

# Engineering a Corneal Stromal Equivalent Using a Novel Multilayered Fabrication Assembly Technique\*

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To overcome the serious shortage of donor corneas for transplantation, alternatives based on tissue engineering need to be developed. Decellularized corneas are one potential alternative, but their densely packed collagen architecture inhibits recellularization *in vitro*. Therefore, a new rapid method of recellularizing these constructs to ensure high cellularity throughout the collagen scaffold is needed. In this study, we developed a novel method for fabricating corneal constructs by using decellularized porcine corneal sheets assembled using a bottom-up approach by layering multiple sheets between cell-laden collagen I hydrogel.

Corneal lenticles were cut from porcine corneas by cryosectioning, then decellularized with detergents and air-dried for storage as sheets. Human corneal stromal cells were encapsulated in collagen I hydrogel and cast between the dried sheets. Constructs were cultured in serum-free medium supplemented with ascorbic acid and insulin for 2 weeks. Epithelial cells were then seeded on the surface and cultured for an additional week. Transparency, cell viability, and phenotype were analyzed by qPCR, histology, and immunofluorescence. Constructs without epithelial cells were sutured onto an *ex vivo* porcine cornea and cultured for 1 week.

Lenticules were successfully decellularized, achieving dsDNA values of  $13 \pm 1.2$  ng/mg dry tissue, and were more resistant to degradation than the collagen I hydrogels. Constructs maintained high cell viability with a keratocyte-like phenotype with upregulation of keratocan, decorin, lumican, collagen I, ALDH3A1, and CD34 and the corneal epithelial cells stratified with a cobblestone morphology. The construct was amenable to surgical handling and no tearing occurred during suturing. After 7 days *ex vivo*, constructs were covered by a neoeepithelium from the host porcine cells and integration into the host stroma was observed.

This study describes a novel approach toward fabricating anterior corneal substitutes in a simple and rapid manner, obtaining mature and suturable constructs using only tissue-derived materials.

**Keywords:** cornea, tissue engineering, decellularization, keratocyte, lenticles

## Impact Statement

New strategies are needed to face the important worldwide shortage of donor tissues for corneal transplantation. This study describes a novel approach based on decellularized sheets of porcine cornea interspaced with cell-laden collagen I hydrogels. These constructs matured into a transplantable tissue.

## Introduction

SEVERAL DISEASES AFFECTING the cornea can lead to blindness and require a corneal transplant to regain sight. Supply of human donor tissue is limited and it is estimated that only 1 cornea is available for every 70 needed,<sup>1</sup> high-

lighting the critical shortage. There is a clear necessity for alternative approaches with tissue engineering being one method of potentially generating transplantable tissue.

Among the promising materials under consideration for fabricating the cornea is decellularized animal tissue. This comprises of the extracellular matrix (ECM) of the cornea

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\*Part of this study has been presented at the 2019 Annual Meeting of the Association for Vision and Ophthalmology (ARVO). The abstract is published in *Investigative Ophthalmology and Visual Science* 60 (9), 4124–4124.

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without any donor cells present to provide a scaffold for cell growth. Decellularized porcine corneas have been transplanted into humans in cases of infectious ulcers with encouraging results.<sup>2,3</sup> However, these acellular scaffolds are composed of densely packed collagen lamellae, and thus recellularization is difficult, making it slow, both *in vitro*<sup>4</sup> and *in vivo*.<sup>5</sup>

In attempts to improve recellularization, human lenticules extracted during myopia correction surgeries have been attached together using a fibrin glue hydrogel to prepare a decellularized scaffold.<sup>6</sup> Although the decellularized lenticules did populate with a small number of cells, the phenotype of these was not characterized.

In another study, thin sheets of decellularized porcine cornea were seeded with stromal cells and multiple layers implanted intrastromally.<sup>7</sup> After 6 months, the corneas had the same thickness as unoperated eyes and some cells were visible between engrafted sheets. Thus, the first study showed the feasibility of binding the decellularized lenticules without using cells, and the second showed the possibility of recellularizing each layer before implantation. The combination of both approaches where cells are embedded in the hydrogel has not been explored previously and sets the basis for this study. This novel approach would allow cells to be incorporated directly into the decellularized cornea while still binding the different layers together.

The aim of this study was therefore to develop a corneal substitute by using sheets of decellularized porcine cornea interspersed with cell-laden collagen hydrogels. The use of collagen rather than fibrin is more physiologically relevant since the cornea is primarily made up of collagen fibrils. These constructs presented high transparency, easy handling after fabrication, high viability, and *in vivo*-like phenotype after a culture period of 3 weeks. Furthermore, constructs containing both stromal and epithelial cells were fabricated, and remained viable when sutured into an *ex vivo* model of anterior lamellar keratoplasty (ALK).

## Materials and Methods

### *Fabrication of acellular matrix sheets*

Porcine eyes were obtained within 24 h from abattoir death. After trimming and under sterile conditions, the eye globes were washed twice with phosphate-buffered saline (PBS), immersed in 2% iodine solution (Videne<sup>®</sup>, Ecolab, Belgium) for 4 min under gentle agitation, and then rinsed three times with PBS for 2 min. The central corneal button from each eye was excised using a 10 mm biopsy punch. Corneal buttons were embedded in optimal cutting temperature medium (OCT; Thermo-Scientific, Ireland), snap-frozen in liquid nitrogen, and cryosectioned on a cryostat (Leica, Germany) to obtain 60- $\mu$ m-thick slices. Slices were washed in PBS to remove OCT and then transferred into a decellularization solution of 0.5% (w/v) sodium dodecyl sulfate (SDS) and 1% (v/v) Triton X-100 (both Sigma-Aldrich, Ireland) in distilled water for 24 h at room temperature.

To promote penetration of the solution and removal of debris, this and further processing was carried out on an orbital shaker. The samples were treated with 10 U/mL of RNase and DNase in 10 mM MgCl<sub>2</sub> solution for 1 h at 37°C (all Sigma), then decontaminated by washing in PBS supplemented with 100 U/mL penicillin, 100 mg/mL strep-

tomycin (both Gibco, Ireland), and 250 ng/mL amphotericin B (Sigma) for another 24 h at room temperature, followed by washing in sterile distilled water three times.

The acellular sheets were air dried in a sterile biohazard cabinet and stored in a sealed container at room temperature until use. Native, decellularized and decellularized, dehydrated and rehydrated sheets were fixed in 4% paraformaldehyde for 15 min at room temperature. They were then embedded in paraffin wax and sectioned on a microtome (Leica). Slides were deparaffinized, rehydrated, and coverslipped using 4',6-diamidino-2-phenylindole (DAPI)-containing aqueous mounting medium (Sigma). Slides were imaged with an epifluorescence microscope (Olympus IX83). The thickness of the sheets was measured in ImageJ from those images ( $n = 20-30$ ). Additionally, the macroscopic appearance and transparency of the dried acellular sheets was assessed by placing them over printed text.

### *DNA content*

Native and decellularized porcine cornea sheets were freeze dried and digested in 3.88 U/mL of papain solution rotating at 60°C for 18 h. DNA content was then quantified using a Quant-iT<sup>™</sup> PicoGreen<sup>®</sup> dsDNA Kit (Invitrogen, Ireland) following the manufacturer's instructions. The plates were read using a spectrophotometer (BioTek<sup>™</sup> Synergy HTX) using 480 nm excitation wavelength and 520 nm emission wavelength.

