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In vitro evaluation of electrospun blends of gelatin and PCL for application as a partial thickness corneal graft

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Abstract: The advent of innovative surgical procedures utilizing partial thickness corneal grafts has created a need for the development of synthetic implants to recreate corneal stromal tissue. This work evaluates electrospun gelatin and polycaprolactone (PCL) scaffolds as a potential biomaterial suitable for use in regeneration of corneal stromal tissue. Electrospun gelatin has been used for many years in tissue engineering; however, post-production modification, such as crosslinking, is usually required to mechanically strengthen such scaffolds. This article aims therefore to compare glutaraldehyde (GA) crosslinked electrospun gelatin scaffolds with electrospun blends of gelatin and PCL at different ratios. Scaffolds were fabricated using electrospinning and characterized by scanning electron microscopy, Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy, and tensile testing. To evaluate biocompatibility, primary human corneal stromal cells (hCSC) were seeded upon the scaffolds to assess adherence, proliferation, and phenotype. Results demonstrated that scaffolds fabricated from mixtures of gelatin and PCL showed increased mechanical strength and plasticity compared to scaffolds fabricated from GA crosslinked gelatin alone. In addition, scaffolds fabricated from PCL and gelatin showed comparable support of hCSC adhesion and proliferation. In conclusion, blended mixtures of gelatin and PCL can be considered as an option in the selection of corneal repair materials in the future. © 2018 The Authors. *Journal of Biomedical Materials Research Part A* published by Wiley Periodicals, Inc. J Biomed Mater Res Part A: 107A: 828–838, 2019.

Key Words: corneal stroma, electrospinning, keratocytes, gelatin, polycaprolactone

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INTRODUCTION

Developments in corneal surgery over the last 15 years have seen a paradigm shift in the way corneal donor tissue is used.[1] Skilled surgeons now have the option to treat corneal blindness using partial thickness corneal transplantation, a highly effective and safe alternative to full-thickness transplantation.[2] For example, Deep Anterior Lamellar Keratoplasty (DALK), is a procedure in which only the anterior portion of the cornea is removed and replaced with a prepared portion of donor corneal tissue. DALK has been shown to deliver clinical benefits relative to full thickness transplantation including fewer post-operative complications while delivering similar refractive outcomes and graft survival rates.[3] The rise of DALK is now driving the development of stromal mimetic alternatives suitable for grafting.[4]

The corneal stroma makes up ~90% of the cornea and is composed of orthogonally stacked lamellae of collagen I fibrils separated by proteoglycans and interspersed with a quiescent cell population known as keratocytes.[5,6] A challenge in developing a successful partial thickness tissue-engineered cornea is mimicking this structure which has been accomplished with some success using electrospinning. Control over electrospinning parameters can yield scaffolds with features in the micro to nano scale similar to the native extracellular matrix.[7] Electrospun scaffolds of natural biopolymers for tissue engineering applications have been successfully reported by numerous groups.[8,9] However, reports

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of protein denaturation post-electrospinning and high cost of goods have limited the translation of such constructs.[10] Gelatin is a natural biopolymer derived from the controlled hydrolysis of collagen and has seen clinical use in a number of medical areas. It has several advantages over collagen, as it can be electrospun without further denaturation, there is less potential for prion transmission and immunogenic and antigenic reactions, and is less expensive.[10,11]

While gelatin appears an ideal material for stromal reconstruction, its solubility in aqueous systems requires a level of post-production modification. Glutaraldehyde (GA) has been frequently used to crosslink structural biopolymers; however, reports of accelerated calcification of such constructs *in vivo*[12] have seen many groups seek alternative strategies for mechanically strengthening electrospun gelatin. These have included chemical crosslinking,[13] coaxial spinning,[14] and blending with less-brittle synthetic polymers.[15–17] Poly-ε-caprolactone (PCL), with its history of use in the body, has been previously used to this end.[18]

The use of GA crosslinked (CL) electrospun gelatin and gelatin/PCL electrospun blends have not previously been reported in corneal tissue engineering. This work evaluates these biomaterial systems in terms of physiochemical and mechanical properties, and ultimately their influence on the phenotype of primary human corneal stromal cells (hCSC), derived from corneal keratocytes. We anticipate that the use of such stromal constructs to be suitable for clinical situations where the endothelium remains intact yet there is a need to support stromal replacement.

MATERIALS AND METHODS

Materials

Unless otherwise specified, materials were purchased from Sigma-Aldrich, United Kingdom.

Electrospinning

Gelatin type A (300 bloom, from porcine skin) and PCL (M_w 80,000) were dissolved in 1,1,1,3,3,3 Hexafluoroisopropanol (HFIP) by stirring at 37°C overnight, to obtain polymer solutions of 8% (*w/v*). Fibrous mats were produced by electrospinning solutions of: 100% gelatin (100:0); 50% gelatin, 50% PCL (50:50); 25% gelatin, 75% PCL (25:75); and 100% PCL (0:100) in HFIP as summarized in Table I. Electrospinning was performed though a syringe fitted with a blunt 18G stainless steel needle (0.84 mm internal diameter) attached to a syringe pump (Harvard Apparatus) set to flow rates between 0.75 and 2 mL/h, at a voltage of 15–20 kV. Fibers were collected on a grounded stainless steel plate (10 × 15 cm) over a working distance of 15–20 cm. After spinning a total volume of 3.5 mL/plate, fibrous mats were detached from the collector plates and placed in a vacuum oven (Heraeus Thermo Vacuum, VT6025) for 24 h to remove residual HFIP. Glass coverslips (2D controls; 400 mm^2) were immersed in 8% (*w/v*) solutions of gelatin and PCL (same ratios as above) for 1 min and solvent removed as described above.

Crosslinking gelatin and gelatin:PCL scaffolds

Electrospun gelatin and gelatin: PCL mats (100:0, 50:50, 25:75, and 0:100) were affixed into CellCrown™ scaffold holders (Scaffdex©, Finland) and placed in a sealed tank (8 × 8 × 5 cm), suspended 3 cm above a 1% (*v/v*) GA solution for 24 h at room temperature (RT). After crosslinking, samples were immersed in 200 m*M* glycine at RT for 1 h to block any free residual GA groups.

