Biomimetic corneal stroma using electro-compacted collagen

Zhi Chen  
*University of Wollongong, zhic@uow.edu.au*

Xiao Liu  
*University of Wollongong, xiaol@uow.edu.au*

Jingjing You

Yihui Song

Eva Tomaskovic-Crook  
*University of Wollongong, evatc@uow.edu.au*

*See next page for additional authors*
Biomimetic corneal stroma using electro-compacted collagen

Abstract
© 2020 Acta Materialia Inc. Engineering substantia propria (or stroma of cornea) that mimics the function and anatomy of natural tissue is vital for in vitro modelling and in vivo regeneration. There are, however, few examples of bioengineered biomimetic corneal stroma. Here we describe the construction of an orthogonally oriented 3D corneal stroma model (3D-CSM) using pure electro-compacted collagen (EC). EC films comprise aligned collagen fibrils and support primary human corneal stromal cells (hCSCs). Cell-laden constructs are analogous to the anatomical structure of native human cornea. The hCSCs are guided by the topographical cues provided by the aligned collagen fibrils of the EC films. Importantly, the 3D-CSM are biodegradable, highly transparent, glucose-permeable and comprise quiescent hCSCs. Gene expression analysis indicated the presence of aligned collagen fibrils is strongly coupled to downregulation of active fibroblast/myofibroblast markers α-SMA and Thy-1, with a concomitant upregulation of the dormant keratocyte marker ALDH3. The 3D-CSM represents the first example of an optimally robust biomimetic engineered corneal stroma that is constructed from pure electro-compacted collagen for cell and tissue support. The 3D-CSM is a significant advance for synthetic corneal stroma engineering, with the potential to be used for full-thickness and functional cornea replacement, as well as informing in vivo tissue regeneration. Statement of Significance: This manuscript represents the first example of a robust, transparent, glucose permeable and pure collagen-based biomimetic 3D corneal stromal model (3D-CSM) constructed from pure electro-compacted collagen. The collagen fibrils of 3D-CSM are aligned and orthogonally arranged, mimicking native human corneal stroma. The alignment of collagen fibrils correlates with the direction of current applied for electro-compaction and influences human corneal stromal cell (hCSC) orientation. Moreover, 3D-CSM constructs support a corneal keratocyte phenotype; an essential requirement for modelling healthy corneal stroma. As-prepared 3D-CSM hold great promise as corneal stromal substitutes for research and translation, with the potential to be used for full-thickness cornea replacement.

Disciplines
Engineering | Physical Sciences and Mathematics

Publication Details

Authors
Zhi Chen, Xiao Liu, Jingjing You, Yihui Song, Eva Tomaskovic-Crook, Gerard L. Sutton, Jeremy Micah Crook, and Gordon G. Wallace

This journal article is available at Research Online: https://ro.uow.edu.au/aiimpapers/4250
Graphical Abstract

- Collagen membrane
- Corneal stromal model
- Biomimetic structure
- Human cornea
- Human corneal stromal cells
- Electro-compaction
- Quiescence
- Keratocytes

**Human corneal stromal cells**

**Electro-compaction**

**Collagen membrane**

**Corneal stromal model**

**Biomimetic structure**

**Human cornea**

**Stroma**

**Quiescence**

**Keratocytes**
Biomimetic Corneal Stroma Using Electro-Compacted Collagen

Zhi Chen, Xiao Liu, Jingjing You, Yihui Song, Eva Tomaskovic-Crook, Gerard Sutton, Jeremy M. Crook, Gordon G. Wallace

ARC Centre of Excellence for Electromaterials Science, Intelligent Polymer Research Institute, AIIM Facility, Innovation Campus, University of Wollongong, Fairy Meadow, New South Wales 2519, Australia.

Save Sight Institute, University of Sydney, Sydney, New South Wales 2000, Australia.


Sydney Medical School, University of Sydney, Sydney, New South Wales 2066, Australia.

Illawarra Health and Medical Research Institute, University of Wollongong, Wollongong, New South Wales 2522, Australia.

Department of Surgery, St Vincent’s Hospital, The University of Melbourne, Fitzroy, Victoria 3065, Australia.

Contacts for corresponding authors:
Jeremy M. Crook (jcrook@uow.edu.au, T +61 2 4221 3011.
Gordon G. Wallace (gwallace@uow.edu.au).

Email for other authors:
Zhi Chen (zc555@uowmail.edu.au); Xiao Liu (xiaol@uow.edu.au); Jingjing You (jing.you@sydney.edu.au); Yihui Song (yson6289@uni.sydney.edu.au); Eva Tomaskovic-Crook (evatc@uow.edu.au); Gerard Sutton (gerard.sutton@vei.com.au).

Keywords: collagen, 3D engineered corneal stroma, biomimetic, human corneal stromal cells.

Abstract

Engineering substantia propria (or stroma of cornea) that mimics the function and anatomy of natural tissue is vital for in vitro modelling and in vivo regeneration. There are, however, few examples of bioengineered biomimetic corneal stroma. Here we describe the construction of an orthogonally oriented 3D corneal stroma model (3D-CSM) using pure electro-compacted collagen (EC). EC films comprise aligned collagen fibrils and support primary human corneal stromal cells (hCSCs). Cell-laden constructs are analogous to the anatomical structure of native human cornea. The hCSCs are guided by the topographical cues provided by the
aligned collagen fibrils of the EC films. Importantly, the 3D-CSM are biodegradable, highly transparent, glucose-permeable and comprise quiescent hCSCs. Gene expression analysis indicated the presence of aligned collagen fibrils is strongly coupled to downregulation of active fibroblast/myofibroblast markers α-SMA and Thy-1, with a concomitant upregulation of the dormant keratocyte marker ALDH3. The 3D-CSM represents the first example of an optimally robust biomimetic engineered corneal stroma that is constructed from pure electro-compacted collagen for cell and tissue support. The 3D-CSM is a significant advance for synthetic corneal stroma engineering, with the potential to be used for full-thickness and functional cornea replacement, as well as informing in vivo tissue regeneration.