### *Degradation test*

*In vitro* enzymatic digestion was performed to determine the resistance to biodegradation. Single decellularized sheets and 100  $\mu$ L collagen gels (cast as described later) were tested by being incubated with 1 mL 20 U/mL collagenase I (Gibco) in PBS at 37°C. Photographs were taken periodically, before the supernatant was collected and fresh collagenase solution added. Collagen content in the supernatant was quantified by measuring the hydroxyproline content through a dimethylaminobenzaldehyde (DMBA) assay.<sup>8</sup>

Briefly, 200  $\mu$ L of supernatant at each time point was digested with 12 M HCl for 18 h at 110°C. These samples were then allowed to evaporate for 48 h at 50°C in a fume hood. After evaporation, the dry samples were resuspended in deionized water and incubated for 20 min at room temperature with Chloramine T and an oxidizing buffer. DMBA reagent was added and incubated for 20 min at 60°C. After cooling, the reactants were read at 570 nm in a plate reader (BioTek Synergy HTX).

### *Cell culture*

Human corneal stromal cells were isolated from the corneal/scleral ring remaining after a corneal transplant in accordance with the Declaration of Helsinki. The corneal/scleral rings were rinsed with sterile PBS and the epithelium and endothelium were carefully removed using a scalpel blade. After a brief wash with sterile PBS, the corneal stroma was diced into small pieces and transferred into a 25-cm<sup>2</sup> culture flask. Media consisted of low-glucose Dulbecco's modified Eagle's medium (DMEM; HyClone, Dublin, Ireland) supplemented with 10% fetal bovine serum and 100 U/mL Penicillin/Streptomycin (Gibco). The stromal pieces were cultured at 37°C and 5% CO<sub>2</sub> in a humidified incubator.

Media were changed regularly until the cells had migrated from the tissue and reached 80–90% confluence before being passaged to allow further expansion. The cells were cryopreserved and used at passage 4.

Human telomerase-immortalized corneal epithelial cells (hTCEpi, Evercyte, Austria) were expanded and cultured using keratinocyte growth medium (PromoCell, Germany), following the manufacturer's instructions.

#### *Construction of an engineered corneal stroma*

Collagen hydrogels were prepared from rat tail collagen I (Corning, New York) as previously described.<sup>9</sup> A 3.5 mg/mL solution was prepared from stock, salt concentration adjusted using 10×PBS and pH raised to neutral by addition of 1 N NaOH. After this neutralization, 10,000 cells/μL gel were embedded in the solution. Twenty-five microliters of solution was deposited on a decellularized corneal sheet and a second sheet placed on top. This process was repeated until there were four gels and five sheets. This construct was then incubated for 30 min at 37°C in a humidified incubator, before being submerged in a media of DMEM/F12 (1:1; HyClone) supplemented with 1×insulin/transferrin/selenium (Gibco) and 100 μg/mL L-ascorbic acid-2-phosphate (Sigma). This medium was chosen as it has been shown to promote a keratocyte-like phenotype.<sup>10</sup> Constructs were fed every second day for 3 weeks.

A total of 50,000 hTCEpi cells were seeded on the construct after 2 weeks and cultured for another week in Keratinocyte growth medium.

#### *Cell viability*

Constructs were incubated with 2 μM Calcein-AM and 4 μM EthD-1 (Biotium, San Francisco, California, USA) for 1 h at 37°C in a humidified incubator. Samples were washed three times in PBS and imaged by laser scanning confocal imaging (Leica SP8, Germany).

#### *Transparency and light transmittance*

The macroscopic appearance of the constructs was assessed by placing them over printed text. Additionally, light transmittance through the constructs after 3 weeks of culture was quantified by measuring the absorbance of light at different wavelengths ranging from 300 to 700 nm that pass through each sample using a microplate reader (BioTek Synergy HTX, Winooski, Vermont, USA). The absorbance of light passing through ultrapure water was used as a control to represent 100% transmittance and this absorbance reading was subtracted from the measured absorbance of each sample to give the change in absorbance due to the sample being present (A). The transmittance of light was calculated using the following formula as described elsewhere<sup>11</sup>:

$$\text{Transmittance (\%)} = \frac{1}{10^A} \times 100$$

#### *Quantitative polymerase chain reaction*

RNA from constructs was extracted using TRIzol, following the manufacturer's instructions (Invitrogen). To increase lysis and release of the genetic material, samples were tritu-

rated using a tissue homogenizer (IKA T10 basic, Germany). Chloroform was added, the samples were vortexed, and then centrifuged for 15 min at 12,000 g at 4°C. The supernatant was transferred into a new tube and an equal volume of iso-propanol added, along with 3 μL of GlycoBlue™ (Invitrogen) to improve precipitation. Samples were kept overnight at –20°C and then centrifuged at 12,000 g for 15 min at 4°C. The supernatant was discarded and 70% ethanol in RNase-free water used to wash the pellet. Samples were centrifuged again, and the supernatant removed before the pellets were air dried. The pellets were dissolved in RNase-free water and RNA yield and purity quantified using a NanoDrop-1000 (Thermo Scientific). All steps were performed on ice.

RNA was converted into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Invitrogen). Real-time polymerase chain reaction was performed using TaqMan Universal Master Mix II and the following TaqMan primers: glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Hs02758991\_g1), aldehyde dehydrogenase 3A1 (ALDH3A1, Hs00964880\_m1), alpha smooth muscle actin (α-SMA) (ACTA2, Hs00426835\_g1), keratocan (KERA; Hs00559942\_m1), collagen 1a1 (COL1; Hs00164004\_m1), lumican (LUM, Hs00929860\_m1), decorin (DCN, Hs00754870\_s1), and CD34 (Hs00990732\_m1). The genes of interest were normalized against GAPDH using the ΔΔCt method. Calculated values were expressed as a power of 2<sup>–ΔΔCt</sup>. For this study, all values were normalized to the serum-expanded cells.

#### *Histological analysis and immunohistochemistry*

After culture, samples were fixed with 4% paraformaldehyde for 15 min at room temperature. They were then embedded in paraffin wax and sectioned on a microtome (Leica). Routine Hematoxylin and Eosin (H&E), Alcian Blue, and Picrosirius Red staining were performed.

Slides were stained for 4 min with Harris Hematoxylin (Sigma), followed by a 10-min wash with running tap water, 30 s in acid alcohol, and further tap water for 5 min. Counterstaining was 2 min with Eosin Y (Sigma). Alcian Blue was used to assess sulfated glycoaminoglycans (sGAG) content; 1% Alcian Blue 8GX (Sigma) in 0.1 M HCl was applied for 5 min, followed by three, 30-s washes in deionized water. Picrosirius Red was used to assess collagen distribution; slides were immersed in Sirius Red (Sigma) in a saturated aqueous solution of picric acid for 1 h followed by 1 min in 0.5% acetic acid. After staining, all slides were dehydrated and coverslips attached using DPX mountant (Sigma).