Scanning electron microscopy including environmental SEM (ESEM)

Electrospun mats were sputter-coated with gold for 5 min at 25 mA (Leica EM SCD005, Germany) and fiber morphologies were assessed using a scanning electron microscope (SEM; JEOL SM 1100, United Kingdom). Images were acquired of three independent batches of electrospun material each in triplicate and analyzed using Image J© v1.47i to determine the mean fiber thickness (mean of 20 measurements for each replicate; 60 measurements in total per batch). Cross-sections of the mats were imaged by SEM to determine thickness (three individual batches; 6–10 measurements per batch). Morphology of fibrous mats (100:0 CL and 100:0) in the hydrated state was assessed using ESEM imaging (Philips XL30 field emission gun ESEM; 3.6 Torr).

Porosity measurements

The porosity of each scaffold was estimated using a published method.[19] In brief, scaffolds were weighed and size measured using a micrometer (Sylvac, Fowler, United Kingdom) to calculate the apparent density [Eq. (1)].

$$\text{apparent density}\left(\frac{\text{g}}{\text{cm}^3}\right)=\frac{\text{mat mass (g)}}{\text{mat thickness (cm)}\times\text{mat area (cm}^2\text{)}} \quad (1)$$

Using bulk densities obtained from the literature, [gelatin 1.3 g/cm^3 (Ref. 20 and PCL 1.14 g/cm^3 (Ref. 21)], the ratio of the apparent and bulk densities of the materials (n = 3 individual batches) was used to estimate the porosity [Eq. (2)].

$$\text{mat porosity}\,(\%)=1-\frac{\text{mat apparent density}\,(\text{g/cm}^3)}{\text{material bulk density}\,(\text{g/cm}^3)}\times 100\% \quad (2)$$

TABLE I. Electrospinning Conditions for Fabrication of Gelatin:PCL Electrospun Scaffolds

Polymer Blend (Gelatin:PCL)	Polymer Conc. (w/w) (%)	Working Distance (mm)	Working Voltage (kV)	Flow Rate (ml/h)	Spinning Volume (ml)	Spinning Time (min)
100:0	8.0	150	12.5	0.75	3.5	280
50:50	8.0	150	15	2.0	3.5	105
25:75	8.0	150	15	0.8	3.5	265
0:100	8.0	200	20	0.8	3.5	265

Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR)

Chemical analysis of the scaffolds was performed by ATR-FTIR spectroscopy (Varian 660-IR FTIR using ATR sampler accessory) over a range of 400–4000 cm^{-1}. Assessment of gelatin and PCL content was made through comparison of the ratio of integrated peak areas (PCL ester peak at 1724 cm^{-1}; gelatin amide I peak at 1652 cm^{-1}). Pseudo-Voigt peak fitting was performed using Fityk v0.9.8 software and using corrected baselines.[22]

Water contact angle

Water contact angle (WCA) was determined using a CAM200 instrument (KSV Instruments Ltd.). Droplets of ultra-pure water (2–10 μL; 18.2 MΩ resistivity at RT) were dispensed upon scaffolds secured to a microscope slide. The Young–Laplace function was used to model and fit the droplet, using two radii of curvature. Mean and standard deviation were calculated from measurements of five different samples for each scaffold or coated glass coverslip.

Mechanical strength

Moduli and elongation at break were measured at a rate of extension of 5 mm/min using an electromechanical universal tester (Instron 50 kN 3342, Canton, MA equipped with Series IX/S software). Rectangular sections of scaffold (300 × 4 mm) were mounted within a sealed acetate frame (minimum n = 3 for each treatment). Effects wof hydration and GA crosslinking were tested by immersion of scaffolds in PBS for 2 h at RT prior to testing. From the stress–strain curves generated, the tensile strength and Young's modulus were determined. The tensile strength was taken as the maximum stress and the Young's modulus was calculated from the linear portion of the stress–strain curve.

Human corneal stromal cell isolation, expansion and culture on scaffolds

Donor human corneas deemed unsuitable for transplantation were acquired from the Manchester Eye Bank following consent obtained from the donors or their relatives and anonymization. Research was approved by the local ethics research committee (07/H0403/140) and in accordance with the tenets of the Declaration of Helsinki. Three independent patient donors (n = 3; in order to take into account patient to patient variability) were used for this study. Cells were extracted from corneoscleral rims by collagenase digestion as previously described.[23] Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% (*v/v*) fetal calf serum, 0.2 m*M* L-glutamine, and 1% (*v/v*) antibiotic-antimycotic (ABAM, 10,000 units/mL penicillin, 10 mg/mL streptomycin; 25 mg/mL amphotericin B; Fibroblast [F] medium). For seeding onto scaffolds, the electrospun mats were mounted into CellCrown scaffold holders (80 mm^2) and sterilized using a 254 nm UVP XX-15W UV lamp for 20 min and soaked in 1% (*v/v*) ABAM solution for 8 h, followed by pre-soaking in F medium for 24 h. hCSC (passage 2) were seeded onto scaffolds at a density 2×10^4 cells/cm^2 in F medium. To drive hCSCs toward a keratocyte phenotype, hCSC were cultured in DMEM/F12 (Lonza, Belgium) supplemented with 1% (*v/v*) ABAM, 1 μg/mL human insulin and 1 m*M* L-Ascorbic acid-2-phosphate; Keratocyte (K) medium). Culture, expansion, and differentiation of hCSCs followed methods previously described.[23,24] Cells were expanded and used in all experiments at passages 2 and 3.

Analysis of cell-scaffold interactions

PrestoBlue® assays (Life Technologies, United Kingdom) were performed on days 1, 3, 6, and 12 according to the manufacturer's protocol to assess cell viability. In brief, scaffolds were washed with Hanks Buffered Saline Solution (HBSS) and incubated with 10% (*v/v*) PrestoBlue stock diluted in HBSS for 30 min at 37°C. Fluorescence at excitation 560 nm/emission 590 nm was measured using a fluorometric plate reader (Tecan Infinite 1000, United Kingdom). Transparency of constructs seeded with 1×10^5 cells/cm^2 hCSC was evaluated by placing over laminated sheets of text (letter A) after 12 days in culture and compared to acellular controls (images captured using an EOS 100D digital camera; Canon, Chichibu, Japan).

