of surface morphology

Scanning electron micrographs (SEMs) of surface morphology of the AD-Col and FD-Col films are displayed in Fig. 1. Compared with the smooth surface of the AD-Col film (Fig. 1A), the FD-Col film has a uniform micro-rough surface (Fig. 1B). Freeze-drying procedure makes the FD-Col's surface produced some subtle convex areas. These unique surface structures of the FD-Col membrane may provide more binding sites for adhesive protein, which is conducive to cell adhesion.

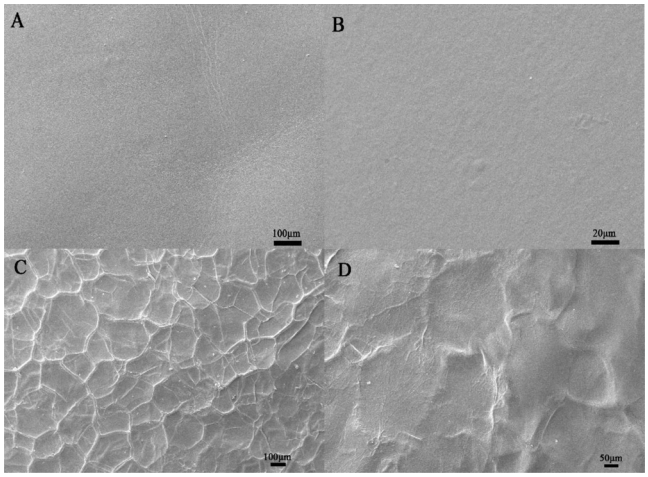

Fig. 1. Surface morphology observed by scanning electron microscopy. SEM images of AD-Col (A) and FD-Col (B).