Biomaterials 35 (2014) 4005–4014

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Heparin-modified gelatin scaffolds for human corneal endothelial cell transplantation

Guoguang Niu [a], Jin-San Choi [a], Zhan Wang [a], Aleksander Skardal [a], Matthew Giegengack [b], Shay Soker [a,*]

[a] Wake Forest Institute for Regenerative Medicine, Wake Forest School of Medicine, Wake Forest Baptist Medical Center, Medical Center Boulevard, Winston-Salem, NC 27157, USA
[b] Department of Ophthalmology, Wake Forest School of Medicine, Wake Forest Baptist Medical Center, Medical Center Boulevard, Winston-Salem, NC 27157, USA

ARTICLE INFO

Article history:
Received 29 October 2013
Accepted 13 January 2014
Available online 6 February 2014

Keywords:
Cross linking
Gelatin
Basic fibroblast growth factor
Heparin

ABSTRACT

Although one of the most transplanted tissues, a shortage of cadaveric corneas for transplantation still exists in the western society and elsewhere. The goal of this study was to develop a biological scaffold to support transfer of cultured human corneal endothelial cells (HCECs) into the anterior chamber of the eye, potentially a replacement for cadaveric donor tissue. A series of transparent scaffolds were fabricated from gelatin and modified with heparin. Mechanical parameters of the scaffolds, such as stiffness, affected cell proliferation, phenotype and cell surface marker expression were determined. The heparin-modified scaffolds had a greater capacity to absorb basic fibroblast growth factor (bFGF) and showed better release kinetics for up to 20 days. The release of bFGF from the scaffolds improved HCECs survival and reduced cellular loss. The scaffolds were flexible and could be folded and implanted in rabbits' eyes, through a small incision in the cornea. The scaffolds adhered to the inner surface of the corneal stroma and gradually integrated with the surrounding tissue. These results indicate that gelatin based corneal scaffolds modified to absorb and release growth factors and seeded with HCECs, might be a suitable alternative for cadaveric cornea transplantation.

1. Introduction

Human corneal endothelial cells (HCECs), which line the inner surface of cornea, are critical for maintaining transparency of the cornea by regulating stromal hydration using ATPase pumps [1,2]. HCECs typically have poor capacity for regeneration when damaged or diseased, as can occur as a consequence of cataract surgery [3], Fuchs' corneal dystrophy [4], diabetes [5], or elevation of intraocular pressure [6]. Instead of regenerating, HCECs increase their size to compensate the wounded area [7]. When cell density falls below the critical level needed to maintain normal corneal hydration, corneal edema occurs and vision gradually clouds [1,8]. In this case, corneal transplantation is required to replace the damaged cornea.

Penetrating keratoplasty (PK) is still the most common keratoplasty procedure worldwide [8] used to treat irreversible opacification of the cornea. However when dealing with diseased HCECs, Descemet's stripping and endothelial keratoplasty (DSEK), has replaced PK as the preferred treatment. In this procedure, the diseased HCECs together with their underlying basement membrane (Descemet's membrane) are physically stripped from the stroma. Cadaveric donor tissue, including a thin layer of posterior stroma, Descemet's membrane and healthy HCECs, is implanted in the recipient eye [8,9]. Compared with PK, DSEK surgery generally results in more rapid rehabilitation, better refractive outcome and fewer post-surgery complications [10]. While DSEK has proven superior to PK for cases of HCEC deficiency/dysfunction, there is room for improvement. Corneal transplants don't last forever, and when they fail it is usually due to loss of the donor HCEC's. It is reported that more than 30% HCECs are lost within the first 6 months of transplantation [8,11]. Rejection is a potential cause of endothelial cell loss. Most pertinent to this study, worldwide there is a deficiency qualified donor tissue. Domestically, while supply of tissue is adequate, there is a range in the quality of corneas available. In general, donor tissue from younger donors as well as donor tissue with higher density of HCEC's are preferable.

As an alternative, cell therapy approaches have been explored, in which the HCECs are isolated from cornea, expanded *in vitro*, and then transplanted in the anterior chamber of the eye to replace the

* Corresponding author. Tel.: +1 336 713 7295; fax: +1 336 713 7290.
E-mail address: ssoker@wakehealth.edu (S. Soker).