Reverse-transcription-quantitative polymerase chain reaction

For gene expression, hCSC were seeded on scaffolds at a density of 1×10^5 cells/cm^2 and cultured for 12 days in K medium. RNA extraction, cDNA synthesis, and qPCR were performed as previously described.[23] Quantitative PCR reactions were performed with a 2 μL cDNA volume using inventoried Taqman Assays (Applied Biosystems, Life Technologies): *GAPDH* (Hs99999905_m1), *CD34* (Hs00990732_m1), *ALDH3A1* (Hs00964880), *ACTA2* (Hs00426835_g1), and *THY1* (Hs00 17816_m1). Amplification was performed using an AB7500 quantitative PCR machine (Applied Biosystems) and analyzed using AB7500 SDS v.2.0 software. Relative expression levels were determined using the $2^{-\Delta\Delta(CT)}$ method and normalized to readings of *GAPDH.* RNA was isolated from hCSC cultured on triplicate samples of each scaffold and pooled; three separate experiments were performed to generate n = 3 data.

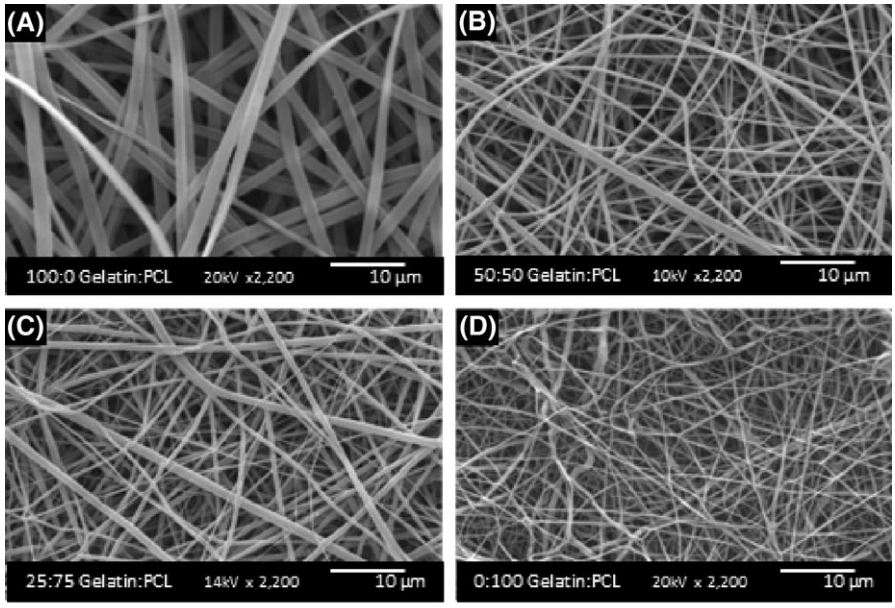
Sectioning and immunostaining of constructs

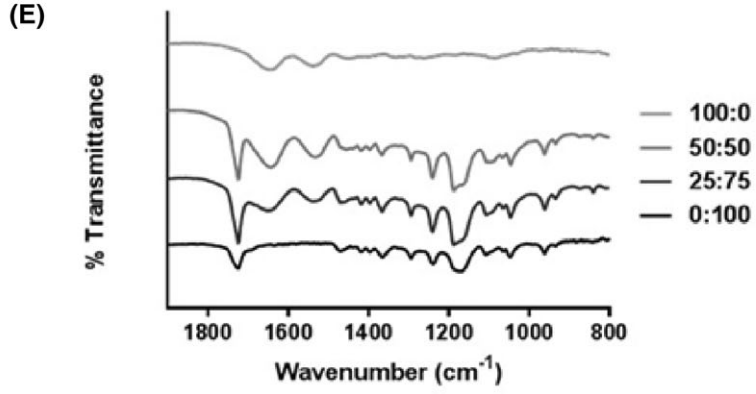
Cell-seeded scaffolds were fixed in 4% (*w/v*) paraformaldehyde at RT for 20 min. Samples were dehydrated though a series of

TABLE II. Antibodies used for Immunocytochemistry

Antibody	Supplier	Dilution
Primary Antibodies		
Polyclonal rabbit anti-*ALDH3A1*	Abcam	1:100
Monoclonal mouse anti-*α-SMA* clone 1A4	Abcam	1:200
Polyclonal rabbit anti-*CD34*	Santa Cruz	1:50
Monoclonal mouse anti-*CD90* clone AF-9	Abcam	1:200
Secondary Antibodies		
Donkey anti-mouse Alexa-Fluor 594	Life Technologies	1:300
Donkey anti-rabbit Alexa Fluor 488	Life Technologies	1:300

graded alcohol solutions in a tissue processor (Leica TP1020) and paraffin-embedded. Sections of 8 μm were cut (Leica RM 2165 microtome) and placed onto microscope slides. Samples were deparaffinized in xylene, rehydrated in a series of graded alcohol solutions, and antigen retrieval achieved in a pH 6.0 sodium citrate buffer at 95°C for 60 min. Immunofluorescent staining was performed as described previously[23] using the antibodies detailed in Table II. Samples were counterstained with 4′,6-diamidino-2-phenylindole (1:200,000), mounted in fluorescent mounting medium (Dako, United Kingdom) and imaged under an Olympus BX51 upright microscope using the associated Olympus CellˆF software.





(F)

Wavenumber	Electrospun scaffold composition			
	100:0	50:50	25:75	0:100
1652cm^{-1}	1.00	0.46	0.32	0
1724 cm^{-1}	0	0.54	0.68	1.00

FIGURE 1. Representative SEM images of gelatin:PCL electrospun scaffolds showing (A) 100:0; (B) 50:50; (C) 25:75, and (D) 0:100. ATR-FTIR spectra of the four electrospun scaffolds showing transmittance in the region of interest between 800 and 1900 cm^{-1} (D). FTIR spectra of 0:100, 25:75, and 50:50 present PCL-related stretching modes present at 1724 cm^{-1} (carbonyl stretching), 1293 $^{-1}$ (C—O and C—C stretching), and 1240 cm^{-1} (asymmetric C—O—C stretching). Spectra of 100:0, 50:50, and 25:75 present protein related bands at 1652 cm^{-1} (amide I) and 1542 cm^{-1} (amide II). A comparison of the peak area ratio of PCL specific peak at 1724 cm^{-1} and gelatin specific peak at 1652 cm^{-1} in the ATR-FTIR spectra of electrospun scaffolds of varying compositions (F).

TABLE III. Characterization of Scaffolds, Describing fiber Diameter, Scaffold Thickness, Porosity, and Water Contact Angle (WCA).