Statement of Significance

This manuscript represents the first example of a robust, transparent, glucose permeable and pure collagen-based biomimetic 3D corneal stromal model (3D-CSM) constructed from pure electro-compacted collagen. The collagen fibrils of 3D-CSM are aligned and orthogonally arranged, mimicking native human corneal stroma. The alignment of collagen fibrils correlates with the direction of current applied for electro-compaction and influences human corneal stromal cell (hCSC) orientation. Moreover, 3D-CSM constructs support a corneal keratocyte phenotype; an essential requirement for modelling healthy corneal stroma. As-prepared 3D-CSM hold great promise as corneal stromal substitutes for research and translation, with the potential to be used for full-thickness cornea replacement.

1. Introduction

Cornea, the transparent tissue at the eye’s anterior surface, serves to direct light entering the eye and as the first protective barrier from the external environment [1]. Corneal disease and damage is a common cause of visual impairment with corneal opacities affecting 4% of the 39 million blind people worldwide [2]. Corneal transplantation remains the main
treatment for severe cornea damage. However, the shortage of suitable corneal tissue donors, transplant rejection, and the increasing risk of transmissible diseases underpin the urgent need for qualified substitutes for corneal tissue replacement [3].

During the past decade, advances in tissue engineering have provided opportunities for corneal regeneration by applying principles of materials science and biological science. Anatomically, the healthy cornea consists of five layers including the anterior epithelium, Bowman’s layer, stroma, Descemet’s membrane and posterior endothelium (Fig. 1A, B).

Damaged corneal surface may be repaired by grafts of human cornea epithelial sheets cultured on collagen [4, 5], silk fibroin [6, 7] and a variety of synthetic hydrogels [8-11]. Similarly, corneal endothelial sheets have been successfully regenerated on various substrates with proof-of-concept pre-clinical application demonstrated by in vivo animal modelling [12-16].

Although methods for corneal epithelial and endothelial bioengineering have been widely reported, more difficult construction of a biomimetic stromal model is yet to be realised [1]. Corneal stroma is the primary component of cornea, accounting for 80%~85% of the entire cornea [17]. Structurally, the stroma is composed of layers of parallel collagen fibrils forming 250-300 flattened lamella. Lamellae are arranged orthogonally and are typically filled with corneal keratocytes (Fig. 1B) [18]. This highly organized structure contributes to the transparency and mechanical strength of cornea, and is therefore key to engineering cornea in vitro [17].

Stroma bioengineering has included cell-free (e.g., decellularized cornea [19], collagen [20], silk fibroin [21]) and cell-laden (e.g., human corneal fibroblasts (HCFs) with collagen [22], silk fibrin [23], or fibrin/agarose [24]) matrices. While several approaches have recapitulated many of the properties of human cornea [22, 23, 25-31], most have failed to provide biomimetic structure. More recently, stroma-mimetic scaffolds have been generated by assembling electro-spun synthetic polymer nanowires [32], stacking patterned silk membranes [33], forming collagen gel composed of aligned fibers in a magnet [25] and
orthogonally stacking stretched compressed collagen [34]. However, electro-spun synthetic
polymer and stacked silk scaffolds only used collagen as a coating or additive to improve the
biocompatibility rather than as the main component providing structural support [32, 33]. The
magnetic technique enables fabrication of scaffolds with similar structure to human cornea
stroma, but requires covalent crosslinking either enzymatically during fibril assembly using
transglutaminase or chemically following multi-layer assembly using 1-ethyl-3-(3-dimethyl
aminopropyl)carbodiimide (EDC)/N-hydroxysuccinimide (NHS) to enhance mechanical
properties [25]. Moreover, to achieve adequate transparency requires incorporation of
proteoglycans and optimization of crosslinking [25, 35].

Here we describe the construction of a biomimetic 3D-CSM using electro-compacted
collagen and primary hCSCs (human corneal stromal cells) (Fig. 1C). Electro-compacted
collagen fibres and films have been described for various applications, including tendon repair
[36], neuronal [37] and skin regeneration [38]. For corneal bioengineering, EDC/NHS
crosslinked EC fibres have previously been shown to support the growth of keratocytes [39].
Herein, we report the native tissue-like structure and function of cell-laden EC constructs,
through investigation of collagen fibril alignment, light transmittance, glucose permeability,
degradeability and cell phenotyping. The studies provide support for the 3D-CSM as a
platform for corneal stroma research and therapy.
2. Materials and methods

