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3D bioprinting of a corneal stroma equivalent

Abigail Isaacson, Stephen Swioklo, Che J. Connon*

Institute of Genetic Medicine, Newcastle University, Newcastle Upon Tyne, UK

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ABSTRACT

Corneal transplantation constitutes one of the leading treatments for severe cases of loss of corneal function. Due to its limitations, a concerted effort has been made by tissue engineers to produce functional, synthetic corneal prostheses as an alternative recourse. However, successful translation of these therapies into the clinic has not yet been accomplished. 3D bioprinting is an emerging technology that can be harnessed for the fabrication of biological tissue for clinical applications. We applied this to the area of corneal tissue engineering in order to fabricate corneal structures that resembled the structure of the native human corneal stroma using an existing 3D digital human corneal model and a suitable support structure. These were 3D bioprinted from an in-house collagen-based bio-ink containing encapsulated corneal keratocytes. Keratocytes exhibited high cell viability both at day 1 post-printing (> 90%) and at day 7 (83%). We established 3D bio-printing to be a feasible method by which artificial corneal structures can be engineered.

1. Introduction

The World Health Organisation estimates that 10 million people worldwide require surgery to prevent corneal blindness as a result of trachoma, with a further 4.9 million suffering from total blindness due to corneal scarring (Whitcher et al., 2001). Even with adequate numbers of prospective cornea donors, a considerable discrepancy exists between the supply and demand of transplantable corneas (Golchet et al., 2000). The unmet clinical need for cornea donors has led to increasing effort in the development of artificial corneal substitutes, which must meet specific criteria if they are to functionally mimic the native cornea.

The cornea serves as the protective, outermost layer of the eye and is responsible for the transmission and refraction of incident light beams that are in turn focused onto the retina by the lens (Eghrari et al., 2015; Meek and Knupp, 2015; Griffiths et al., 2016). Its near-perfect spherical anterior surface, together with the index of refraction change at the air/tear film interface, account for approximately 80% of the total refractive power of the human eye (Ruberti and Zieske, 2008). The ability to recapitulate the rotational symmetric curvature necessary for optical refractive power is therefore fundamental to the design framework that exists for engineering functional corneal substitutes (Muller et al., 2001; Ruberti and Zieske, 2008).

It is the distinct arrangement of collagen lamellae in the corneal stroma that is responsible for maintaining the strength and shape of the cornea (Farrell and McCally, 2000). The corneal stroma comprises somewhere between 200 and 250 lamellae that are assembled heterogeneously throughout its depth, with the lamellae in the mid to posterior stroma lying in parallel while those in the anterior stroma are interwoven with one other (Hogan et al., 1971). The complexity of corneal microstructure presents an ongoing challenge when using traditional tissue engineering methods that focus on assembling corneal extracellular matrix (ECM) in vitro. As such, the replication of human corneal geometry remains to be fully realised within the context of tissue engineering.

The ability to construct biosynthetic corneal models would be useful for a number of applications, and this has been achieved in recent years where, for example, corneal models have been required for the characterisation of corneal cellular regeneration (Li et al., 2003) as well as for modelling corneal fibrosis (Karamichos et al., 2014). In these instances, corneal models were assembled using different techniques; the study by Li et al. made use of plastic contact lens molds into which hybrid collagen hydrogels were injected and crosslinked, while Karamichos et al. plated human corneal fibroblasts (HCFs) onto six-well plates bearing porous polycarbonate membrane inserts that were left in culture over a period of weeks. The former method enabled the fabrication of a curved corneal surface onto which corneal epithelial cells could be seeded, cultured and eventually implanted, while the latter relied upon cell proliferation and ECM secretion to render an in vitro 3D model that could be used to study fibrosis reversal in the cornea.