For immunofluorescent staining, slides were rehydrated followed by an antigen-retrieval step. Samples were first blocked with 1% bovine serum albumin (BSA) and 10% donkey serum in 0.5% Triton X-100 for 1 h at room temperature. The following primary antibodies were used and incubated overnight at 4°C: Collagen I, Decorin, Keratocan, ALDH3A1, Lumican, CD34, and α-SMA. After incubation, three, 5-min washes with 1% BSA in PBS were performed to remove the unbound antibody. Secondary antibody was then incubated for 1 h at room temperature followed by again washing three times for 5 min with 1% BSA in PBS. Coverslips were attached using DAPI-containing aqueous mounting medium (Sigma). Slides were imaged using laser scanning confocal microscopy (Leica SP8). Table 1 contains

TABLE 1. ANTIBODIES AND ANTIGEN RETRIEVAL METHODS FOR IMMUNOFLUORESCENT STAINING

Primary antibody	Catalogue number (brand)	Dilution	Antigen retrieval method	Secondary antibody (dilution)
ALDH3A1	Ab76976 (Abcam)	1:200	Heat-mediated citrate buffer pH 6 (30 min at 95°C)	Donkey anti-rabbit-AlexaFluor® 488 (1:200)
$\alpha$ -SMA	Ab7817 (Abcam)	1:100	Heat-mediated citrate buffer pH 6 (30 min at 95°C)	Goat anti-mouse AlexaFluor 488 (1:200)
Keratocan	HPA039321 (Atlas Antibodies)	1:50	Heat-mediated citrate buffer pH 6 (30 min at 95°C)	Donkey anti-rabbit-AlexaFluor 488 (1:200)
CD34	Ab81289 (Abcam)	1:250	Heat-mediated citrate buffer pH 6 (30 min at 95°C)	Donkey anti-rabbit-AlexaFluor 488 (1:200)
Decorin	Ab189364 (Abcam)	1:133	Heat-mediated citrate buffer pH 6 (30 min at 95°C)	Rabbit anti-goat-AlexaFluor 594 (1:200)
Lumican	Ab168348 (Abcam)	1:100	Heat-mediated citrate buffer pH 6 (30 min at 95°C)	Donkey anti-rabbit-AlexaFluor 488 (1:200)
Collagen I	Ab90395 (Abcam)	1:400	Enzymatic-mediated 0.25 U/mL Chondroitinase ABC (1 h at 37°C)	Donkey anti-mouse AlexaFluor 488 (1.5:200)

$\alpha$ -SMA, alpha smooth muscle actin.

details of the immunofluorescent staining procedure. Secondary antibody only staining was performed as negative control.

Constructs containing epithelial and stromal cells were fixed for 15 min in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 during 10 min at room temperature, and subsequently stained with 0.5  $\mu$ g/mL Phalloidin-TRITC (Sigma) and 1  $\mu$ g/mL DAPI (Sigma) for 1 h protected from light. Samples were then imaged using laser scanning confocal microscopy (Leica SP8). They were further processed for wax embedding and H&E staining, as previously described.

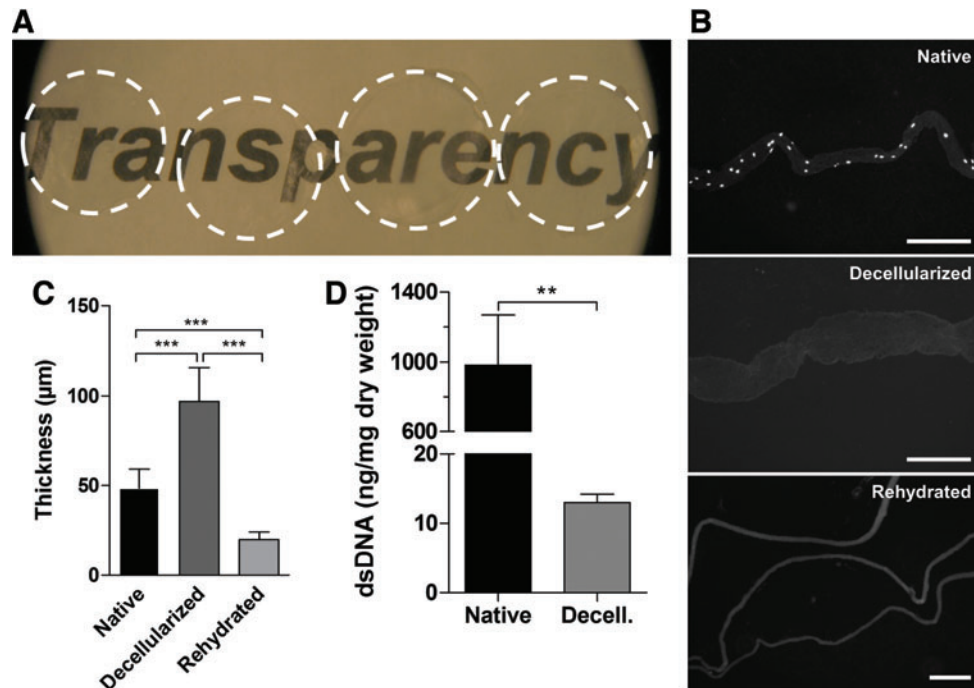
#### Implantation of constructs onto an ex vivo porcine cornea

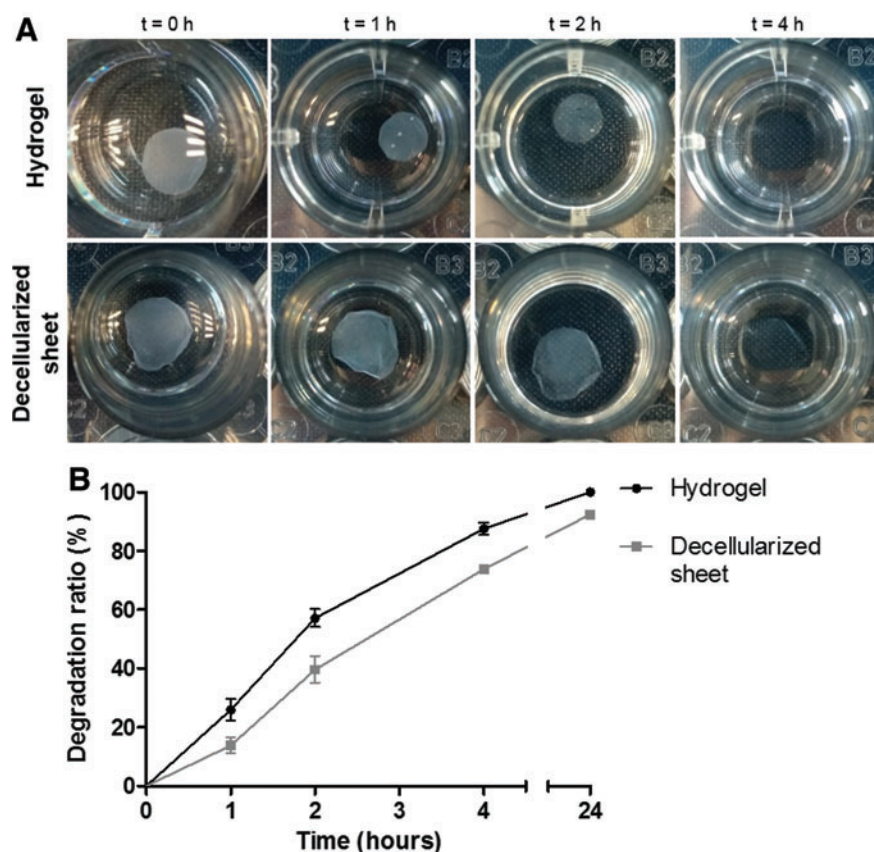
Porcine eyes were obtained within 24 h from abattoir death. A 6-mm-diameter defect of approximately half of the