2.1. Fabrication of electro-compacted collagen film and conventional collagen gel

The method for the electro-compaction of collagen was modified from previous published reports [40, 41]. Acid soluble monomeric collagen type I solution (97%) from bovine hides (Advanced Biomatrix, San Diego, CA, catalogue # 5010-D, 6mg ml\(^{-1}\)) was dialyzed against ultrapure water (18.2 MΩ-cm) at 4°C for 48h to remove salts. The resultant collagen solution was diluted into 0.25mg ml\(^{-1}\) and loaded into the electro-compaction cell (Fig. 1D) that comprised a stainless steel plate as cathode and ITO glass plate as anode. Silicone rubber rims (15mm in diameter and 2mm in thickness) were printed using a 3D-Bioplotter System (Envision TEC) and applied as spacers. Nylon rims were cut from nylon membrane filters (Whatman 7404-009; LOT: D112754; thickness:150-187 um) using a laser engraver (Universal Laser Systems) and loaded into the cell as collectors for EC films.
Applying 5v for 300s, the collagen solution separated into a lower collagen film and an upper aqueous phase. The upper aqueous phase was then replaced with PBS and incubated at room temperature (RT) for 18h before the film was collected (Fig. 1C). The thickness of each EC film was measured using an Atomic Force Microscope (Park Systems XE-Bio) after the surface water was gently blotted off [40]. Experiments were performed at RT using silicon probes (BRUKER, DNP-S10). Membrane thickness was measured at the edge of each sample [40]. Conventional collagen (CC) gels were dehydrothermally prepared according to the manufacture’s protocol. Briefly, the collagen solution was mixed with 10x PBS and the pH adjusted to 7.0–7.5 at a final concentration of 3.5 mg ml⁻¹. The solution was then gelated by incubating at 37 °C for 2 hr in the electro-compaction chamber (15mm in diameter, 2mm height).

2.2. Human corneal stromal cell culture

Human corneal tissue obtained from the NSW tissue bank was used for hCSC isolation, as approved by the Human Research Ethics Committee of NSW South Eastern Sydney Local Health District (HREC ref no: 15/251; approval date: 3 September 2015) and the Human Research Ethics Committee of University of Wollongong/Illawarra Shoalhaven Local Health District (HE15/483; approval date: 1 December 2015). Briefly, the epithelium, Descemet’s membrane and endothelium were sequentially removed, and the stroma was cut into ~1 cm³ pieces. The pieces were cultured in 35mm petri dishes (Thermo Fisher Scientific, USA). Drops of DMEM/F12 with 5% FBS were added to the surface of the stromal explants, left in the biological safety cabinet for 15 min before being transferred into a 37°C, 5% CO₂ humidified incubator for 2h. A further 1mL DMEM/F12 with 5% FBS was added and explants were incubated overnight, after which floating explants were removed. Stromal cells grown from the explants were observed after 2-3 days incubation. Culture medium was changed every 2 days and cells were passaged upon reaching 70%-80% confluence and cryo-
preserved in -80°C for future use. Cells were expanded in DMEM/F12 supplemented with 10% FBS and 1% penicillin/streptomycin. As described previously [28, 32], cells were stabilized in serum-free medium (SFM) (DMEM/F12 (phenol red-free) 1:1, 1% (v v\(^{-1}\)) 100x Insulin-Transferrin-Selenium (ITS), 0.01% (v v\(^{-1}\)) 10M phosphoascorbic acid and 0.01% (v v\(^{-1}\)) 100 ng/nL FGF-2) for 3 days before further use. All cell-based experiments were performed with passage 3~4 hCSCs.

2.3. Fabrication of corneal stromal constructs

hCSCs were seeded at 2.5 x 10\(^4\) cells cm\(^{-2}\) on EC films and incubated for 12 hr to allow cell adhesion. The direction of current going through the electro-compaction chamber has been marked on the filter membrane rim (Fig. S2), indicating the direction of aligned collagen fibrils to provide guidance when orthogonally stacking single layer EC into 3D-CSM. Four hCSCs-seeded films were then successively stacked, one on top of another with collagen fibrils of each layer orthogonally arranged with the direction of collagen fibrils marked on the nylon membrane (Fig. S2) and 3D-CSM were weighed down by sterilized acrylic rims in SFM medium to allow complete integration of EC films (Fig. S1A). The constructs were incubated in SFM at 37 °C and the medium was changed every two days.

2.4. Scanning electron microscopy

Scanning Electron Microscopy (SEM) was performed using a JSM-7500FA LV Scanning Electron Microscope, operating at 10 kV. Samples were fixed using 2.5% (v v\(^{-1}\)) glutaraldehyde in PBS at 4 °C for 2 hr, followed by 3 x 1 min washes in PBS. Samples were then dehydrated through a series of ethanol solutions (1 x 10 min in 30%, 50%, 75%, 90%, followed by 2 x 10 min in 100%) and then critical point dried with a Leica CPD030 Critical Pt Dryer. Samples were mounted on the specimen holder and sputter coated with 10 nm gold using a Dynavac Sputter Coater, followed by observation. The images were analysed for
directionality of EC and CC fibrils using ImageJ software. The preferred orientation/angles of fibrils in the images are reported (0° is the East direction, and the orientation is counterclockwise). Isotropic patterns are expected to give a flat histogram, whereas those with a preferred orientation tend to give a histogram with a peak at that angle.

2.5. Rheology

Rheology was performed using a TA AR-G2 rheometer at RT with a 12 mm diameter parallel plate. An initial strain sweep test was performed to determine the linear viscoelastic (LVE) range, where G’ (elastic modulus) and G” (viscous modulus) were independent of the strain amplitude (Fig. S3). The oscillatory measurements were then performed at strain 1% in the frequency range 0.01-100 HZ. Non-electro-compacted collagen solution (NEC) (0.25mg ml⁻¹), electro-compacted collagen before (ECCB) and after (ECCA) PBS treatment were tested.