3D bioprinting is a technique that has garnered notable interest for tissue engineering applications for its ability to direct the hierarchical assembly of 3-dimensional biological structures for tissue construction (Mironov et al., 2006). Since its advent, 3D bioprinting has made

* Corresponding author. Institute of Genetic Medicine, Faculty of Medicine, Newcastle University, International Centre for Life, Newcastle Upon Tyne, NE1 3BZ, UK.
E-mail address: Che.Connon@ncl.ac.uk (C.J. Connon).

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possible the layer-by-layer deposition of biological materials in a prescribed pattern corresponding with the anatomy of an organotypic model (Zhang and Zhang, 2015). This model is usually acquired from clinical images such as CT and MRI scans and is used as a means by which to generate the fundamental printing paths on which 3D bioprinting depends; these are expressed in the form of a unique G-code that can be computed automatically by 3D printing software at resolutions specified by the chosen print parameters (Murphy and Atala, 2014). The ability to replicate features such as concavity, undercuts and convoluted patterns is therefore a function of the complexity of two-dimensional figures, such as points, lines and circles (Chia and Wu, 2015). The final, post-printing stage involves the cell-mediated remodelling of the printed biological construct in the presence of appropriate physiological cues to ensure that it develops suitable biomechanical, structural and functional properties (Jakab et al., 2010; Mironov et al., 2011).

In this study, we examined the feasibility of generating complex 3D bioprinted corneal stroma equivalents using pneumatic 3D extrusion bioprinting. Printed constructs were anatomically analogous to a human corneal model derived from the topographic data of an adult human cornea, acquired *in situ* post-refractive surgery. Several low viscosity bio-ink combinations were tested for their printability prior to cell incorporation. Printing accuracy was evaluated by quantifying central and peripheral thickness of the corneal construct and the viability of encapsulated corneal keratocytes was evaluated on days 1 and 7 post-printing. Overall, our study provides a basis for further research into the use of 3D bioprinting for the generation of artificial, biological corneal structures for regenerative medicine applications.

2. Materials and methods

2.1. Digital model and support structure generation

A patient-specific digital corneal model constructed using a rotating Scheimpflug camera with a Placido disk and discretised by the Finite Element Method (FEM) was used (Simonini and Pandolfi, 2015). The vertical and horizontal diameters of the model measured 12.377 mm × 12.385 mm, respectively, while its thickness measured approximately 500 μm at the centre and 823 μm towards the periphery. The corneal model was used as a template with which to build a digital support structure on AutoCAD 2017 (version 20.1) in order to facilitate the 3D bioprinting process. This was made possible by sealing the rim of the model cornea with a planar circle (r = 6.5 mm) such that it then resembled a dome; the modified model was then subtracted from the centre of one of the square faces of a digital cuboid (23.7 mm × 23.7 mm x 6 mm) that was designed to sit neatly inside a 35 mm Petri dish. The resulting support structure was exported as an STL file and 3D-printed at a resolution of 100 μm with white Acrylonitrile Butadiene Styrene (ABS) using a CEL Robox 3D printer.

2.2. G-code export and printing setup

The 3D printing software Slic3r (1.2.9) was configured with an INKREDIBLE bioprinter (Cellink AB, Gothenburg, Sweden). A stereolithography (STL) file of the corneal model was imported onto Slic3r, from which versions of G-code were subsequently exported. Printing speed was set at 6 mms^{-1} and 30G high precision blunt needles (CELLINK, AB) were used in all experiments.

2.3. Bio-ink preparation

Sodium alginate (Acros Organics brand, ThermoFisher Scientific, U.K.) and methacrylated type I collagen (PhotoCol®, Advanced Biomatrix, USA) were used to prepare all bio-inks. Methacrylated collagen was first dissolved in acetic acid and subsequently neutralized with sodium hydroxide at 4 °C, following the supplied preparation protocol. Six bio-inks were formulated in total, two of which comprised 3% (w/v) sodium alginate and 8 mg/ml methacrylated collagen only. The final four bio-inks, termed Coll-1 to Coll-4, had various combinations of methacrylated collagen mixed with 2% (w/v) sodium alginate to the following ratios: (i) Coll-1: one part 8 mg/ml collagen to two parts alginate; (ii) Coll-2: one part 8 mg/ml collagen to three parts alginate, (iii) Coll-3: one part 6 mg/ml collagen to two parts alginate; and (iv) Coll-4: one part 6 mg/ml collagen to three parts alginate.

