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Hyaluronic acid concentration-mediated changes in structure and function of porous carriers for corneal endothelial cell sheet delivery

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ABSTRACT

In this study, the effects of hyaluronic acid (HA) concentrations (0.05–1.25 wt.%) on the properties of porous carriers for corneal endothelial tissue engineering were investigated. The pore size and porosity gradually increased with decreasing solid content. However, at relatively low HA concentration (i.e., 0.05 wt.%), the material samples contained small interior pores and a dense surface skin layer, probably due to no gas bubble effect on the stirring processing of porous microstructures of freeze-dried polysaccharide hydrogels. The carriers prepared from 0.25 wt.% HA solution had the highest freezable water content and oxygen and glucose permeability among the samples evaluated. Results of cell viability assays and quantitative real-time reverse transcription polymerase chain reaction analyses showed that the HA concentration-related alteration of porous microstructure dictates the compatibility of biopolymer carriers with corneal endothelial cell (CEC) cultures. In vivo studies demonstrated that the CEC sheet/HA carrier construct implants are therapeutically efficacious in the reconstruction of endothelial scrape-wounded corneas. It is concluded that the polysaccharide concentration is the major factor for affecting the processing of carriers and their structure and function. Porous hydrogels prepared from 0.25 wt.% HA solution are capable of delivering bioengineered CEC sheets to the posterior surface of cornea.

1. Introduction

Corneal endothelium is located on the posterior surface of cornea and helps control tissue hydration and water transport. Penetrating keratoplasty is the most commonly used surgical procedure for corneal diseases due to endothelial dysfunction such as pseudophakic bullous keratopathy and Fuch's endothelial dystrophy [1]. To produce the corneal endothelial equivalents, the author and other researchers have bioengineered corneal endothelial cell sheets by using thermo-responsive polymer coatings [2–4]. Given that the thermally detached cell sheets are fragile, intraocular cell grafting is difficult to achieve without involving any supporting carrier materials. In the author's laboratory, the gelatin carriers have been described for improving the retention of cell sheet transplants at the site of injury and enhancing tissue regeneration in animals with endothelial dysfunction [5]. To further alleviate the physiological adverse events associated with the intracameral implantation of dense structured gelatin carriers, the freeze-dried porous hydrogel materials are being developed as next-generation cell delivery systems [6]. However, in terms of biomaterials for clinical ophthalmic applications, hyaluronic acid (HA) is a well-known viscoelastic agent extensively used in cataract surgery [7] and deep lamellar keratoplasty [8]. Therefore, this biomaterial may be suitable for intraocular cell delivery carrier components.

HA is a generally represented as a linear anionic polysaccharide comprised of repeating disaccharide units of D-glucuronic acid and *N*-acetyl-D-glucosamine. It has high capacity for lubrication and water retention. As the only non-sulfated glycosaminoglycan of the body, HA is widely distributed in connective tissue, synovial fluid, and aqueous and vitreous humor of the eye. For ocular tissue engineering applications, HA has been studied for its role on regulating cell behaviors. Gomes et al. have shown that HA is a naturally occurring extracellular matrix molecule able to promote human corneal epithelial cell migration and its interaction with cell surface CD44 receptor is likely to contribute to the beneficial effects on corneal wound healing [9]. Due to its ability to improve cellular adhesion, proliferation, and migration, HA can also be efficiently utilized for functionalization of *N*-isopropylacrylamide and acrylic acid *N*-hydroxysuccinimide copolymeric carriers to facilitate noninvasive delivery of retinal pigment epithelial cell suspensions into the subretinal space for treatment of retinal degenerative diseases such as age-related macular degeneration and diabetic retinopathy [10]. By taking advantage of the favorable cell adhesion on biomaterial carriers, carbodiimide cross-linked HA hydrogels have previously been used as bioengineered corneal endothelial cell (CEC) sheet carriers [11].

Here, to further meet the demand of intraocular cell delivery carriers, the porous HA hydrogels were fabricated by a simple stirring process

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combined with freeze-drying method as described in the previous paper [12]. The gas bubble produced by a simple stirring operation has been found very beneficial to enlarge the pore dimension of freeze-dried carriers [6]. In the engineering of freeze-dried porous materials, the polymer concentration is known to be one of the key parameters to determine the microstructures [13–15]. This motivates the author to investigate the effects of HA concentrations (0.05–1.25 wt.%) on the properties of porous carriers for corneal endothelial tissue engineering. The aqueous HA solutions of different concentrations were subjected to stirring at 25 °C and subsequent freezing at −20 °C. Following lyophilization, the hydrogel materials were modified by means of carbodiimide chemistry. Microstructural characterization of HA samples was performed by both scanning electron microscopic analyses and porosity measurements. In vitro degradation tests were also conducted to determine the cross-linking-mediated stability of polysaccharide molecules. The freezable water content of HA hydrogels and glucose permeation through the carrier materials were evaluated to document any biopolymer concentration-dependent abnormalities in mass transfer patterns. In addition to cell viability and metabolic activity assays, the gene expressions of membrane transport proteins in CECs were verified to screen the biocompatibility of HA carriers. The CEC sheet delivery performance of porous HA carriers was investigated by using a rabbit model of corneal endothelial dysfunction. Clinical observations were made in all animals to better understand the therapeutic potential of cell/biopolymer construct implants for corneal endothelial reconstruction.

