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Characterization of the modified chitosan membrane cross-linked with genipin for the cultured corneal epithelial cells

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ABSTRACT

Objectives: To modify a chitosan membrane (CM) by cross-linking the chitosan with genipin, a naturally occurring cross-linker extracted from *Gardenia jasminoides* fructus, with the aim of developing a new cell culture support and to observe the phenotypes of cultured human corneal epithelial cells (HCECs) on genipin-cross-linked chitosan membrane (GCM).
Methods: We tested the cross-linking characteristics and mechanical strength of the GCM. CMs modified by cross-linking with different concentrations of genipin were prepared to investigate the rate of membrane degradation. The biocompatibility of the GCMs was investigated by determining the viability of HCECs cultured on them in vitro. The morphology of the HCECs cultured on CM or GCM was analyzed by confocal microscopy and scanning electron microscopy (SEM). Immunocytochemical staining was conducted to determine the phenotypes of the cultured cells.
Results: The fixation index of the GCM was 31 ± 3% after treatment of CM with 0.5 mM genipin. A stress–strain test showed that the GCM could tolerate three times the mechanical force of noncross-linked CM. The biodegradation rate of GCM was much slower than for CM. A 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay showed that cell viability was not affected by cross-linking with 5.0 mM genipin. SEM showed that the cultured HCECs adhered to and grew well on the surface of the GCM. Immunocytochemical staining showed keratin 3 (K3) and connexin 43 (Cx-43) immunoreactive HCECs on the GCM and their proliferative ability was not significantly affected by strong immunoreactivity of Ki-67 and p63 markers.
Conclusions: GCM has potential as a scaffold for corneal epithelium in ocular surface surgery and greater mechanical strength and slower degradation than unmodified CM.

1. Introduction

Corneal epithelium renewal is an essential process for protecting the inner part of the cornea from pathogens and various noxious environmental agents. Despite its excellent regenerating ability, several pathological conditions may delay or destroy the normal corneal epithelial healing process, such as chemical burns, corneal ulcers, dry eyes, and Stevens–Johnson syndrome, and result in a persistent epithelial defect. Therefore, maintenance of corneal homeostasis is one of the most important issues for tissue engineering on the ocular surface.

Amniotic membrane (AM) has been widely used as temporary dressing or permanent graft to promote corneal wound healing and can play a vital role in ocular surface reconstruction surgery [1–3]. However, AM should be harvested from eligible seronegative (hepatitis B virus, hepatitis C virus, syphilis, human immunodeficiency virus) maternal donors after cesarean delivery with full consent and any procedures involved should be approved by an Institutional Review Board. Nonetheless, the potential risk of infection by an unknown or undetectable infectious etiology after receiving AM transplantation (AMT) cannot be completely excluded [4]. Furthermore, the AM may degrade within 10–14 days after use in acute Stevens–Johnson syndrome [5]. Therefore, more than one

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AMT surgery may be required. Corneal melting after AMT because of uncontrollable inflammation is another troublesome situation that clinicians may face.

To overcome these problems, several new biomaterials have been developed, including fish-scale collagen [6], chitosan membrane (CM) [7], and keratin-modified chitosan membranes [8]. Chitosan, a natural polymer derived from chitin, which is usually found in the shells of invertebrates and insect cuticles, has been widely applied in biomaterials. Our previous work indicated that CM could share the advantages of AM for preserving the phenotype of corneal epithelial progenitor cells [7]. Because of its easy accessibility, biocompatibility [9], antibacterial properties [10], and biodegradability, CM is highly competitive with AM. Chitosan is currently used in various biomedical applications, including as hemostatic agents [11], drug delivery [12,13], absorbable suture materials, and tissue engineering [14].

Cross-linking reagents are commonly applied in tissue engineering scaffolds to improve the mechanical strength and degradation properties of biopolymers. Genipin, which can be extracted from the fructus of *Gardenia jasminoides*, is a natural cross-linker for various biomaterials such as gelatin, collagen, and chitosan. Genipin has been widely investigated for biological applications because of its natural origin and low cytotoxicity. Genipin-cross-linked chitosan (GCM) increases ultimate tensile strength [15] with slower biodegradability and improved biostability [16] compared with unmodified chitosan.

As new technology continues to develop, promising biomaterials may provide an alternative for individually tailored surgery in various circumstances. Our previous study demonstrated that CM is a promising biomaterial that can be substituted in every potential AM application [7]. In the present study, we modified CM using a naturally occurring cross-linker, genipin, to generate a new scaffold with enhanced strength and slower biodegradability compared with unmodified CM when culturing human corneal epithelial cells (HCECs). Moreover, the degree of cross-linking, swelling ratio, biodegradation properties, and biocompatibility of the GCM, and viability, immunohistochemistry, and morphology of the HCECs cultured on GCM were characterized. We demonstrated that GCM may be used as an alternative tissue engineering scaffold for ocular surface reconstruction surgery.