2.6 Microindentation

The mechanical properties of 3D-CSM and human cornea stroma was carried out using an EZ-S mechanical tester (Shimadzu, Japan) using an indentation technique. Samples were tested in the compression mode with a 10 N load cell at a cross-head speed of 0.1 mm min⁻¹. The Young’s modulus (E₂) of the sample was calculated using eq. (1). [42, 43]

\[ F = \frac{8}{3} r E_2 d \]  
\[ \text{eq. (1)} \]

Here, F is the applied force, r is the radius (0.495 mm) of the indenter tip, and d is the indentation depth. Data in the linear range were used to calculate E₂.

2.7. Biodegradation
3D-CSM were incubated in SFM that was replaced every 2 days and the dehydrated weights were recorded at days 1, 7 and 14 (n = 6). The average and variance of the dry weight of all samples were calculated. The residual mass% was calculated according to the following equation: Residual mass% = (dehydrated mass / initial dehydrated mass) x 100%. (Eq. 2)

2.8. Light transmission

Transparency of samples for visible light was evaluated by measuring total transmittance using a ColourQuest XE (HunterLab, USA) spectrophotometer. CIE L*a*b* (CIELAB) system are used to describe the values, the value L (luminosity) represents the level of light or dark ranging from white (L = 100) to dark (L = 0) [44].

2.9. Glucose permeability

Glucose permeability studies were carried out using two-compartment diffusion chambers with mechanical stirring at 100 rpm at RT, according to published protocols [45, 46]. 3D-CSM were mounted between the two chambers (permeate chamber and receptor chamber) without leaking. The permeate chamber was filled with glucose solution (10 mg ml\(^{-1}\)) and the receptor chamber was sampled every 5 min. The samples were then assessed by spectrophotometry at 540 nm using a glucose assay kit (GAGO-20; glucose oxidase/peroxidase reagent and O-dianisidine dihydrochloride reagent; Sigma-Aldrich) with a UV-3600 spectrophotometer (Shimadzu).

2.10. Live/dead human corneal stromal cell analysis

Calcein AM and propidium iodide (PI) staining were used to assess live and dead hCSCs respectively. Following 3 x 30 s washes in PBS, samples were stained with Calcein-AM (0.5 mg L\(^{-1}\)) and PI (0.3 mg L\(^{-1}\)) in PBS at 37 °C for 15 min. Live (green) and dead (red) cells were then observed using a Leica TSC SP5 II Confocal Microscope.
2.11. Human corneal stromal cell proliferation analysis

PrestoBlue (Invitrogen) assay was performed according to the manufacturer’s instructions. 3D-CSM were incubated with 10% PrestoBlue reagent (v:v⁻¹) in culture medium for 30 min at day 1, 3, 5, 7 and 14 day. The media were collected, and transferred into 96-well plates to measure the fluorescence of the test reagent using a FLUO star Omega Microplate Reader with the excitation/emission wavelengths set at 560/590 nm for Presto-Blue.

2.12. Immunocytochemistry

Samples were washed with PBS and then fixed in 3.7% paraformaldehyde in PBS for 30 min at room temperature. Following 3 x 5 min washes in PBS, samples were permeabilized with 0.3% Triton X-100 in PBS supplemented with 5% (v:v⁻¹) BSA overnight at room temperature and washed again. Samples were then incubated with conjugated antibodies KI67 (Invitrogen, LOT1372558A), unconjugated primary antibodies keratocan (HPA039321, Sigma-Aldrich), alpha-SMA (A2547, Sigma-Aldrich) at 4°C overnight. Rinsed with 0.1% Triton X-100 in PBS, samples with unconjugated primary antibodies were incubated with Alexa Fluor tagged secondary antibodies (Alex Fuor 488, Invitrogen A21206; Alex Fluor 594, A21203) for 1h at 37°C, followed by washing and counterstaining with DAPI, rinsed with PBS and observed using a Leica TSC SP5 II Confocal Microscope. For F-actin staining, samples were permeabilised with 0.1% Triton X-100 for 10 min after fixation and then stained with Alexa 488-Phalloidin (ThermoFisher Scientific, Waltham, MA, USA) in1% BSA for 40 min at RT in dark and the nuclei were visualized using DAPI before observation.

2.13 Cell orientation analysis

The morphology of the cells (n=180) on the EC and CC membrane was analysed with ImageJ. The angle between the long axis of the cell and the direction of aligned collagen fibrils was
defined as the orientation angle [47, 48]. An angle of 0° corresponds to complete orientation. The smaller the angle, the more the cell is oriented with the feature direction. The angle on CC in which fibrils are disorganized was defined using the same direction of aligned fibrils/current to make a comparison. The directionality analysis of the cells on EC and CC were also conducted using ImageJ. The preferred orientation/angles of cells in the images are reported in the manner mentioned above (section 2.4).