Polymer Blend (Gelatin:PCL)	Fiber Thickness (μm)	Scaffold Thickness (μm)	Porosity (%)	WCA (°)
100:0	2.20 ± 1.02	75.03 ± 16.06	65.57 ± 12.74	40.30 ± 5.46
50:50	0.89 ± 0.58	73.62 ± 10.05	73.28 ± 19.23	55.51 ± 19.38
25:75	0.63 ± 0.22	76.00 ± 12.21	63.94 ± 16.89	44.47 ± 8.92
0:100	0.62 ± 0.47	62.15 ± 22.44	88.75 ± 3.31	85.37 ± 6.87

All measurements are represented by mean ± SD ($n = 3$).

Statistical analysis

Results are expressed as mean ± SEM unless otherwise described. Statistical significance for material characterization studies was estimated by one-way ANOVA tests in GraphPad Prism v6.01 followed by Tukey *post hoc* test to compare groups. Cell data were analyzed by two-way ANOVA followed by a Bonferroni *post hoc* test. Levels of significance were expressed as p values versus the control ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

RESULTS

Fibrous scaffolds composed of gelatin and PCL were fabricated

SEM images showed gelatin scaffolds (100:0) were composed of ribbon-like fibers [Fig. 1(A)], distinct from the rounded fibers observed for PCL (0:100) scaffolds [Fig. 1(D)]. Both 50:50 and 25:75 scaffolds showed signs of ribboning within the matrix [Fig. 1(B,C)]. Characterization of scaffolds in terms of fiber and scaffold thickness, porosity and WCA are presented in Table III.

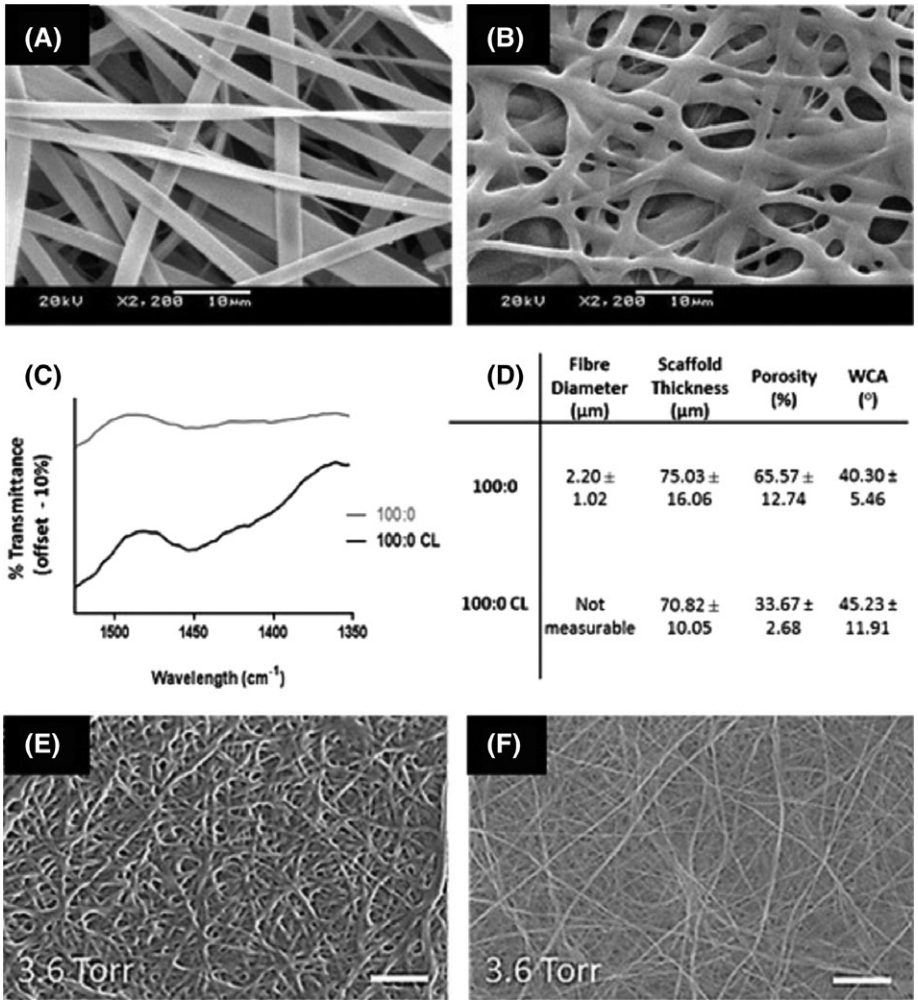


FIGURE 2. Characterization of GA crosslinked (CL) 100:0 (Gelatin:PCL) electrospun scaffolds. (A) Representative SEM images of 100:0 (Gelatin:PCL) electrospun scaffold (prior to crosslinking); (B) Representative SEM image of GA CL 100:0 (Gelatin:PCL) scaffold; (C) FTIR trace of 100:0 and 100:0 CL (Gelatin:PCL) scaffolds showing characteristic aldimine peak at 1450 cm^{-1}. (D) Comparison of physical properties of GA CL 100:0 and 100:0 (Gelatin:PCL) scaffolds. All measurements represented as mean ± SD ($n = 3$). Representative environmental scanning electron microscopy ESEM images of electrospun scaffolds (E) GA CL 100:0 (Gelatin:PCL) scaffold and (F) 0:100 (Gelatin:PCL) scaffold. Scale bar 20 μm.

Fiber thickness was notably larger in the 100:0 scaffolds (2.20 ± 1.02 μm), relative to the other scaffolds containing PCL (0.89 ± 0.58 to 0.62 ± 0.47 μm). Scaffold thicknesses for all formulations were relatively consistent (62–76 μm).

WCA of the electrospun scaffolds showed a marked difference between scaffolds made from the pure materials (100:0–40.3° ± 5.46 and 0:100–85.4° ± 6.9). However, no relationship between material composition and WCA was observed in scaffolds generated from blends of the two materials (50:50 55.5° ± 19.4 and 25:75–44.5° ± 8.9). In assessing the porosity of the scaffolds, no relationship was observed between material composition and porosity (100:0–65.6%; 50:50 73.3%; 25:75–63.4%; 0:100–88.75% porosity).