depth of the cornea was made using a trephine. Lamellar dissection was performed using a blunt crescent knife (Beaver-Visitec International, UK). Constructs after 3 weeks of culture (without epithelial cells) were sutured in place with 4 or 8, 10–0 nylon sutures. The corneas were resected with 5 mm of scleral rim and placed onto a hemispherical agarose mold (2% agarose w/v). Corneas were cultured for 7 days in a humidified chamber in 4 mL of serum-free DMEM/F12, supplemented with 4% dextran (MW 450,000–650,000, Sigma) to limit swelling. The medium was added to the level of the limbus, leaving the cornea at the air/liquid interface. Medium was changed daily, with three drops added onto the corneal surface to limit drying.

To determine re-epithelialization from the host tissue fluorescein staining was performed. Four–five drops of 1% fluorescein sodium salt (Sigma) in PBS solution were added

**FIG. 1.** Characterization of decellularized sheets. **(A)** Macroscopic appearance of the dried acellular sheets, showing high transparency. **(B)** DAPI staining of a native sheet, a sheet after decellularization, and three sheets after dehydration and rehydration, showing the absence of cell nuclei after decellularization (scale bar = 200  $\mu$ m). **(C)** Thickness measurements of sheets: native, after decellularization, and after dehydration and rehydration. **(D)** dsDNA quantification of native and decellularized sheets.  $^{***}p \leq 0.001$ ,  $^{****}p \leq 0.0001$ . DAPI, 4',6-diamidino-2-phenylindole. Color images are available online.





**FIG. 2.** Resistance to biodegradation. (A) Macroscopic visualization and (B) quantitative analysis of degradation. Color images are available online.

to cover the surface of the corneas, after 1 min of incubation at room temperature, sterile PBS was used to wash the corneas until no more dye was visibly leaching. In the dark, samples were exposed to blue light from a handheld slit lamp (15090—PSL, Reichert) and imaged with a cell phone. This was performed at 2 and 7 days after implantation. After culture, corneas were fixed overnight in formalin and processed for wax embedding and H&E staining, as previously described.

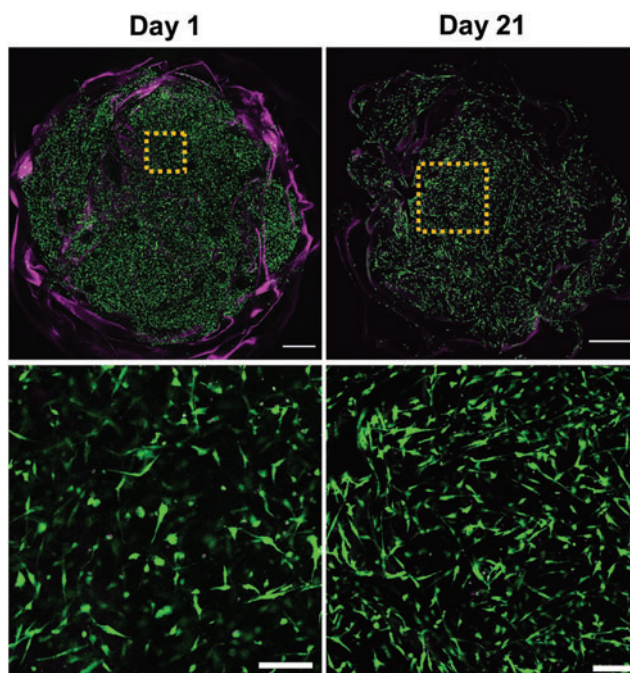
#### Statistical analysis

All experiments were performed three times with a minimum of three replicates. Unpaired two-tailed t-test and two-way analysis of variance were employed, and statistical significance accepted at a level of  $p < 0.05$ .

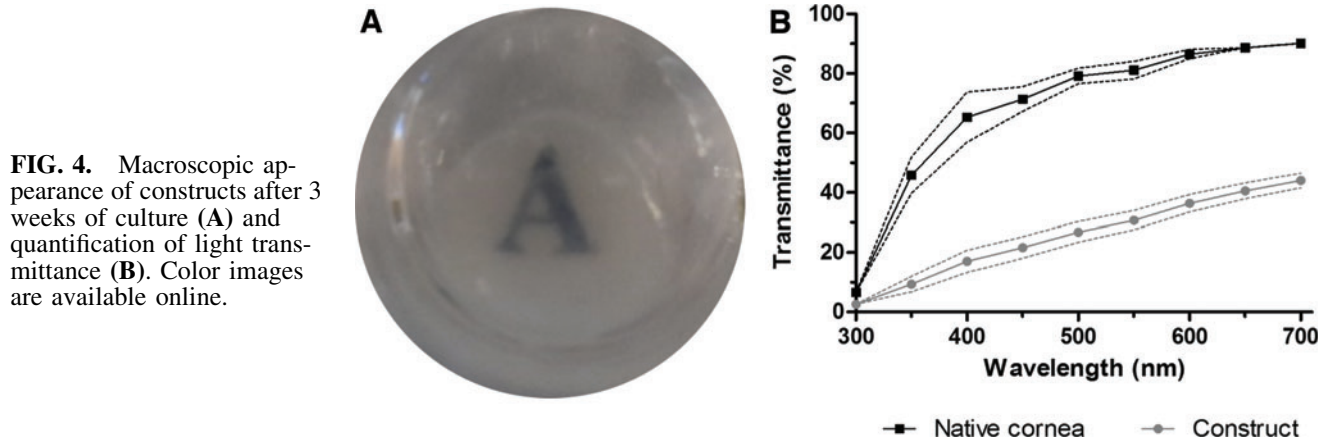
## Results

#### Characterization of decellularized sheets

The decellularized sheets obtained were highly transparent (Fig. 1A). Sections stained with DAPI presented no cell nuclei after decellularization (Fig. 1B). Thickness measurements showed that the decellularization procedure had a swelling effect on the sections and that, when sections were air dried and rehydrated, they did not recover their original thickness (Fig. 1C). Quantification of dsDNA showed successful decellularization, with significantly lower levels of DNA remnants than the native tissue (Fig. 1D). Native sheets had  $982.8 \pm 286$  ng/mg of DNA per dry tissue (average  $\pm$  standard deviation), while decellularized sheets presented  $13 \pm 1.2$  ng/mg of DNA per dry tissue (average  $\pm$  standard deviation).



**FIG. 3.** Cell viability in constructs after fabrication and after 3 weeks in culture (live = green, dead = magenta). Bottom row insert from top row at higher magnification. Top scale bar = 1 mm, bottom scale bar = 200 μm. Color images are available online.