2.14. Real-time quantitative PCR (RT-qPCR)

After 14 days culture, 3D-CSM were rinsed with PBS to remove medium. Total RNA was then isolated using Aurum™ Total RNA Mini Kit on ice according to the manufacturer’s protocol. For EC, hCSCs were seeded on the top after gels were prepared. Cells seeded on tissue culture plastic (TCP) were used as controls. hCSCs grown on EC film or TCPs were directly treated with lysis buffer, while 3D-CSMs constructs were de-assembled into single/separate layers using tweezers prior to lysis. The quantity and purity of RNA was assessed using a NanoDrop 2000c Spectrophotometer (Thermo Scientific). RNA was reverse transcribed to cDNA using iScript™ Reverse Transcription Supermix (Bio-rad, 1708841) according to the manufacturer's instructions followed by assessment of cDNA. RT-qPCR was performed for 40 amplification cycles using SYBR™ Select Master Mix for CFX (catalogue #4472942) on a Bio-Rad CFX real time instrument. hCSCs grown on TCP was used as a reference point for comparison. 18S was used as the endogenous control for normalisation of expression levels. The data were analyzed using the delta-delta Ct method. Recorded data was averaged and represented as a mean value ± the calculated standard error. Primers for genes used were purchased from Sigma Aldrich and primer sequences can be seen in Table S1.

2.15. Statistical analysis
Experimental data were analyzed statistically using ANOVA with Tukey post hoc test. Homogeneity of variance tests were performed to confirm statistical assumptions were met for ANOVA. P-values less than 0.05 were considered to be significant (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, n=3). IBM SPSS statistics 25 was used for all analyses.

3. Results

3.1. Fabrication of electro-compacted collagen films

Since the thickness of native human cornea is 400-600 μm [49], 0.25 mg ml-1 collagen solutions were selected to prepare electro-compacted films (144 ± 52 um) (Fig. S4), four of which were combined to create the corneal stromal model of similar thickness as that of native human corneal stroma (518±91um) (Fig. S1B). The current density for electro-compaction of collagen was monitored, with the initial current density being 0.7-0.8 mA cm$^2$ and this continuously decreased to 0.33mA cm$^2$ before plateauing at 100 sec, when the compaction of collagen was completed. The voltage, however, was applied for 300 sec to avoid incomplete compaction (Fig. 2A). The toughness and flexibility were provisionally evaluated by repeatedly pricking the sample using tweezers (Fig. SVideo). Rheological testing was carried out to confirm the effect of electro-compaction and PBS incubation. $G'$ (storage modulus) and $G''$ (viscous modulus) vs. frequency for non-electrocompacted collagen solution (NEC), electrocompacted collagen before (EC) and after (ECCA) PBS treatment are shown in Fig. 2B. With the same initial collagen concentration, the $G'$ of all samples was continually higher than $G''$ in the frequency range, with $G'$ weakly dependent on frequency. The $G'$ of EC was significantly higher (5-fold) than that of NEC. Compared with EC, a slight increase (20%~50% depending on frequency) of $G'$ was observed on ECCA.

SEM images demonstrated highly-condensed and aligned collagen fibrils (Fig. 2C-F), further detailed below (see 3.4). The quantitative directionality analysis also supports directional
alignment of collagen fibrils in EC (Fig. S5A), exhibiting a preferential directionality (60°~80°). Analysis of collagen fibrils in CC shows a relatively flat histogram without specific peaks (Fig. S5B).
hCSCs were stabilized for 3 days in SFM before seeding onto electro-compacted collagen films and continuing to culture in the serum media [28]. As expected, the presence of the nuclear proliferation marker Ki67 decreased for cells cultured in SFM, while constant, high level Ki67 was detected for cells cultured in FBS media (Fig. S6). Similarly, PrestoBlue-based analysis demonstrated higher cell proliferation of hCSCs in FBS compared to SFM (Fig. S7). Importantly, Calcein AM (acetoxymethyl) and Propidium Iodide staining showed high cell viability in corneal stromal model from day 1 to day 14, with uniform distribution of hCSCs across EC layers (Fig. 3A). Moreover, consistent with cell culture in SFM, immunophenotyping showed hCSCs at day 14 post-seeding did not express Ki67 in the 3D-CSM (Fig. 3B). Cell viability was corroborated by PrestoBlue assay, which showed stable metabolically active cell cultures that did not expand over time (P>0.05) (Fig. 3C).

RT-qPCR showed negligible expression of myofibroblast/fibroblast markers α-SMA and Thy-1 transcripts for 3D CSM- and 2D EC-hCSCs, with levels being comparatively higher for cells cultured on TCP (P<0.001)(Fig. 4A, B). By contrast, expression of keratocyte marker-ALDH3 was upregulated for 3D CSM-hCSCs samples compared with 2D EC-hCSCs.
and TCP-hCSCs (P<0.01) (Fig. 4C). Following 14 days culture, immunophenotyping revealed the presence α-SMA while no apparent keratocan expression (Fig. S8).

3.4. Alignment of collagen fibrils and human corneal stromal cell orientation

Assessment of EC films by SEM demonstrated highly-condensed and aligned collagen fibrils running parallel to the direction of current through the electro-compaction chamber (Fig. 2C-F and Fig. 5B). In contrast, collagen fibrils of conventional collagen (CC) films were disordered. The calculated orientation angle of hCSCs on EC (26°±22°) was significantly lower (n=180, P<0.05) than cells on CC (51°±24°), indicating the orientation of hCSCs was directed by the aligned collagen fibrils of EC. Moreover, EC exhibited smaller fibrils (30~50 nm diameter) compared to CC (100~150 nm diameter) (Fig. S9), with the former being similar to that of native human cornea (31-34 nm) [50]. To investigate whether the orientation of hCSCs is affected by aligned collagen fibrils, F-actin staining was employed to visualise cells cultured for 12 hr on EC and CC films. The collagen fibrils of EC films
appeared to influence hCSC orientation, with cells clearly aligned with collagen fibrils (Fig. 5A). In contrast, hCSCs on CC films were patently disordered (Fig. 5A), consistent with the underlying substrate topography (Fig. 5B). The quantitative directionality analysis shows that while hCSCs on EC exhibit directional alignment (60°~ 80°), those on CC did not follow the directionality of specific angle (Fig. S5C and D). For hCSCs on EC, the peak is in accordance with the directionality analysis of EC fibrils (section 3.1).