ATR-FTIR was used to confirm the chemical composition of the scaffolds. Figure 1(E) displays the key region of interest presenting the percentage transmittance for each scaffold offset by 10%. Gelatin related amide bands at 1652 cm^{-1} (amide I) and 1542 cm^{-1} (amide II) were observed in all gelatin containing scaffolds (100:0, 50:50, and 25:75). PCL-related ester bands were confirmed in 0:100, 25:75, and 50:50 scaffolds at 1724 cm^{-1} (carbonyl stretching), 1293 cm^{-1} (C—O and C—C stretching), and 1240 cm^{-1} (asymmetric C—O—C stretching). To assess the ratio of ester to amide, FTIR peaks at 1724 and 1652 cm^{-1} were integrated [Fig. 1(F)]. The 50:50 and 25:75 scaffolds were observed to have distinctly different ratios of the two bands (50:50–0.46; 25:75–0.32). To enhance the mechanical properties (and prevent dissolution of the gelatin in later cell culture studies) of 100:0 gelatin:PCL scaffolds, samples were CL over GA vapor for 16 h. SEM images of the 100:0 scaffold before [Fig. 2(A)] and after crosslinking [Fig. 2(B)], show a distinct change in the fibrous morphology of the scaffold and as a consequence, fiber diameters in the CL scaffold were not measurable. ATR-FTIR analysis of the scaffolds before and after GA crosslinking shows an increase in transmittance percentage at 1450 cm^{-1} corresponding to an aldimine group [Fig. 2(C)]. After crosslinking, the scaffolds became notably less porous (100:0–65.6%; 100:0 CL–33.7%); however, there was little difference observed between scaffold thickness (100:0–75.03 μm ± 16.1; 100:0 CL–70.82 μm ± 10.05) [Fig. 2(D)]. ESEM images were acquired of both CL gelatin (100:0 CL) and PCL (0:100) scaffolds in a partially hydrated state [Fig. 2(E,F)] confirming a fibrous structure.

Hydration influences the mechanical properties of the scaffolds

Tensile testing of scaffolds with four different ratios of gelatin:PCL, before and after crosslinking for all samples and following hydration is shown in Figure 3. In the dry state, the tensile modulus (E) decreased with increasing PCL content ranging from 136 MPa ± 61 (100:0) down to 15 MPa ± 5 (0:100) [Fig. 3(A)]. In the hydrated state, this

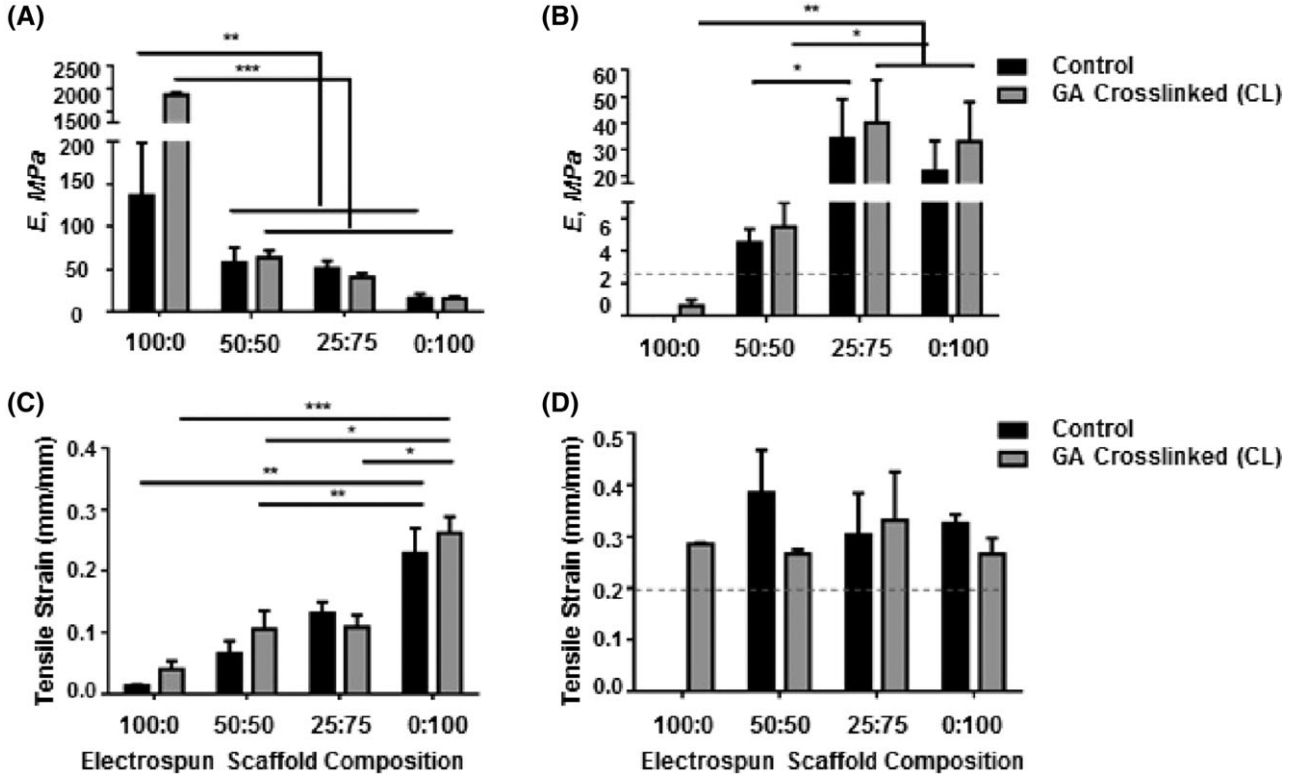


FIGURE 3. Mechanical testing of electrospun scaffolds. Young's Modulus (A,B) and elongation at break (C & D) of scaffolds before (control) and after glutaraldehyde (GA) crosslinking (CL), both in dry (A,C) and hydrated states (B,D). Data presented as mean ± SEM, $n = 4$. (— denotes literature values for the lower limit of full thickness corneal tissue.[25] $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

trend reversed and the modulus increased with increasing PCL content [Fig. 3(B)]. The 0:100 and 25:75 scaffolds recorded similar moduli in both wet and dry states (*Dry* 25:75–40.6 MPa ± 15.2; 0:100–15.5 MPa ± 2.3; *Wet* 25:75–39 MPa ± 8; 0:100–33 MPa ± 7. The 50:50 and 100:0 formulations exhibited significantly lower moduli when hydrated, with 100:0 scaffolds possessing a modulus of 136 MPa ± 61 while dry, but when wet the material was too fragile to measure. The moduli of the 50:50 scaffolds changed from an average of 57 MPa ± 18 while dry to 4 MPa ± 0.2 once hydrated.