Degradation was slower in the decellularized sheets than the collagen hydrogels. After 4 h of incubation in collagenase solution, hydrogels appeared to completely degrade, while decellularized sheets were still visible (Fig. 2A). Even after 24 h, remnants of the sheets were still present. When quantified (Fig. 2B), 57% of the hydrogels was degraded by 2 h, 87% by 4 h, and 100% after 24 h. In comparison, 39%, 73%, and 92% of the decellularized sheets were degraded after 2, 4, and 24 h, respectively. After 48 h, sheets had degraded completely.

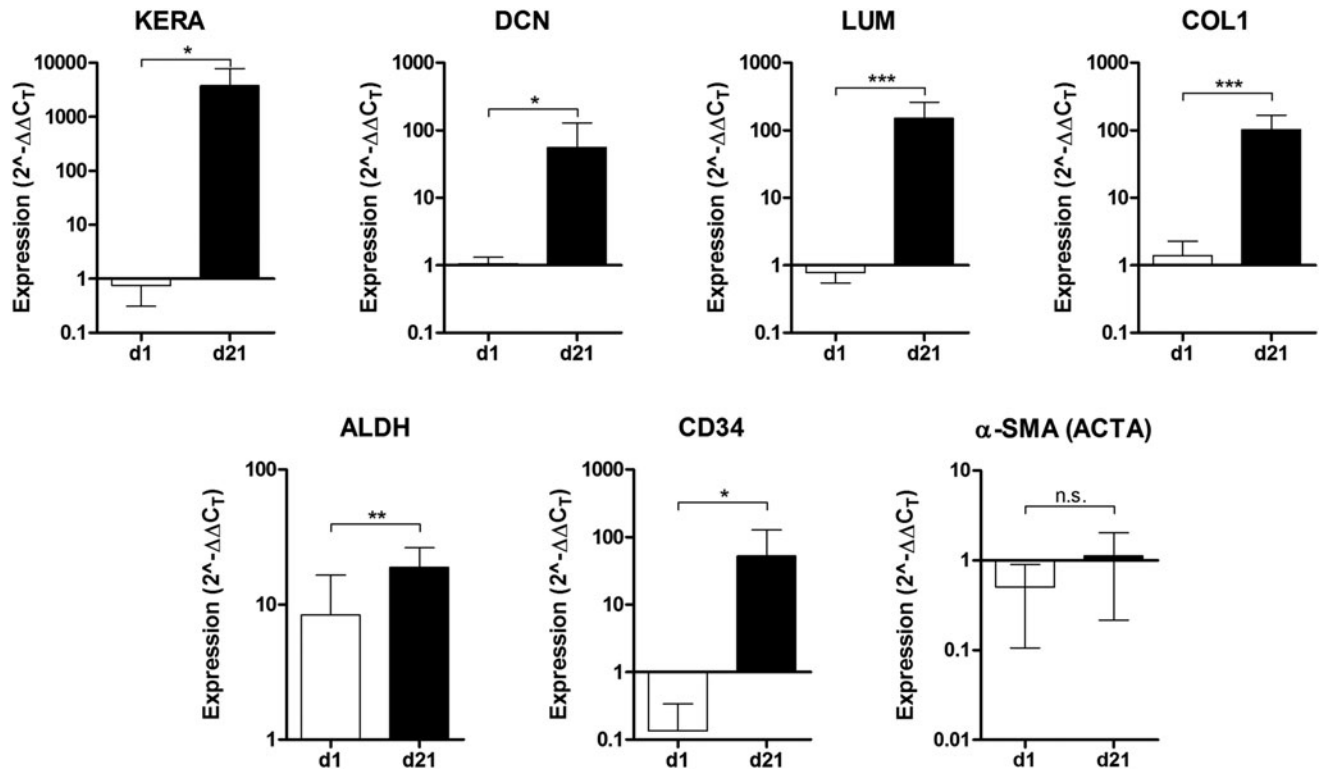
#### Corneal stromal equivalent viability and transparency

Tissue constructs were obtained by stacking dried sheets between cell-laden collagen hydrogels. Human corneal stromal cells remained viable in the constructs throughout the culturing

period, even in serum-deprived conditions (Fig. 3). After 1 day in culture, some cell spreading could be detected within the scaffold. After the whole 3-week culture period, most cells displayed an elongated morphology with visible processes. Furthermore, even in this short period, constructs had acceptable optical properties when assessed macroscopically (Fig. 4A). When quantified, they allowed less light transmittance than the native cornea, but did allow >40% of transmittance in the longer wavelengths of the visible spectrum (Fig. 4B).

#### Corneal stromal equivalent cell phenotype

Characteristic markers of corneal stromal ECM and corneal stromal cell phenotype were analyzed by quantitative polymerase chain reaction (qPCR) (Fig. 5). Keratocyte markers



ALDH3A1 and CD34 were significantly upregulated as the time in serum-free conditions increased. All ECM components analyzed were also highly upregulated. Typical small leucine-rich proteoglycans, keratocan, lumican, and decorin that are found in the corneal stroma, were expressed significantly higher. The expression of collagen I, the most common collagen in the corneal stroma, was significantly upregulated. Furthermore, the fibrotic marker  $\alpha$ -SMA was not significantly upregulated.

Constructs were further analyzed through histology and immunohistochemistry (Fig. 6). The thickness of the constructs decreased significantly during the culturing period, from  $245.2 \pm 66.50 \mu\text{m}$  to  $187.9 \pm 27.44 \mu\text{m}$ , presumably due to the cells remodeling the collagen hydrogel. Constructs presented cells distributed evenly in all hydrogel layers, as seen by H&E staining (Fig. 6A). Picrosirius Red staining for collagen showed differential staining in the hydrogel portion and the decellularized sheets (Fig. 6B). Despite the loss of sGAG during decellularization, upon subsequent culture, cells produced their own ECM in the hydrogel sections (Fig. 6C).

Immunohistostaining corroborated the results obtained from qPCR with constructs staining positive for ALDH3A1, CD34, keratocan, lumican, decorin, and collagen I, whereas  $\alpha$ -SMA remained negative (Fig. 6D).