2.4. Cell culture

Corneal keratocytes are the most abundant cell type in the corneal stroma, itself comprising 80% of corneal thickness, and were therefore deemed a suitable cell type for bio-ink formulation. Human corneal stromal cells were isolated, as previously described (Gouveia and Connon, 2013), from cadaverous human corneal tissue (male/female, age 60–80 years and with no prior history of corneal diseases or ocular trauma, research consent given) obtained from NHS Blood and Transplant (NHSBT) through a service level agreement with Newcastle-upon-Tyne Hospitals NHS Foundation Trust, U.K.. Briefly, the epithelia-depleted corneal tissues were finely chopped using a scalpel, transferred to DMEM/F12 medium (ThermoFisher Scientific) supplemented with 5% fetal bovine serum (FBS; BioSera, Labtech International, U.K.), 2 g/L (450 units/mL) collagenase type-1 (ThermoFisher Scientific) and incubated at 37 °C under continuous rotation for 5 h, followed by incubation with 0.25% trypsin-EDTA (ThermoFisher Scientific) for 10 min. The isolated corneal stromal cells were plated onto tissue culture flasks (Greiner Bio-One, U.K.) and maintained using DMEM/F12 medium supplemented with 5% FBS and 1% penicillin/streptomycin (ThermoFisher Scientific). Media were changed every 2–3 days, and cultures were maintained until reaching 70–80% confluence. At this point fibroblasts underwent serum starvation for a period of 3 days to promote their differentiation into keratocytes. During this time, cells were cultured in serum-free medium comprised of DMEM/F12 with 1×10^{-3} M ascorbic acid (Sigma-Aldrich, U.K), 1 × ITS (Sigma-Aldrich), and 1% penicillin/streptomycin. Cells were used at passage 3.

2.5. 3D bioprinting and optimisation

A pneumatic, dual extruder 3D bioprinter was used to print corneal stromal equivalents. This was calibrated so that bioprinting began from the centre of the support and then outwards and upwards towards the rim. Following calibration, the hollowed-out section of the bespoke 3D printed plastic support was filled with gelatine slurry in order to facilitate the printing of low viscosity collagen and alginate bio-inks while maintaining printability; the gelatine slurry was prepared using the Freeform Reversible Embedding of Suspended Hydrogels (FRESH) starter kit and protocol provided by Allevi (USA). The support was then returned to the printing plate for the duration of the printing process. Corneal structures were extruded at air pressures of 180 KPa, 15 KPa, 40 KPa, 20 KPa, 15 KPa and 10 KPa for bio-inks comprising 3% alginate alone, 8 mg/ml methacrylated collagen alone, Coll-1, Coll-2, Coll-3 and Coll-4, respectively. A phosphate buffered saline (ThermoFisher Scientific) (PBS)-based slurry and a calcium chloride (Sigma-Aldrich) ($CaCl_2$)-based slurry were prepared for use with collagen and alginate bio-inks respectively, with the latter additionally used for the printing of composite bio-inks. Corneal structures printed using alginate-based bio-inks were crosslinked with 100 μl of 1% (w/v) $CaCl_2$ and were immediately incubated at 37 °C for 8 min, while structures printed from collagen alone were incubated for 30 min at 37 °C. The gelatine slurry was aspirated after incubation leaving behind printed and stabilised constructs that were then detached from the support and stored in PBS thereafter. Corneal structures printed with composite bio-inks were crosslinked in 100 μl $CaCl_2$ and incubated at 37 °C for 8 min; the $CaCl_2$ was then aspirated and replaced with an equal volume of PBS and incubated for a further 20 min at 37 °C. Serum-free medium was used in