Crosslinking of the 100:0 scaffold resulted in the increase of the Young's modulus significantly in the dry testing regime [Figure 3(A)], with increasing modulus from 136 MPa ± 6 to 1858 MPa ± 47 (CL). Non-CL100:0 scaffolds in the hydrated state could not be tested due to a loss of integrity of the scaffold after hydration [Figure 3(B)]. Crosslinking had little effect on the mechanical properties of the 50:50, 75:25, and 0:100 scaffolds in both dry and hydrated states. When compared to full thickness cornea tensile testing (3–13 MPa[25]), 50:50 scaffolds appeared to most closely fit the observed range with a modulus of 5.5 MPa ± 0.8. Elongation at break in the dry testing regime increased with increasing PCL content ranging from 0.014 ± 0.003 mm/mm (100:0) up to 0.229 ± 0.100 mm/mm [0:100; Figure 3(C)]. PCL 0:100 scaffolds were able to reach over 20% extension before failure, irrespective of hydration [Figure 3(C,D)]. Elongation at break of gelatin containing scaffolds was seen to increase with hydration; 0.066 ± 0.044 mm/mm when dry (50:50) to 0.386 ± 0.082 mm/mm when hydrated [Figure 3(D)].

Fibrous scaffolds were biocompatible for hC*S*C with PCL scaffolds containing gelatin supporting a quiescent keratocyte phenotype

The biocompatibility of 100:0 CL scaffolds along with 50:50; 25:75; and 0:100 scaffolds were compared in terms of hCSC adhesion and proliferation on the scaffolds. Over a 5 h period, significantly more hCSC adhered to 100:0 CL scaffolds than the 0:100 scaffolds observed through a difference in relative fluorescence of 514 ± 51 Relative Fluorescence Units (RFU) for cells cultured on 100:0 CL relative to 277 ± 37 for cells on 0:100 scaffolds [PrestoBlue; Figure 4 (A)]. However, there were no significant differences between hCSC adherence to 100:0 CL, 50:50 or 25:75 scaffolds [Figure 4(A)]. hCSC were able to proliferate over a 12 day

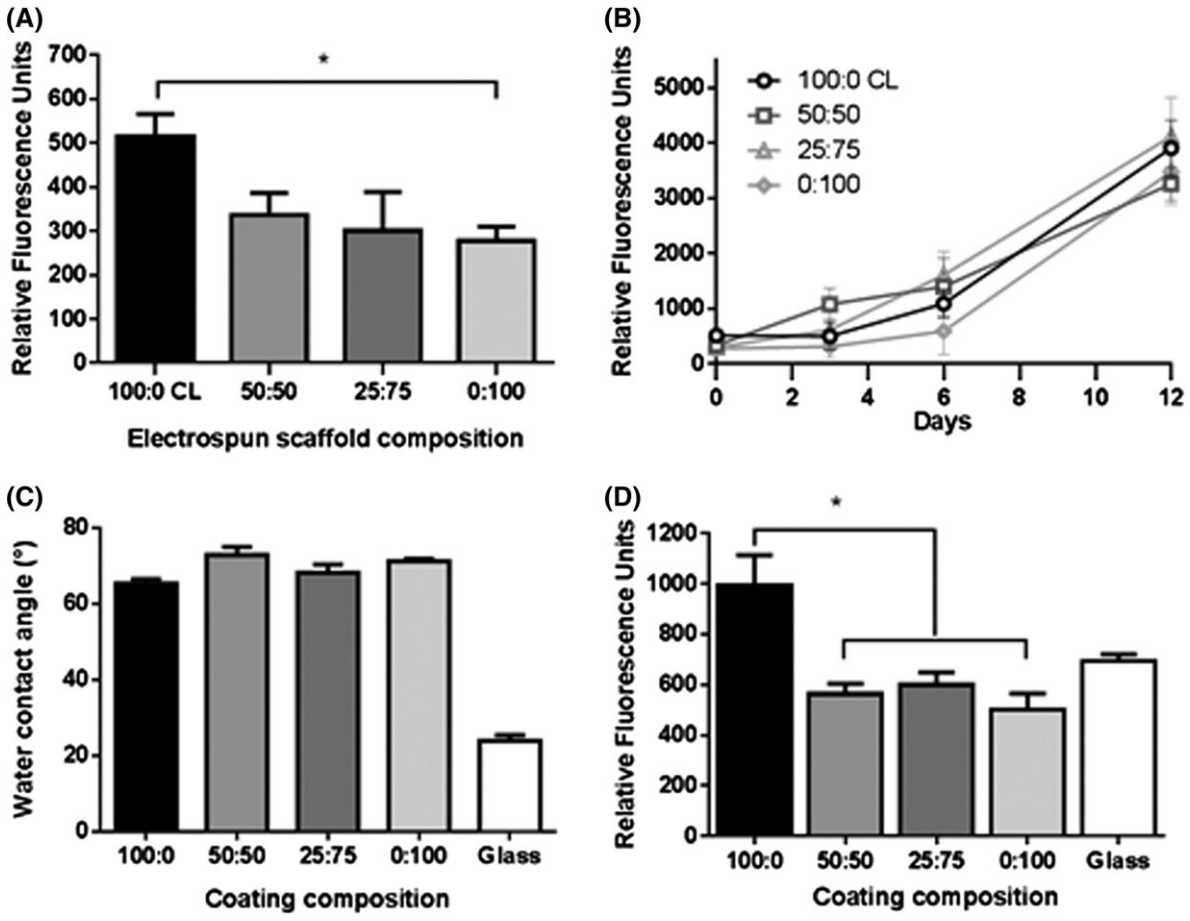


FIGURE 4. (A) hCSC adhesion to electrospun scaffolds 5 h post-seeding as measured by the PrestoBlue assay. Values represented by mean ± SEM, $n = 4$, $^*p < 0.05$; (B) hCSC proliferation on electrospun membranes over 12 days. Values represented by mean ± SEM, $n = 2$ ($n = 2$; triplicate scaffolds in each experiment); (C) Water contact angle of gelatin and PCL surfaces after dip coating procedure. Mean ± SEM, $n = 5$; (D) Adhesion of hCSC on coated glass coverslips after 5 h. Mean ± SEM, ($n = 3$; triplicate scaffolds in each experiment), $^*p < 0.05$.