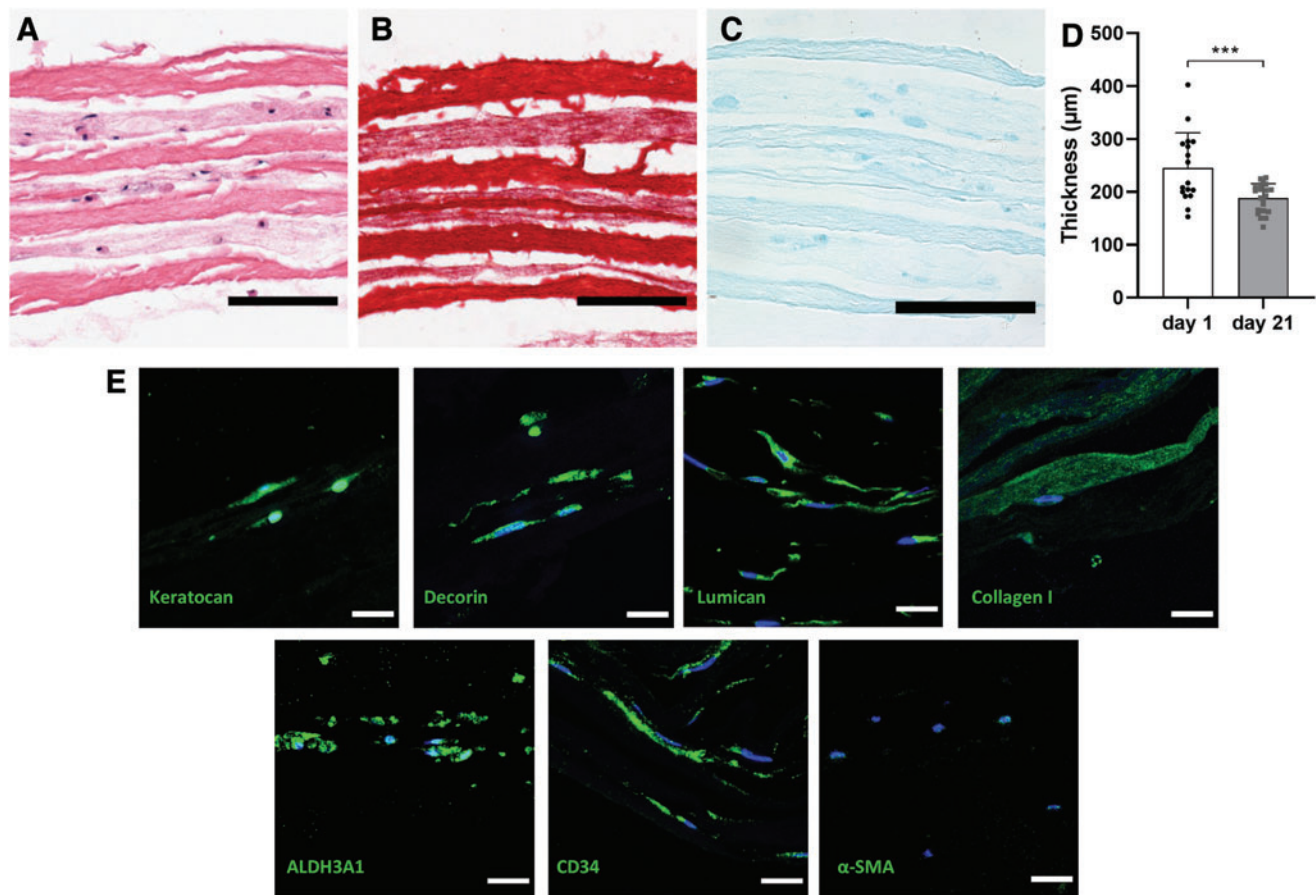
#### Epithelial cell growth on corneal stromal equivalents

Corneal epithelial cells were cultured on the top layer of the constructs (Fig. 7). A coculture was successfully obtained using this method with stromal cells detected in the middle and lower layers. Epithelial cells had attached to the construct surface and formed a tight epithelium presenting a typical cobblestone morphology (Fig. 7E). Some degree of stratification was visible in H&E-stained sections (Fig. 7F, G).

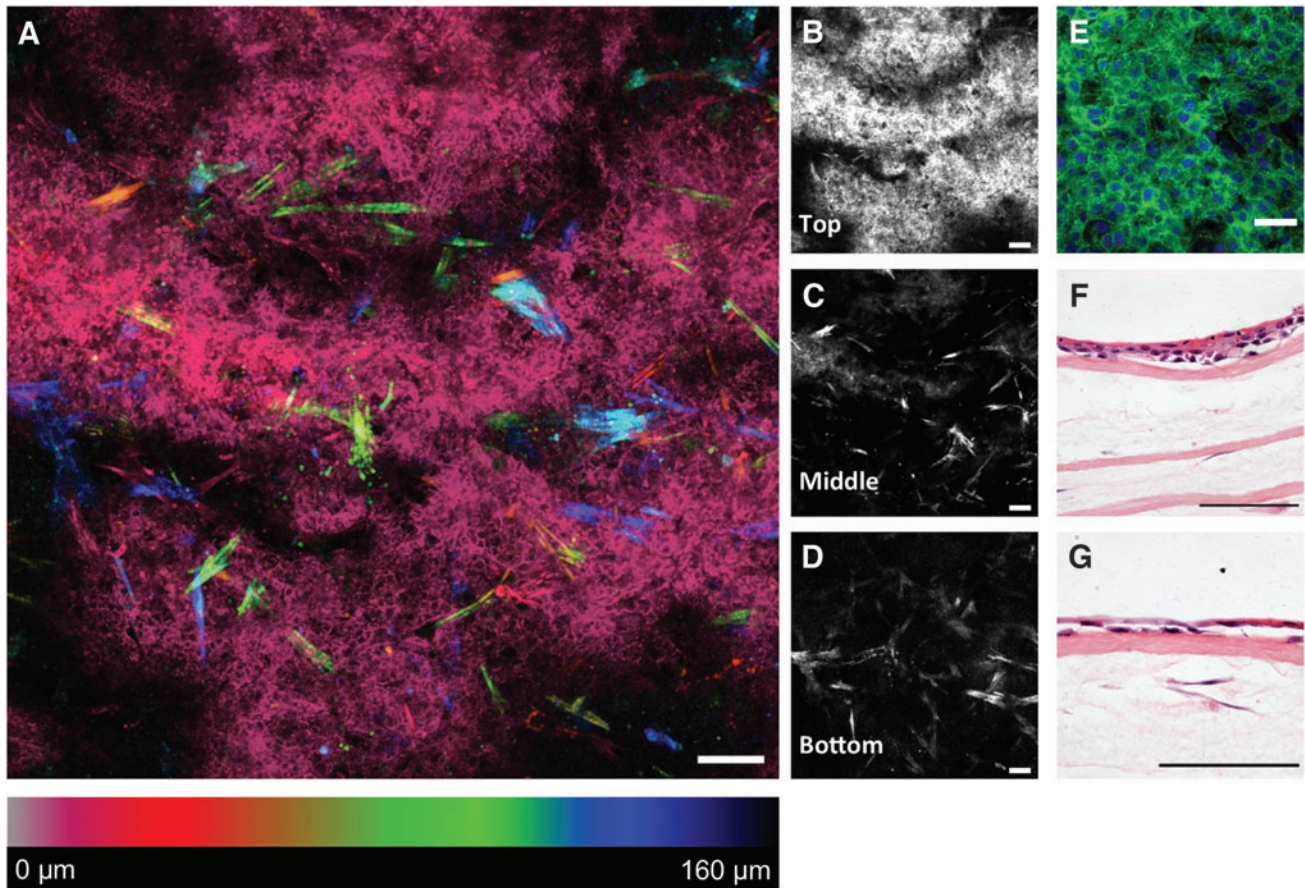
#### Implantation of corneal stromal equivalent in an *ex vivo* anterior lamellar keratoplasty model

Constructs were successfully implanted onto porcine corneas in an *ex vivo* ALK model (Fig. 8). Constructs were amenable to surgical handling and suturing without tearing (Fig. 8A). Fluorescein staining is commonly used to assess corneal epithelial wounds. If the staining is positive (yellow), it indicates an area with damaged or absent epithelium (barrier function compromised); whereas a negative staining is an indication of a healthy epithelium as its barrier does not allow the penetration of the dye.