3.5. Orthogonally arranged layers of human corneal stromal cells within corneal stromal constructs

The hCSCs within 3D-CSM were principally arranged orthogonally from one layer to another as evident by F-actin labelling of cells (Fig. 6A). The multilayer structure of 3D-CSM was confirmed by SEM (Fig. 2E, F) and the 3D distribution of hCSCs was confirmed by confocal microscopy, with cells observed on different planes from the top to the bottom of a 3D-CSM, being orthogonally arranged from one layer to the next (Fig. 6B).
3.6. Mechanical property, transparency, glucose permeability and degradability of corneal stromal constructs

Presently, the Young’s modulus of 3D-CSM and human corneal stroma are $0.23\pm0.01$ kPa and $23.05\pm7.43$ kpa, respectively. 3D-CSM remained highly transparent for the duration of study and the integrity of all 3D-CSM was well maintained (Fig. 7A). No significant changes were observed on the glucose permeability coefficient and mass of human cornea stroma over time. Nevertheless, a small decrease in dehydrated mass was observed at day 7 and day 14, although 80% of the initial mass of 3D-CSM remained at day 14 (Fig. 7D).

$L^*$ (luminosity) of 3D-CSM was evaluated using a ColourQuest XE spectrophotometer. While the $L^*$ was retained for all samples tested with or without cells (P>0.05), predictably the presence of hCSCs marginally impaired light transmission (P<0.05,
87–89% vs 81–83% respectively) (Fig. 7B). The water content of 3D-CSM was consistently around 80%. No significant change on transmittance and water content were observed at day 1, day 7 and day 14 (Fig. S10).

The glucose permeability coefficient of 3D-CSM was tested and monitored up to day 14 (Fig. 7C), demonstrating greater permeability than that of native human cornea.

4. Discussion

The rate of electro-compaction to form a collagen film can be adjusted by changing the applied voltage [40]. Compared to previous reports, the optimized current density presently employed resulted in more rapid film formation [36, 51, 52], and without obvious
bubble generation [51]. Importantly, high current densities can lead to bubble generation on
the cathode due to H₂ and on the anode due to O₂ formation. Both can diminish the integrity
of the collagen films generated. On the other hand, insufficient current density results in
incomplete compaction. Incubating the films in PBS for 18 hr post-electro-compaction results
in improved mechanical properties [53]. The resultant films were robust enough to physically
transfer with the nylon ring prepared as per the experimental method. The constantly higher G’
than G” for NEC, EC and ECCA indicates that the elastic behaviour dominates the viscous
behaviour. The significantly higher G’ of EC to NEC confirmed successful compaction of
collagen. The slight increase of G’ was observed on ECCA was in accordance with a previous
report of mechanical enhancement of electro-compacted collagen after PBS treatment [53].

Despite previous reports of fabrication and bio-application of 2D EC collagen films
[40, 41, 51, 54, 55], none describe an association between the direction of collagen alignment
and current. Our approach has the potential to be applied for bioengineering other tissues (e.g.
muscle and skin.) whereby aligned collagen fibres can improve the biological performances of
engineered scaffolds [56-58].

Interestingly, wounding of native cornea is characterised by quiescent corneal
keratocytes becoming apoptotic and/or activated by transforming into mitotically active
fibroblasts/myofibroblasts that express fibrotic extracellular matrix, culminating in scar
deposition and tissue opaqueness [59]. As such, maintenance of clear constructs is consistent
with the hCSCs in 3D-CSM being quiescent and healthy. Furthermore, the cornea plays a
critical role in maintaining normal vision as the transparent anterior part of the eye. Therefore,
transparency of 3D-CSM and consequently component biomaterials is essential to
recapitulate native tissue function, albeit also important for observing constituent cell
behaviour [60].
The avascular nature of natural cornea necessitates favourable scaffold permeability which facilitates nutrient diffusion from the aqueous humor to the cornea surface [46]. For example, effective diffusion of glucose provides sufficient energy for corneal metabolism and is critical for the wellbeing of constituent stromal cells [61]. Remarkably, a small increase in permeability over time was indicated for our construct, which may be correlated with the above described small but measurable reduction in tissue mass that lead to decreased thickness. Although the increase of permeability over time does not impede the transfer of glucose of cornea, whether it would be advantageous once implanted in vivo will require further study.

The maintenance of hCSCs with a specialised keratocyte phenotype is essential for modelling corneal stroma. The ability to transform the keratocytes into proliferating fibroblasts is necessary, however, for tissue engineering. Prior to culturing on electro-compacted collagen films, we cultured hCSCs in serum-containing media to induce fibroblasts that are proliferative, followed by stabilisation in serum-free media (SFM) with supplements to restore a dormant keratocyte phenotype [62]. Although hCSCs in SFM exhibited a flattened fibroblastic morphology, this is consistent with a previous report of non-proliferating corneal fibroblasts showing decreased Ki67 staining when cultured in Insulin-transferrin-sodium supplemented Dulbecco's Modified Eagle Medium up to 7 days (Fig. S6) [63].