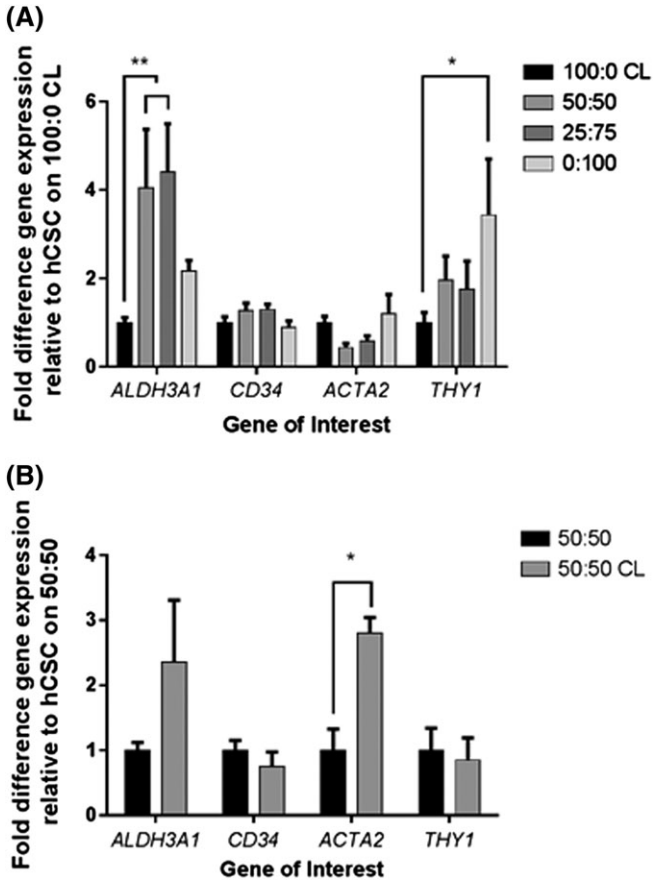


FIGURE 5. hCSC gene expression in response to electrospun scaffolds measured by qPCR. (A) hCSC gene expression after 12 days culture upon four electrospun scaffolds in K media. All data normalized to GAPDH and displayed relative to 100:0 CL. Data represented by mean relative quantitation ± SEM, (n = 3; triplicate samples); (B) hCSC gene expression after 12 days culture upon 50:50 scaffolds with and without GA crosslinking. All data normalized to GAPDH and displayed relative to 50:50. Data represented by mean relative expression ± SEM, (n = 3) triplicate samples.*$p < 0.05$, **$p < 0.01$.

period on all four electrospun scaffolds at similar rates [Figure 4(B)].

Assessment of the effects of surface chemistry on adhesion, without potential differences in topography, was performed using glass coverslips dip-coated in HFIP solutions of each of the formulations [confirmed by WCA measurements; Figure 4(C)]. A similar hCSC adhesion experiment was performed [Figure 4(D)] and similar trends were observed on the coated glass coverslips as observed on the fibrous scaffolds. A difference in relative fluorescence determined in the PrestoBlue assay of 994 ± 103 for cells cultured on 100:0 CL coated glass coverslips was observed relative to 501 ± 54 for cells on 0:100 coated glass coverslips. However, in contrast to the data generated with the fibrous scaffolds, the 100:0 CL coated glass also supported significantly higher hCSC adhesion than 50:50 or 25:75 coated glass.

qPCR was used to assess gene expression of two markers of the quiescent (non-scarring) keratocyte phenotype, ALDH3A1 and CD34, and two markers of the activated (scarring) fibroblast phenotype, *ACTA2* (α-SMA) and *THY1* (CD90). Gene expression data were compared to cells cultured on the gelatin scaffold rather than the native cornea stromal tissue as the cells had been cultured *in vitro* prior to these experiments. Overall relative gene expression responses to the electrospun substrates were small with a fourfold increase being the greatest response observed in all experiments [Figure 5 (A)]. Statistically significant increases in *ALDH* expression were observed in hCSC cultured on the both 50:50 and 25:75 scaffolds relative to 100:0 CL. hCSC cultured on 0:100 scaffolds expressed significantly more *THY1* than those on 100:0 CL. To assess the direct effect of crosslinking on the phenotype of hCSC, cells were cultured on 50:50 scaffolds with and without crosslinking and phenotype was assessed [Figure 5(B)].

Scaffolds 50:50 and 50:50 CL differed little in terms of *CD34* and *THY1* expression in this experiment. *ACTA2* expression was the largest and only statistically significant difference recorded which was increased when cells were cultured on CL samples [Figure 5(B)]. This phenomenon was explored further though assessing protein expression by immunofluorescence (Figure 6). Cells were clearly situated in a layer on top of the scaffolds and hCSC cultured on 50:50 scaffolds which had not been CL were seen to express higher levels of CD34 and CD90. *α-SMA* appears to be expressed to a higher degree by hCSC cells cultured on 50:50 CL scaffolds. Macroscopic images captured after 12 days culture with or without hCSCs in K medium showed that 100:0 CL scaffolds were more transparent than any PCL containing scaffold (Figure 7). No differences were observed between the other scaffold types with or without hCSC.

DISCUSSION

A global shortage of corneal donor tissue, coupled with increasing levels of adoption of DALK has resulted in a need for partial thickness corneal tissue grafts.[1] The development of tissue-engineered stromal replacements hold promise to supplement the donor tissue pool in addition to producing standardized constructs. We anticipate that the use of such stromal constructs to be suitable for DALK procedures used in clinical situations where the endothelium remains intact yet there is a need to support stromal replacement. Such constructs would allow the surgeon flexibility to replace the stroma layer by layer depending on the depth of the injury. Re-epithelialization would then occur from the patient's own limbal stem cell reserve or in combination with limbal stem cell transplantation.

Our approach proposes the use of polymeric platforms, produced by electrospinning, as biomimetic constructs for corneal tissue regeneration. More specifically, this article compares the attributes of gelatin and PCL electrospun blends relative to chemically CL gelatin as a novel way to produce suitable candidates for stromal replacement. This work represents the first time an electrospun composite of synthetic and natural polymers has been evaluated as a candidate material for corneal stromal tissue engineering.