In this study, positive staining was seen on the constructs after 2 days of implantation, while the presence of only small islands of positive fluorescein staining at day 7 indicated that



**FIG. 6.** Histological examination of constructs at day 21, staining with (A) H&E (s = sheet, h = hydrogel), (B) Picrosirius Red, and (C) Alcian Blue (scale bar =  $100 \mu\text{m}$ ). (D) Thickness measurements of constructs at day 1 and 21. (E) Immunohistofluorescent staining of Keratocan, Lumican, Decorin, Collagen I, ALDH3A1, CD34, and  $\alpha$ -SMA (green), and cell nuclei (blue), scale bar =  $20 \mu\text{m}$ . H&E, Hematoxylin and Eosin. Color images are available online.



**FIG. 7.** Anterior cornea constructs with epithelium. (A) Depth color-coded Z-stack, cells at the surface (epithelium) appear *pink/red*, whereas stromal cells in deeper areas appear from *orange* to *blue*; (B–D) single images of the Z-stack (scale bar = 100  $\mu\text{m}$ ); (E) higher magnification of the epithelium F-actin (*green*) and nuclei (*blue*), scale bar = 10  $\mu\text{m}$ ; (F, G) H&E-stained sections show a degree of epithelial stratification (scale bar = 100  $\mu\text{m}$ ). Color images are available online.

neighboring epithelial cells colonized the surface of the scaffold (Fig. 8B). Histological analysis through H&E showed good integration of the construct onto the host bed and at its periphery, while the surface was covered in a neoe epithelium (Fig. 8C).

## Discussion

To overcome the severe worldwide shortage of donor corneal tissue for transplantation, tissue engineering approaches are being developed. In this study, a multilayered construct fabricated with decellularized cornea sheets and cell-laden collagen hydrogels is described. These corneal stroma tissue equivalents presented high cell viability, transparency, expression of stromal cell markers, and supported epithelial regeneration.

Decellularization of the stromal sheets was successful, with values of dsDNA remaining well below the recommended maximum concentration of 50 ng/mg of dry tissue to minimize adverse host reactions.<sup>12</sup> While a shorter decellularization protocol to that previously described was used in this study, levels of DNA were lower.<sup>13</sup> This is probably due to the thinner tissue allowing improved penetration of the detergents.

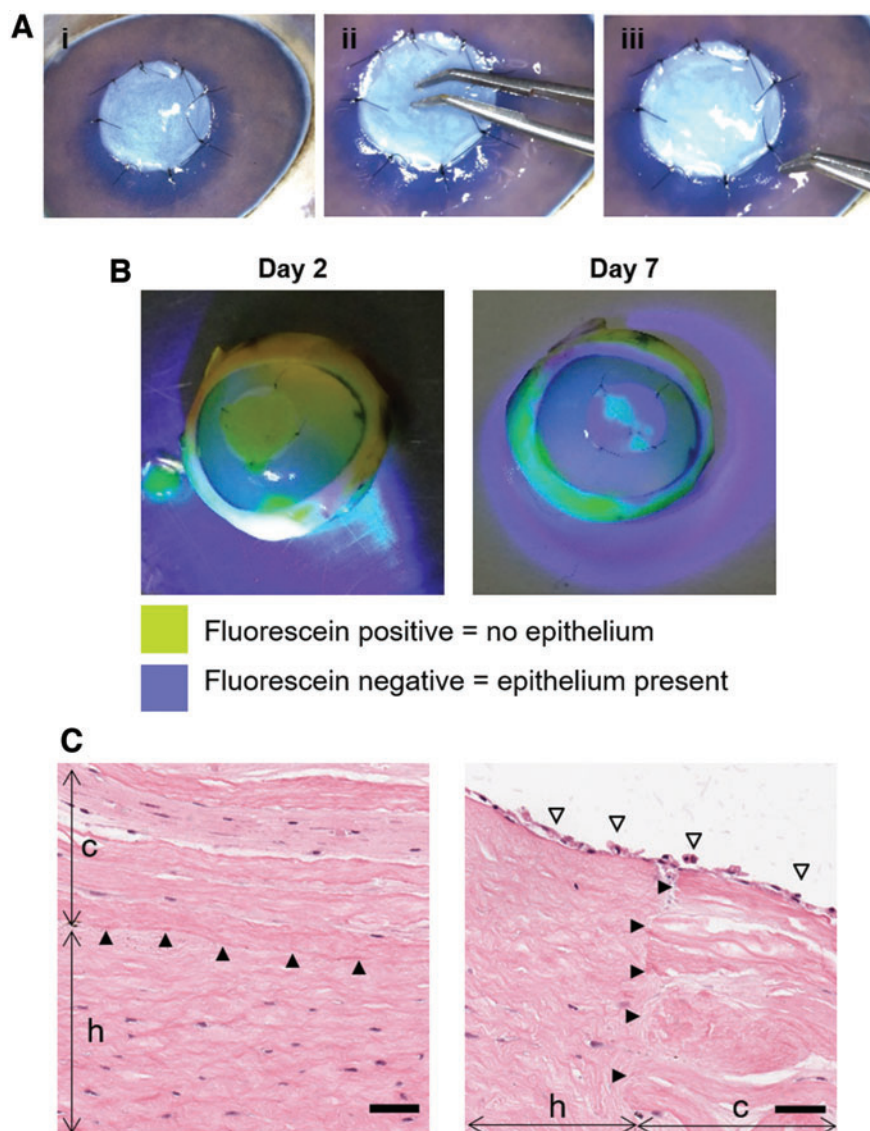
Swelling occurred during the decellularization process, which has been previously reported.<sup>14–18</sup> Once the endothe-

lium is damaged or removed, the cornea swells. Furthermore, SDS is an aliphatic molecule and it binds to proteins by its hydrophobic domain that results in increased negative charge, which attracts water and leads to swelling.<sup>19</sup> To counteract this, osmoregulators, such as dextran,<sup>13,20</sup> glycerol,<sup>20</sup> gelatin, and sodium chloride<sup>21</sup> can be used to reduce swelling. Unsurprisingly, dehydration resulted in a reduction in thickness, however, the thickness only partially recovered upon rehydration. This could be attributed to the loss of sGAG during the decellularization process that would normally attract water molecules into the tissue.<sup>22,23</sup>

In this study, the sheets were dehydrated by air drying in a laminar flow hood and did not fully recover their thickness after rehydration. This would lead to a denser collagen network than found in the native tissue.