2. Materials and methods

2.1. Materials

Hyaluronic acid sodium salt was obtained from Kewpie (Tokyo, Japan) as a dry powder. It was made by fermentation method and was highly purified. According to information from the supplier, the HA samples used in this study had a weight-average molecular weight of around 1100 kDa. 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC), hyaluronidase type V from sheep testes (1770 units/mg), glucose, and glucose assay kit (glucose oxidase/peroxidase reagent and o-dianisidine reagent) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Balanced salt solution (BSS, pH 7.4) was obtained from Alcon Laboratories (Fort Worth, TX, USA). Phosphate-buffered saline (PBS, pH 7.4) was purchased from Biochrom AG (Berlin, Germany). Medium 199, gentamicin, Hanks' balanced salt solution (HBSS, pH 7.4), trypsin-ethylenediaminetetraacetic acid (EDTA), and TRIzol reagent were purchased from Gibco-BRL (Grand Island, NY, USA). Collagenase type II was purchased from Worthington Biochemical (Lakewood, NJ, USA). Fetal bovine serum (FBS) and the antibiotic/antimycotic (A/A) solution (10,000 U/ml of penicillin, 10 mg/ml of streptomycin, and 25 μg/ml of amphotericin B) were obtained from Biological Industries (Kibbutz Beit Haemek, Israel). All the other chemicals were of reagent grade and used as received without further purification.

2.2. Preparation and characterization of porous HA carriers

The aqueous HA solutions of 0.05–1.25 wt.% were prepared by dissolution of HA powder in double-distilled water. Before lyophilization at −55 °C for 2 days, the aqueous solution was cooled to 25 °C, stirred with a rate of 350 rpm for 20 min, and frozen at −20 °C for 24 h to form ice crystals.

All fabricated HA hydrogel sheets were further cross-linked by immersing in an acetone/water mixture (85:15, *v*/*v*, pH 4.75) of 10 mM EDC [16]. The cross-linking reaction was allowed to proceed at 25 °C for 2 days. The samples were thoroughly washed with double-distilled water to remove excess EDC and urea by-product. Using a 7-mm-diameter corneal trephine device, the hydrogel sheets were cut out to create carrier disks (~700 μm in thickness). In this study, the carrier materials prepared from 0.05, 0.25, and 1.25 wt.% HA solutions were designated as HA005, HA025, and HA125, respectively.

Specimens were prepared for scanning electron microscopy (SEM) as described previously [17]. Small pieces of the hydrogel disks were mounted onto stubs and gold coated in a sputter coater (Hitachi, Tokyo, Japan). The cross-section and surface morphologies of the HA carriers were examined using a Hitachi S-3000 N SEM with an accelerating voltage of 10 kV. Fifty different pores were randomly selected, and the average pore diameters were calculated. Results were averaged on four independent runs.

The solvent replacement method was used for porosity measurements [18]. Each HA disk was first dried to constant weight (W_i) in vacuo. The test samples were immersed in absolute ethanol overnight, blotted with tissue paper to remove excess ethanol on the surface, and weighed (W_f) immediately. The porosity (%) was calculated as $((W_f - W_i) / V\rho) \times 100$, where V is the volume of the hydrogel disk and ρ is the density of absolute ethanol. Results were averaged on four independent runs.

2.3. In vitro degradation tests

To measure the extent of degradation, each test HA disk was first dried to constant weight (W_i) in vacuo and was immersed in BSS containing 400 units/ml hyaluronidase at 34 °C (physiological temperature of the cornea) with reciprocal shaking (50 rpm) in a thermostatically controlled water bath. Degradation medium was replaced weekly with fresh buffer solution containing the same concentration of enzyme [19]. At specific time intervals, the samples were taken out and washed with double-distilled water. The degraded samples were further dried in vacuo and weighed to determine the dry weight (W_d). The in vitro degradability (%) was calculated as $((W_i - W_d) / W_i) \times 100$. Results were the average of five independent measurements.

2.4. Determination of freezable water content

Differential scanning calorimetry (DSC) measurements were used to examine the states of water in the HA carriers. The disk samples were placed in a DSC cell (TA Instruments, New Castle, DE, USA), cooled to −20 °C to freeze the swollen hydrogels, and heated to 20 °C at a heating rate of 5 °C/min under a nitrogen gas flow. The amount of freezable water was evaluated from the DSC endothermic ice-melting profile of the frozen hydrogel [15]. The enthalpy of melting (ΔH_m) obtained by integration and normalization is in unit of J/g of swollen hydrogel. Temperatures and enthalpies of melting of the samples were calibrated using pure water as the standard. The latent heat of water is 333.5 J/g of pure water. The gram of freezable water per gram of swollen HA hydrogel (W_{fH}/W_s) was calculated as $\Delta H_m/333.5$. Results were the average of five independent measurements.

2.5. Glucose permeation measurements

Glucose permeation studies were performed at 34 °C using a horizontal glass diffusion cell (PermeGear, Hellertown, PA, USA) having two stirred chambers with sampling ports [20]. The donor chamber was filled with a 6.9 μmol/ml (the glucose concentration of aqueous humor in rabbit) glucose solution in BSS (3 ml) and receptor chamber with BSS (3 ml). After immersion in BSS until fully swollen, the HA samples were placed between the two chambers. During the measurements, all solutions were stirred continuously to provide uniform solute distribution and to reduce boundary layering of glucose. After 8 h, the receptor chamber was sampled and analyzed using a glucose assay kit following the manufacturer's instructions. Photometric readings at 540 nm were measured with a spectrophotometer (Thermo Scientific, Waltham, MA, USA) and compared with a standard curve of known glucose concentrations. Results were averaged on six independent runs.