Gene expression analysis by RT-qPCR indicated that 3D CSM-hCSCs were more keratocyte-like compared to TCP-hCSCs, with upregulation of keratocyte-marker ALDH3 and concomitant down-regulation of myofibroblast-markers α-SMA and Thy-1. 2D EC-hCSCs similarly expressed low to negligible levels of α-SMA and Thy-1 transcripts, while both genes were upregulated for TCP-hCSCs. Taken together, these results are consistent with the presence of collagen fibrils favouring hCSCs with a keratocyte phenotype rather than
activated fibroblasts [64], and is supported by reports of up-regulation of keratocan and ALDH3 expression of bovine corneal keratocytes cultured on collagen vitrigel with highly condensed collagen fibrils [65], as well as suppression of α-SMA using aligned collagen fibrils compared to TCP and non-aligned fibrils [66]. Interestingly, ALDH3 is a corneal crystallin, which provides protection against ultraviolet radiation, is important for maintaining cellular transparency, and is normally highly secreted by corneal keratocytes and to a lesser extent by corneal fibroblast and myofibroblasts [67]. ALDH3 expression by hCSCs within 3D-CSM is therefore consistent with constructs remaining transparent. In addition, higher ALDH expression by 3D CSM-hCSCs compared with 2D EC-hCSCs suggests a 3D environment favours gene expression, and is consistent with a previous report of 3D collagen sponge similarly supporting ALDH3 expression of corneal keratocytes compared to 2D collagen and TCP [64]. However, following 14 days culture, most wells for RT-qPCR failed to detect the signal of keratocan (only 2 well detected in 3 independent experiments (triplicates each) after 39 cycles), which indicated negligible level of keratocan expression and was consistent with the immunofluorescence result that no apparent keratocan signal observed (Fig. S8). The qPCR and immunophenotyping thus together corroborated that the 3D-CSM can increase the expression of ALDH3 with no apparent effect on that of keratocan.

As the main component of human corneal stroma, collagen type I has been widely investigated for corneal tissue engineering, either by direct mixing with corneal stromal cells prior to gelation [22] or stromal cell-seeding onto collagen-coated films that are stacked [33]. However, conventionally prepared type I collagen hydrogel lacks an appropriately organized structure thereby necessitating mixing with synthetic or other mechanically superior materials for structural integrity [1]. To the best of our knowledge, robust and consistent biomimetic 3D-CSM composed of pure collagen and hCSCs have rarely been described. Nicolas and colleagues successfully fabricated orthogonal collagen scaffolds with a highly stroma-mimetic structure using an intense magnetic field, but it required further covalent crosslinking for...
requisite mechanical properties [25, 35]. It is important to consider the effects of topographical cues on cell behaviour when developing substrates for corneal bioengineering. For instance, the depth and width of grooves influence cell orientation of human corneal fibroblast [68]. For stromal engineering, aligned fibres can induce keratocyte-like morphology and phenotype [32]. Varied techniques have been applied to create topographies that direct the orientation and phenotypic state of corneal stromal cells, including soft lithography [69], electro-spinning [32], and direct-write assembly [70]. Most reports, however, describe surfaces in micron rather than nano-scale dimensions, with collagen usually applied merely as an additive for improving biocompatibility. In this study, the as described EC films consisted of pure collagen without addition of any other materials and toxic reagents. Moreover, the aligned collagen fibrils of the films acted as a guidance for cell orientation and elongation, eliminating the need for topographical or other modification. However, some limitations should be noted. Firstly, the distribution of collagen fibrils in human corneal stroma substantially contributes to the mechanical properties of human cornea [71]. The fibrils are stretched and can reinforce the tissue when a force is applied to the cornea, determining the mechanical strength of cornea. Ideally, bioengineered corneal stroma should be able to maintain curvature under intraocular pressure [72]. The EC membrane was merely mechanically enhanced by treating with PBS, which is non-toxic and simple but cannot achieve adequate mechanical strength. The Young’s modulus of 3D-CSM was significantly lower than that of human corneal stroma and previous reports [73, 74], in the range of dehydrothermally crosslinked collagen corneal stromal equivalent [75]. Secondly, the 3D-CSM can undergo delamination as the whole structure is not fully integrated. These two issues lead to difficulty with in vivo evaluation and clinical application and may be addressed by improving the preparation of 3D-CSM using crosslinking and more advanced fabrication technique. Last but not least, the collagen fibrils in 3D-CSM are homogeneously aligned and orthogonally arranged in different layers. However, the arrangement of collagen fibrils in
corneal stroma are more complex, with collagen fibrils in the prepupillary area being more closely packed than in the peripheral cornea. Also, deeper layers are more organized than superficial area and the collagen fibrils may circumferentially form into an annulus as approaching the limbus [6, 7]. This relatively more complex arrangement of collagen fibrils in the prepupillary area and limbus requires further development of fabrication method.

Future clinical application of the 3D-CSM will require the use of a biocompatible crosslinker to substantially improve the mechanical properties and enhanced integration of the whole structure. Furthermore, future work should entail more in-depth cellular and molecular analyses as well as in vivo studies. Finally, advanced biofabrication devices and related EC techniques, such as the use of an electro-compaction chamber with biomimetic curvature, would benefit the development of therapeutic tissues, inclusive of increasing the complexity of collagen fibril distribution, more analogous to native tissue.