Alternative dehydration techniques such as freeze drying or critical point drying could be used but these also introduce new limitations. While freeze drying has been previously used to obtain dried sheets of decellularized cartilage,<sup>24</sup> preliminary investigations by our group using stromal sheets showed that freeze drying did not affect the thickness but inhibited the transmission of light through the tissue. Critical point drying was not used as it could lead to degradation of the ECM following exposure to ethanol. The addition of a protecting agent to limit shrinkage was not





**FIG. 8.** Implantation of constructs without epithelium onto an *ex vivo* porcine cornea. **(A)** Surgical procedure: (i) implant sutured in place using 10-0 nylon sutures, (ii) pressing onto the implant to show strength of construct and suture points and (iii) pulling of sutures to demonstrate construct does not tear. **(B)** Fluorescein staining of operated porcine corneas after implantation and culture. **(C)** H&E analysis of implanted constructs, showing good integration of construct (c) with the host (h) (arrowheads) at the center and the edge of the construct. Epithelium (empty arrowheads) from the host has grown onto the construct (scale bar = 50  $\mu\text{m}$ ). Color images are available online.

explored in this study, but could potentially assist in controlling the sheet thickness.<sup>25</sup> While other researchers have used freshly decellularized lenticules,<sup>6,26,27</sup> in this study, dried lenticules were used because of their ease in handling, room temperature storage, and possibility of terminal sterilization, such as gamma irradiation.

We hypothesized that cell delivery through a gel would be a gentle, but effective, way to allow the inclusion of cells at all depths. Collagen I was chosen since it is the primary type of collagen found in the corneal stroma.<sup>28</sup> Our approach showed homogeneous cell distribution throughout the construct. In addition, the collagen hydrogel proved to be an excellent binding agent of the dehydrated decellularized sheets, which were attracted by the moisture of the hydrogel.

The use of the decellularized sheets provided stability to the construct, making it more robust and easier to handle than a gel alone. Hong *et al.* reported a carrier for limbal epithelial stem cells based on embedding a single sheet of decellularized human cornea in a collagen gel that was further plastically compressed.<sup>29</sup> With this approach they increased the suturability and resistance to biodegradation of the construct.

In our study, the sheets resisted degradation more than the hydrogels, giving an advantage for the strength and retention of an implant. Furthermore, when corneal stromal cells are embedded in collagen gel alone and cultured under free-floating conditions, they contract greatly.<sup>30</sup> By removing the serum from the medium, this phenomenon can be diminished,<sup>30</sup> but the present study shows that contraction can be controlled by anchoring the collagen gel to a stronger material,<sup>31</sup> in this case, the decellularized sheets. The differences in construct thickness reported during the culture period could indicate remodeling or compaction of the collagen hydrogel, conferring the constructs more mechanical stability. Moreover, additional crosslinking, such as UV-riboflavin, genipin, or transglutaminase, could further improve mechanical properties.

Layer-by-layer is an approach that has been used for stromal replacement constructs, since it aims to replicate the lamellar structure of the corneal stroma. Wilson *et al.* fabricated a stromal model by orthogonal stacking of layers of cell-seeded, aligned, electrospun PLDLA mesh, using collagen gel to bind them together.<sup>32</sup> Ghezzi *et al.* reported

constructs based on orthogonally stacked cell-seeded patterned silk films, without the aid of any binding material.<sup>33</sup> Che *et al.* have recently described the fabrication of stromal equivalents by stacking cell-seeded ultrathin amniotic membrane.<sup>34</sup> In this case, an extended culture period of 8 weeks allowed the stromal cells to produce ECM to bind the layers. The constructs reported in our present study were fabricated with materials found in the native stroma, which might present advantages for clinical translation.

Decellularized porcine corneas have been used to treat patients suffering from corneal ulcers<sup>2,3,21</sup> showing the feasibility of employing xenogeneic material. The use of discarded tissue from myopia correction procedures is an option to use human material<sup>6,27</sup> as well as donated corneas unsuitable for transplantation due to low endothelial cell count, for example.<sup>26</sup>

In the present study, the keratocytes were primarily found in the gel. It is known that *in vivo* remodeling of thin decellularized stromal tissue is faster than that of thicker tissues.<sup>7</sup> 3 weeks of culture might not have been enough time to allow the cells to repopulate the sheets by migrating from the gels. However, the reduction in construct thickness observed can be an indication of cells remodeling the collagen hydrogel. It is necessary to remark that the nonrecovery of sheet thickness after dehydration could have led to collagen fiber compaction, which might make the sheets more dense and, therefore, more difficult for the cells to penetrate. Moreover, the culture conditions used in this study have been optimized for the recovery of a keratocyte phenotype and these cells are known to migrate slowly.<sup>10</sup>

We would expect that given enough time, once the keratocytes have remodeled the collagen gel, they would migrate to the decellularized sheets as a suitable substrate. In the future, using corneal stromal stem cells (CSSCs) instead of corneal fibroblasts in serum-free conditions might improve the outcome further since CSSCs present a more keratocyte-like phenotype,<sup>35</sup> produce a more natural ECM<sup>36</sup> and possess antiscarring activity.<sup>37</sup> These cells have also been shown to support limbal epithelial stem cells in a compressed collagen I stromal model.<sup>38</sup>

Several studies have developed hydrogels as corneal substitutes, which required implantation using overlying sutures as they are not sufficiently strong to be implanted using running sutures<sup>39–42</sup> or relied on the use of fibrin glue.<sup>43</sup> Alternative strategies such as the one reported by Hong *et al.* increased suturability by embedding a decellularized human corneal lenticule in a compressed collagen gel.<sup>29</sup> These authors also report the fabrication of anterior stromal equivalents by embedding keratocytes in a plastically compressed gel, composed of a collagen I and decellularized corneal ECM mixture, with epithelial cells on the surface.<sup>44</sup> In the approach we took, stacking the decellularized sheets with the normally unsuturable collagen gels provided a construct that could be sutured with conventional interrupted 10-0 sutures.

Constructs at the time of implantation had a thickness of ~190 µm, suitable for ALK and superficial ALK. These procedures are employed to treat anterior stromal defects, such as scarring, opacification, or ulceration resulting from infection, inflammation, trauma, or inadequate healing after surgical refractive procedures.<sup>45,46</sup> Patients suffering from lattice, granular, or Reis/Bückler corneal dystrophies could also benefit from the use of these constructs.<sup>45,46</sup> If

thicker or thinner constructs would be needed, more or less layers, respectively, should be stacked when fabricating the constructs.

The optical properties of the presented constructs were suboptimal. However, studies report a recovery of transparency upon implantation, once the hydration state of the construct matches the native rather dehydrated nature.<sup>18,47</sup> Furthermore, decellularized cornea ECM-derived hydrogels could be employed in future studies since these are more transparent than rat tail collagen hydrogels.<sup>48</sup>

Decellularized stromal lenticules similar to the ones described in this study have been used to culture corneal endothelial cells.<sup>49–52</sup> While not in the scope of this study, the constructs described could also be seeded with endothelial cells to obtain a full-thickness laboratory-grown corneal substitute.

In summary, the feasibility of fabricating an anterior cornea equivalent using only tissue-derived materials was demonstrated, with good cell viability, optical properties, and cell phenotype. These were assembled in a rapid process that allowed regular and dense cell distribution, independent of cell migration. These corneal substitutes show translatability since they can be sutured and support regeneration of the epithelium in an *ex vivo* model.

#### Disclosure Statement

No competing financial interests exist.

#### Funding Information

The research is supported by funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (grant agreement no. 637460) and from Science Foundation Ireland (15/ERC/3269). Human eye tissue was kindly supplied through the Tissue Bank of the Irish Blood Transfusion Service, Dublin, after appropriate consent.

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Received: January 23, 2020

Accepted: April 29, 2020

Online Publication Date: June 25, 2020