0142-9612/$ – see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2014.01.033

diseased cells. A number of studies have reported the success of HCEC isolation from the limbal Descemet's membrane, and the maintenance of appropriate cell morphology and function *in vitro* during expansion [1,12,13]. However, the HCEC layer is very thin (about 5 μm in thickness) and fragile, making it difficult to transfer the cell layer directly into eye. Two methods have been investigated: The first is to harvest an HCEC cell sheet using a thermal reversible culture surface, and then transfer the cell sheet into the eye [14,15]. The second method is to seed HCECs directly on a thin scaffold, and then transfer this cell-scaffold construct into the eye [12]. While both techniques have shown promise, the second method is attractive due easier handling provided by the more robust scaffold. Furthermore, this method successfully produces a flat HCEC monolayer after transplantation.

The ideal scaffold for HCEC transplantation should be transparent, permeable to water and nutrients, non-cytotoxic, biodegradable and have appropriate mechanical properties. Specifically, the scaffold should be sufficiently durable for easy handling, yet pliable enough to be folded to accommodate the transplantation procedure. In addition, the scaffold must support HCEC growth, function, survival, and be easily integrated into the surround tissue. Currently, several biologically-derived scaffolds have be considered for HCEC transplantation, such as decellularized corneal stroma [12], amniotic membrane [16], collagen matrices [17,18], silk films [19], chitosan–polycaprolactone blends [20], hyaluronic acid [21], and gelatin sheets [22]. Biological scaffolds are generally non-cytotoxic and can support cell survival, however their optical or mechanical properties often do not meet the requirements for clinical use. Additionally, the sources of these materials are often inconsistent, limiting their use. However, gelatin has shown promise, having been extensively used in the pharmaceutical and medical field due to its compatibility with cells and suitable biodegradability *in vivo*. Many HCEC transplantation studies primarily focus on ability of the scaffold to successfully carry the cells to the transplantation site, while ignoring the interaction of HCECs with the scaffolds. In some cases, the properties of the scaffold, such as stiffness and surface chemistry, affect cell behavior [23,24]. For instance, epithelial and 3T3 fibroblastic cells displayed normal morphology, while on soft scaffold cells were irregularly shaped [25]. At this point in time the literature has yet to address the effect of mechanical properties of scaffolds on HCEC behavior.

In this study, we describe the fabrication of flexible thin gelatin gel (TGG) scaffolds for transplantation of HCECs. The mechanical properties were optimized to allow folding of the scaffolds, in order to be implanted through a small incision in the eye, while maintaining an intact cell layer on the scaffold. The scaffolds were further functionalized with the addition of heparin to improve binding of growth factors that will support HCEC growth. To demonstrate clinical applicability, the scaffolds were successfully implanted in the anterior chamber of a rabbit eye.

2. Materials and methods

2.1. Preparation of scaffold

Thin gelatin gel (TGG) scaffold was prepared via a two step process: first, a gelatin film was obtained by solution casting gelatin type A (Thermo Fisher Scientific, Rockford, IL) as previously described [26]; next, the gelatin film was cross-linked with Ethyl(dimethylaminopropyl) carbodiimide (EDC, Thermo Fisher Scientific) and N-hydroxysuccinimide (NHS, Thermo Fisher Scientific) with molar ratio 1.0 in pH = 5.5 phosphate buffered solution. Films were created with different molar ratios of EDC to gelatin's amine group of 2.5, 5 and 10. The cross-linking reaction was carried out for 24 h at room temperature, after which the crosslinked scaffold was washed thoroughly with DI-water to remove excess crosslinking agents. The crosslinking degree of scaffolds was evaluated by comparing the change in amine content of gelatin before and after crosslinking as described [27].

For heparin-modified gelatin scaffolds, heparin sodium salt (Sigma–Aldrich, St. Louis, MO) was dissolved in the 10.0 wt% gelatin solution, and the heparin-gelatin film was prepared and cross-linked as described above (EDC/amine molar ratio = 5.0). The percentage of heparin in the gelatin film was 2.0 wt% (TGG-Hep-2), 5.0 wt% (TGG-Hep-5) or 10.0 wt% (TGG-Hep-10). After crosslinking, the heparin content in the scaffolds was determined according to the method previously described [27].

Acellular human cornea stroma was prepared as reported [12]. Discarded human corneas were obtained from Ocular Systems, Inc. (Winston-Salem, NC).

2.2. Characterization of scaffolds

2.2.1. Optical property

The light transmission of scaffolds was evaluated with a UV–Vis spectrophotometer (UV-2500, Shimadzu, Columbia, MD). In brief, scaffolds were immersed in Dulbecco's Phosphate Buffered Saline (DPBS, Thermo Fisher Scientific) for 24 h at 37 °C, and light transmittance was assessed in the range between 400 nm and 750 nm.

2.2.2. Mechanical properties

Dog bone shaped samples, 4 mm wide at the narrowest point with a length of 18 mm, were punched from wet scaffolds for mechanical testing. The mechanical properties were measured using tensile extension by a uniaxial load test machine (Instron5544, Instron Corporation, Issaquah, WA) with an extension rate of 10.0 mm/min. The scaffold's stress–stain curves were recorded, according to which the Young's modulus and the tensile strength and strain at break were obtained. Three to five samples were measured for each scaffold allowing the mean and standard deviation to be calculated.

2.2.3. Microstructure of scaffolds

The scaffolds were immersed in DI-water for 24 h at 37 °C and then lyophilized. The dried scaffolds were sputter-coated with a thin gold layer, and their microstructures were observed using a scanning electron microscope (SEM, Model S-2260N, Hitachi Co. Ltd., Japan). SEM images were acquired and the pore size of scaffolds was analyzed using Image-Pro Plus software (Media Cybernetics, Bethesda, MD). Three SEM images were taken at different locations for each sample and 15 pores were randomly selected for measurements. The mean and standard deviation of the pore diameters were calculated subsequently.

2.2.4. Diffusion permeability

Permeability of the scaffold was evaluated with fluorescently-labeled Dextran with molecular weight of 4 kDa and 70 kDa (Thermo Fisher Scientific). Scaffolds were prepared on Transwell® membrane inserts for 6-well plates with 8.0 μm pore size (BD Bioscience, MD, USA) as described in Section 2.1. Two ml of containing 50 μg/ml FITC-Dextran was added to the luminal chamber of insert, and 2 ml of DPBS blank was added to the basal chamber. FITC-Dextran then diffused from the luminal chamber to the basal chamber through the scaffold. The solution in the basal chamber was removed for measurement and replaced with fresh DPBS at predetermined time points, and the FITC-Dextran content of the removed aliquots was quantified using a plate reader (Spectra Max M5, Molecular Devices, Sunnyvale, CA). The permeability coefficiency (P_{app}) and the diffusion coefficiency of scaffold (D) were calculated according to Eqn. (1) and Eqn. (2), respectively, where ΔQ is the change in quantity of FITC-Dextran over a fixed change in time (Δt), A and T are the surface area and thickness of scaffold, and C is the initial concentration of FITC-Dextran in the luminal chamber.

$$P_{app} = [(\Delta Q)/(\Delta t)]/(A \times C_0) \quad (1)$$

$$D = P_{app} \times T \quad (2)$$

2.2.5. Equilibrium water content

Scaffolds were immersed in DI-water for 1 day at 37 °C, after which the surfaces of the scaffolds were gently blotted dry with tissue paper, and then weighed. Afterwards, the wet scaffolds were dried completely by lyophilization, and then reweighed. The total equilibrium water content (W_e) of scaffolds was calculated according to Eqn. (3), where W_{wet} and W_{dry} are the wet weight and the dry weight of the scaffolds, respectively [28].

$$W_e = \left(W_{wet} - W_{dry}\right)\Big/W_{wet} \times 100\% \quad (3)$$

2.3. In vitro release kinetics of bFGF from scaffolds

To analyze the release kinetics of bFGF from the scaffolds, bFGF was first incorporated into the scaffolds, by immersing circular scaffolds (9.0 mm diameter and 250 μm in thickness) in 0.2 ml of DPBS containing 50, 100 or 250 ng/ml of bFGF for 16 h at 4 °C. Afterwards, the scaffolds were rinsed twice with 0.2 ml DPBS to remove the excess bFGF. Then another 0.2 ml of PBS was added to each sample and incubated at 37 °C. At predetermined time points, all of the DPBS was removed for measurement and replaced with fresh DPBS. The amount of bFGF released from scaffold at each time point was analyzed using an ELISA immunoassay kit for basic