2. Materials and methods

2.1. Materials

Chitosan was obtained from Sigma–Aldrich (St. Louis, MO, USA), acetic acid and glacial acetic acid from Tedia (Fairfield, OH, USA), sodium tripolyphosphate (TPP) from Wako (Osaka, Japan), 24- and 96-well plates from Costar (Lowell, MA, USA), HAMF12/Dulbecco's modified Eagle's medium (DMEM/F12) from Invitrogen (Carlsbad, CA, USA), lysozyme from Calbiochem (Merck, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and other chemicals from Sigma–Aldrich.

2.2. Preparation of the chitosan membrane

Deacetylated chitosan (85%) was dissolved in 1% acetic acid solution to prepare chitosan solutions (2%, w/v), which were stirred at 60 °C overnight. The solutions were passed through a sieve mesh (0.5 μm) and then spread onto a 100-μm-thick glass plate. The nascent membrane on glass plate was then immersed in 0.5 N NaOH at room temperature until the membrane detached from the glass plate. Then, the membrane was rinsed with distilled water, soaked in distilled water for 24 h, and lyophilized.

2.3. Cross-linking processes and characteristics

Cross-linking was conducted following previously reported methods with some modification [16]. Briefly, chitosan solution (2%, w/v) was prepared by dissolving 1 g of chitosan powder in 1% (v/v) aqueous acetic acid (50 mL) with stirring overnight until complete dissolution. Subsequently, genipin powder was added into a 20 mL solution of 1% chitosan with stirring for 4 h at 37 °C to produce the following concentrations of genipin: 0.5 mM, 1 mM, 2 mM, 2.5 mM, and 5 mM. Each solution was prepared using the same method as used for chitosan membrane, as described above. Subsequently, the membrane was washed with distilled water, soaked in distilled water for 24 h, and lyophilized. Each CM and GCM was cut into 8 mm diameter circles before experiments. The cross-linking characteristics of the membranes were determined by measuring their fixation index in a ninhydrin (NHN) assay. The fixation index was defined by the ratio: NHN reactive amine (CM–GCM)/NHN reactive amine (CM). The samples were lyophilized for 24 h and then weighed. Each sample group weighing 4 mg was soaked in 1 mL ddH_2O for 1 h. Then the CM and 0.5 mM GCM were heated with a NHN solution for 10 min at 100 °C. After heating with NHN, the optical absorbance of the solution was recorded at 570 nm using a multifunction microplate reader (SpectraMax M5 Molecular Devices) [17].

2.4. Biodegradation properties

Lysozyme (50 mg) was added into 500 mL PBS buffer with stirring until complete dissolution. CM and GCM samples were lyophilized for 24 h and weighed. Each sample was placed into a tube, 1.5 mL of lysozyme solution was added, and the tubes were incubated at 37 °C. Membrane samples were collected on days 4, 7, 10, 14, 17, and 21. The samples were washed three times in PBS buffer, and then lyophilized and weighed. Each experiment was conducted in triplicate.

2.5. Swelling characteristics

The swelling characteristics of the GCM and noncross-linked CM were determined by immersing dried membranes (0.002 g) to swell in PBS. At specified times (5, 10, 15, 20, 25, and 30 min), samples were obtained and blotted with filter paper to absorb excess water from their surface. The swelling ratio (Q) of the CM and GCM samples was calculated using the following formula: $Q = (Ww - Wd)/Ww \times 100\%$, where Ww is the weight of the swollen samples and Wd is the weight of the dried samples [18]. Each experiment was conducted in triplicate.

2.6. Stress–strain test

The mechanical features of the membranes were determined using a stress–strain test (Benchtop Materials Testing Machines, Tinius Olsen). In brief, a uniform area of 10 mm × 0.5 mm of membrane (CM and GCM) was mounted lengthwise between two screw-tightened brass grips and subjected to load between 0 and 9 N at 0.1 mm/s. During the testing procedure, the force stayed parallel to the stretching direction to ensure sample deformation was minimized. The test membrane fracture was recorded when the first decrease in load was detected during extension.

2.7. Culture of human corneal epithelial cells

HCECs obtained from the ATCC were cultured in keratinocyte-SFM (Gibco, Tokyo, Japan) containing 5 ng/mL EGF, bovine pituitary extract, 5 μg/mL insulin, 0.5 μg/mL hydrocortisone (Sigma),