5. Conclusion

The 3D-CSM represents the first example of 3D stacked engineered corneal stroma using pure electro-compacted collagen (EC). Light transmittance, glucose permeability and degradability verify the model’s potential to emulate healthy corneal stroma. Our studies also affirm previous reports that 2D aligned collagen fibrils and 3D stromal-mimetic collagen models can affect the phenotype of hCSCs in vitro by introducing topographical cues to support a corneal keratocyte phenotype; as required for both healthy cornea and derivative repair-cell phenotypes necessary for wound healing. Therefore, as-prepared 3D-CSM hold significant promise as corneal stromal substitutes, with the potential to be used for full-thickness cornea replacement as well as informing in vivo cell and tissue function and regeneration.
Authorship contribution statement

ZC, XL, GGW and JMC conceived the study. ZC executed the experiments, with support from JY, YS and ETC. ZC and JMC wrote the paper and all authors read, commented on and approved the paper. JMC and GGW are co-senior corresponding authors.

Declaration of Competing Interest

The authors have no financial interests to disclose.

Acknowledgements

The authors wish to acknowledge funding from the Australian Research Council (ARC) Centre of Excellence Scheme (CE140100012) and Professor Gordon Wallace acknowledges the support of the ARC through an ARC Laureate Fellowship (FL110100196). Also, the authors acknowledge the support of the Australian National Nanofabrication Facility (ANFF) Materials node, use of facilities at the UOW Electron Microscopy Centre, Dr. Johnson Chung and Steven Posniak for training and technical advice for RT-qPCR, Alireza Talaei for developing the glucose diffusion chamber, Dr. Andrew Nattestad for technical advice to use the UV-3600 spectrometer, and Dan Yang and Prof. Michael Higgins for providing technical advice and training for AFM.

References


Figure legends

Fig. 1. Schematics of human cornea, fabrication of EC film, and biomimetic 3D-CSM. (A-B) Schema of human cornea comprising five layers from top to bottom and component orthogonally arranged collagen fibrils. (C) Fabricated 3D-CSM handled by tweezers. (D-E) Schema of electro-compaction to form collagen films that are then collected within filter membrane rims. (F-G) Schema of seeding EC films with hCSCs that are assembled orthogonally, layer-by-layer, to construct 3D-CSM.

Fig. 2. Current density change over time and characterizations of EC collagen films and 3D-CSM. (A) Current response (i-t curve) of electro-compaction of collagen. (B) Elastic modulus (filled symbols) and viscous modulus (open symbols) versus frequency for collagen samples before and after electro-compaction, with and without PBS treatment. (C-D) SEM micrographs of the surface of 3D-CSM, showing alignment of collagen fibrils in the direction of the arrow. (E-F) SEM micrographs of cross sections of 3D-CSM.

Fig. 3. hCSC survival and proliferation in SFM in 3D-CSM (A) Time course of live (Calcein AM) and dead (propidium iodide; PI) hCSC staining in 3D-CSM. (B) hCSCs expressed negligible levels of cell proliferation marker Ki67 at day 14 post-seeding; (C) Time course of hCSC proliferation (quantified by PrestoBlue assay) in 3D-CSM up to day 14.

Fig. 4. Comparative gene expression by RT-qPCR of hCSCs within 3D-CSM or on 2D EC and TCPs after 14 days culture. (A, B) Myofibroblast/fibroblast markers α-SMA and Thy-1, respectively. (C) Keratocyte marker ALDH3 Gene expression is normalized to 18s and expressed relative to TCP. *P≤0.05, **P≤0.01, ***P ≤ 0.001. n=3.
Fig. 5. Effect of collagen fibril alignment on hCSC orientation, with direction of fibril alignment indicated by the arrows. (A) F-actin labelling of hCSCs on EC and CC revealed cell orientation was influenced by the alignment of collagen fibrils (scale bar: 300 μm). (B) SEM micrographs of the surface topographies of EC and CC respectively, with collagen fibrils of EC aligned with the direction of current (arrow) employed for electro-compaction, while those of CC were disordered.

Fig. 6. Orthogonally arranged layers of hCSCs within 3D-CSM (scale bar: 300 μm). (A) F-actin labelling of hCSCs in 3D-CSM showed orthogonal arrangement of cells in different layers (denoted by dashed arrows). (B) Alignment of hCSCs in the topmost to the bottommost layer of a 3D-CSM.

Fig. 7. Assessment of transparency, glucose permeability and degradability of 3D-CSM and human cornea stroma cultured in SFM for up to 14 days. (A) Photomicrographs of translucent 3D-CSM. (B) Total visible light transmittance of 3D-CSM. (C) Glucose permeability of 3D-CSM and human cornea stroma. (D) In vitro degradability shown as decreasing dehydrated tissue mass over time of 3D-CSM and human cornea stroma.
Figure 1

Click here to download high resolution image
Figure 7
Click here to download high resolution image

(A) No cells | With cells

Day 1

Day 3

Day 7

Day 14

(B) [Graph showing comparison between 'With cells' and 'Without cells']

(C) [Graph showing data for Day 1, Day 7, and Day 14]

(D) [Graph showing data for different conditions]
Supp Figure 4

Click here to download high resolution image
Supp Figure 7
Click here to download high resolution image

![Bar graph showing fluorescence (560/590 nm) over days with FBS and SFM](image-url)
Supp Figure 8
Click here to download high resolution image
Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: