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Ultrathin chitosan–poly(ethylene glycol) hydrogel films for corneal tissue engineering

Berkay Ozcelik [a], Karl D. Brown [b], Anton Blencowe [a], Mark Daniell [b], Geoff W. Stevens [a], Greg Qiao [a,*]

[a] Department of Chemical and Biomolecular Engineering, The University of Melbourne, Victoria 3010, Australia
[b] Centre for Eye Research Australia (CERA), Royal Victorian Eye and Ear Hospital, Peter Howson Wing, Victoria 3002, Australia

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ABSTRACT

Due to the high demand for donor corneas and their low supply, autologous corneal endothelial cell (CEC) culture and transplantation for treatment of corneal endothelial dysfunction would be highly desirable. Many studies have shown the possibility of culturing CECs in vitro, but lack potential robust substrates for transplantation into the cornea. In this study, we investigate the properties of novel ultrathin chitosan–poly(ethylene glycol) (PEG) hydrogel films (CPHFs) for corneal tissue engineering applications. Cross-linking of chitosan films with diepoxy-PEG and cystamine was employed to prepare ~50 μm (hydrated) hydrogel films. Through variation of the PEG content (1.5–5.9 wt.%) it was possible to tailor the CPHFs to have tensile strains and ultimate stresses identical to or greater than those of human corneal tissue while retaining similar tensile moduli. Light transmission measurements in the visible spectrum (400–700 nm) revealed that the films were >95% optically transparent, above that of the human cornea (maximum ~75%), whilst in vitro degradation studies with lysozyme revealed that the CPHFs maintained the biodegradable characteristics of chitosan. Cell culture studies demonstrated the ability of the CPHFs to support the attachment and proliferation of sheep CECs. Ex vivo surgical trials on ovine eyes demonstrated that the CPHFs displayed excellent characteristics for physical manipulation and implantation purposes. The ultrathin CPHFs display desirable mechanical, optical and degradation properties whilst allowing attachment and proliferation of ovine CECs, and as such are attractive candidates for the regeneration and transplantation of CECs, as well as other corneal tissue engineering applications.

1. Introduction

Corneal endothelial cells (CECs) are specialized, polygonal-shaped cells that reside on the inner surface of the cornea within the aqueous chamber [1]. CECs are responsible for actively pumping fluids across the cornea to maintain corneal transparency [1]. Human CECs do not regenerate in vivo and upon loss of their function corneal transplantation is required to restore vision [1,2]. Various factors can lead to the loss of function of the CEC layer, including ageing, trauma and disease [1,3]. Once the number of these cells reduces below a critical value, the cornea loses its optical clarity due to oedema, which subsequently leads to blindness [1]. Various transplantation methods are available, ranging from the replacement of the whole cornea to the replacement of only the diseased cell layer [4–7]. As a result of the highly invasive nature of full-thickness corneal transplants, less invasive methods have been developed. For example, Descemet's stripping endothelial keratoplasty (DSEK) [8,9] involves removing the Descemet's membrane, along with the non-functioning CECs, from the cornea via a small incision into the anterior chamber. Subsequently, a thin layer of donor corneal endothelium supported by a section of the stroma is inserted into the anterior chamber to replace the CECs [8]. Since only a thin layer of tissue is replaced via a small incision, the technique is less invasive, healing rates are more rapid and the chances of infection are greatly reduced [9]. As with all types of corneal transplantation techniques utilizing donor tissue, there are also risks of rejection and graft failure with the DSEK procedure [9–11]. As such, development of an autologous transplant for the treatment of corneal endothelial dysfunction would be ideal.

A wide variety of substrates have been investigated for the regeneration and potential implantation of CECs [12–19]. In particular, collagen-based materials demonstrate desirable properties for the attachment and proliferation of CECs but generally have tensile strengths inferior to that of the human cornea [12–14]. The effects of harvesting, isolation and purification negatively impact upon the mechanical properties of collagen, whereby they are rapidly degraded due to disassembly of the natural structure and cross-links present in vivo [20]. Ultimately, this hinders their application in surgical procedures such as DSEK, which are physically demanding and require robust substrates for implantation. In addition, since most natural polymers are sourced from animals,

* Corresponding author.
E-mail address: gregghq@unimelb.edu.au (G. Qiao).

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there are potential risks in regards to disease transmission [21,22]. Other materials, such as hyaluronic acid, amniotic membrane and silk fibroin, have also been investigated as potential substrates for CEC regeneration and transplantation with promising results [23–25]. These substrates have desirable tensile properties, support CEC attachment and proliferation, and were able to be implanted in vivo. However, there are certain issues associated with the use of such materials as implantable substrates. Hyaluronic acid requires modification for structural integrity and chemical stability [26] and silk fibroin has issues related to immune responses in vivo [27,28]. In addition, amniotic membrane, which is obtained from human donors in most cases, carries risks associated with disease transmission [29]. Furthermore, variation in the transparency of amniotic membranes depending on the source and location has been reported, which is a significant consideration for ophthalmic applications [30].

Rafat et al. recently demonstrated that cross-linking of collagen composites with a poly(ethylene glycol) (PEG)-dialdehyde derivative yielded thick hydrogel films (~500 μm) that displayed desirable properties for corneal tissue engineering [31]. Interestingly, the incorporation of small amounts of chitosan (1 wt.% relative to collagen) into the composites led to significant improvements in the strength and elasticity of the hydrogels. The hydrogels were subsequently used to replace the majority of the cornea in animal subjects via deep lamellar keratoplasty and displayed good biocompatibility over a period of 120 days. Liang et al. also demonstrated that chitosan, gelatin and chondroitin sulfate composite membranes are capable of supporting the regeneration of rabbit CECs, thus highlighting the suitability of chitosan-based materials for corneal engineering applications [20].

Chitosan is a linear polysaccharide consisting of randomly distributed glucosamine and *N*-acetylglucosamine repeat units and is derived from *N*-deacetylation of chitin, the world's second most abundant polysaccharide after cellulose [32]. Chitosan's biocompatible, biodegradable and non-toxic nature has resulted in significant interest in its use within the biomedical field [33,34]. In addition, its antimicrobial and antifungal properties, and low immunogenicity make chitosan an attractive polymer for medical applications [35,36], particularly wound healing [37,38]. Furthermore, chitosan has also been extensively investigated as a material for various tissue engineering applications. As a result of chitosan's many amine and hydroxyl functional groups, it can be readily cross-linked and modified to afford hydrogels suitable for drug delivery, cartilage and skin regeneration and adipose tissue engineering [39–43].

PEG is a water-soluble, hydrophilic polymer that has been widely explored for biomedical applications due to its non-toxic, minimal immunogenicity and anti-protein fouling properties [44–47]. As the US Food and Drug Administration (FDA) has approved this polymer for use in drug and cosmetic applications [44], PEG has also been investigated to prepare hydrogel scaffolds for tissue engineering applications [48]. Many in vitro and in vivo studies have demonstrated the desirable properties of PEG, as well as the cell and tissue penetration and vascularization capability of scaffolds fabricated using PEG [49–52].

Several studies have investigated the combination of chitosan and PEG to prepare potential tissue engineering scaffolds [53–55]. As such, combining the advantageous properties of both PEG and chitosan, we herein report the fabrication of ultrathin, biocompatible and biodegradable chitosan–PEG hydrogel films (CPHFs) for the regeneration and implantation of CECs. The CPHFs are prepared via the cross-linking of chitosan films with diepoxy-PEG and cystamine. The judicious introduction of disulphide-containing cystamine during the PEG cross-linking step imparts additional points of degradation within the films as well as branching for the cross-linking. The resultant optically transparent and permeable films have excellent mechanical properties that surpass the tensile strength of the human cornea whilst retaining similar elastic moduli, and, as such, are well suited to ophthalmic applications. Their mechanical properties combined with a thicknesses of just 50 μm make these films particularly desirable for minimally invasive implantation procedures resembling DSEK. In vitro studies revealed that the CPHFs supported the proliferation of CECs from sheep sources, thus demonstrating their potential for corneal tissue engineering.

2. Experimental

2.1. Materials

Chitosan from fresh shrimp shells (*Pandalus borealis*) (190–310 kDa; ~80% deacetylated), cystamine dihydrochloride (98%), poly(ethylene glycol) diglycidyl ether (PEGDGE) (M_n = 526 Da), collagen Type I from calf skin (0.1% solution, sterile filtered), phosphate-buffered saline (PBS) tablets, glucose assay kit (Sigma GAGO-20: glucose oxidase/peroxidise reagent and *O*-dianisidine dihydrochloride) and albumin–fluorescein isothiocyanate conjugate from bovine (albumin-FITC), insulin, transferrin, selenium, 4′,6-diamidino-2-phenylindole (DAPI) fluorescent stain, Triton X-100, and dextran $M_r \approx 500000$ were obtained from Sigma–Aldrich. Dulbecco's Modified Eagle's Medium:Nutrient Mixture F12 (DMEM:F12), antibiotic–antimycotic, epidermal growth factor (EGF), fetal calf serum (FCS), Alexa Fluor 488 goat anti mouse IgG, trypsin, and EDTA was obtained from Invitrogen. Anti-Na^+/K^+-ATPase (β2-subunit) monoclonal IgG produced in mouse clone M17-P5-F11 was obtained from Santa Cruz Biotechnology. Thermanox tissue culture plastic (TCP) coverslips were obtained from NUNC. D-Glucose (anhydrous) was obtained from Chem-Supply. Cystamine dihydrochloride was neutralized according to a previously published procedure [52]. Tetrahydrofuran (THF) (Honeywell, 99.99%), sodium hydroxide (Merck, >99%), acetic acid (Chem-Supply, >99.7%) were used as received. Milli Q water (18.2 MΩ cm) was obtained from a Millipore Synergy Water system. Poly(vinyl chloride) (PVC) sheets were obtained from Bunnings Warehouse, Australia. Deuterium oxide (99.8% D) was purchased from Cambridge Isotopes and used as received. Matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI ToF MS) matrices (α-cyano-4-hydroxycinnamic acid (α-CHCA) (⩾99.5%), *trans*-2-[3-(4-tert-butylphenyl)-2-methyl-2-propenylidene]malononitrile (DCTB) (⩾99.0%), 2,5-dihydroxy benzoic acid (DHB)), and cationization agent (NaTFA (99.999%)) were purchased from Aldrich and used as received.

2.2. Instrumentation

Thermogravimetric analysis (TGA) was conducted using a Perkin-Elmer Diamond TGA/DTA with Pyris Thermal Analysis Software. Environmental scanning electron microscopy (E-SEM) was carried out using a FEI Quanta FEG 200 Enviro-SEM with the samples mounted on carbon tabs. UV–Vis light transmittance measurements were carried out using a Shimadzu UV-1800 UV–Vis scanning spectrophotometer. A side-by-side diffusion cell with magnetic stirrers was obtained from PermeGear Inc. Mechanical testing was conducted using an Instron Microtester 5848 equipped with Bluehill material testing software. Spectral reflectance measurements were obtained using a Filmetrics thin-film measurement system with the F20-XT configuration. Immunofluorescence imaging was carried out using a Olympus BX61 system with computer-assisted stereographic tomography (CAST) (Olympus, Japan). ^{1}H nuclear magnetic resonance (NMR) spectroscopy was performed using a Varian

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