Gelatin was chosen on account of its bioactivity, biodegradability, non-antigenicity, current use in clinical applications, stability during electrospinning, and commercial availability at

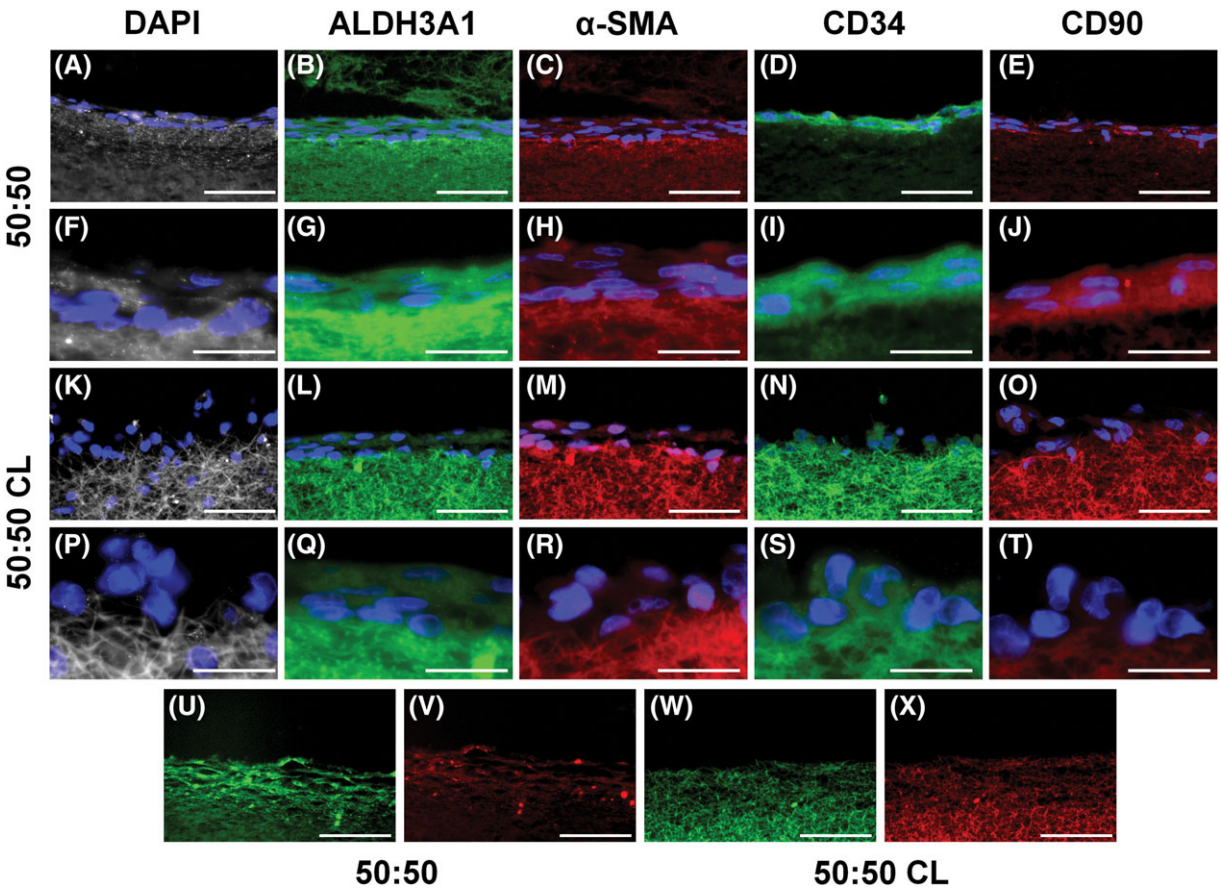


FIGURE 6. Immunofluorescent staining of hCSC cultured on (A–J) 50:50 (G50) and (K–T) 50:50 CL (G50 CL) scaffolds for 12 days in K media. Scaffolds were fixed with paraformaldehyde, wax embedded and sectioned at 8 μm. Sections were stained for either (B, G, L, Q) ALDH3A1; (C, H, M, R) α-SMA; (D, I, N, S) CD34; and (E, J, O, T) CD90; (A, F, K, P). Unstained scaffolds, imaged by auto-fluorescence. All sections counterstained with DAPI. Auto-fluorescence of unstained scaffolds (seeded with cells but not subjected to immunostaining) (U, V—50:50; W, X—50:50 CL) under (U, W) FITC filter; (V, X) TRITC filter. (A–E; K–O; U–X) 400× magnification (scale bar: 50 μm); (F–J; P–T) ×600 magnification (scale bar: 20 μm).

low cost.[10,11] PCL was chosen as an alternative to GA crosslinking to provide strength to gelatin-based scaffolds as it is biocompatible, biodegradable and has been extensively explored for applications in tissue engineering.[18] The longer degradation time of PCL (anticipated to be 6–12 months based on our knowledge of PCL-based scaffolds) was attractive for the production of fibrous materials to contrast our experience with other polymers, such as poly(lactic-co-glycolic acid) (PLGA), which rapidly lose integrity when wet. The lack of transparency of PCL was not considered an issue for clinical applications as polymer degradation and tissue remodeling would allow for restoration of sight; we show that the scaffolds are opaque and this would be sufficient for perception of light/dark following surgery. We therefore wanted to explore the novel application of PCL-gelatin blended scaffolds in ophthalmology.

In optimized electrospinning conditions, gelatin produced ribbon-like fibers upon deposition in line with previous reports,[17,26] whereas rounded fibers were achieved in all PCL containing scaffolds.[27,28] Fiber diameter of gelatin-based scaffolds was seen to decrease and the WCA increased through introducing PCL. Other investigations of gelatin:PCL blends have not reported such fiber diameter trends although the WCA data agrees with other work.[27–29] All these studies, including our findings reported here, produced fibers an order of magnitude greater than the collagen fibrils found *in vivo* (31–34 nm) but are similar in diameter to lamellae structures, which are ~1 to 2 μm thick.[6,30] The distinct material compositions of the different gelatin:PCL blends were successfully validated though ATR-FTIR. The presence of both amide and ester bands in the 25:75 and 50:50 gelatin:PCL scaffolds confirmed that both materials were present at the surface of the scaffolds, as described in the literature.[30,31]

The high solubility of gelatin in aqueous solutions meant that crosslinking was required. Crosslinking of 100:0 gelatin scaffolds with GA resulted in the webbing of individual fibers resulting in a significant drop in the porosity of the scaffold. Uniaxial tensile testing showed that GA crosslinking of 100:0 scaffolds significantly increased the modulus and elongation at break. The effect of GA treatment on the 50:50 and 25:75 scaffolds was not significant, suggesting that the mechanical properties of PCL dominate in this system. All scaffolds in the hydrated state were capable of withstanding the same level of strain before failure. The Young's modulus of the healthy human cornea has been previously reported to be about 3 MPa,[25,32] which corresponds to the measured

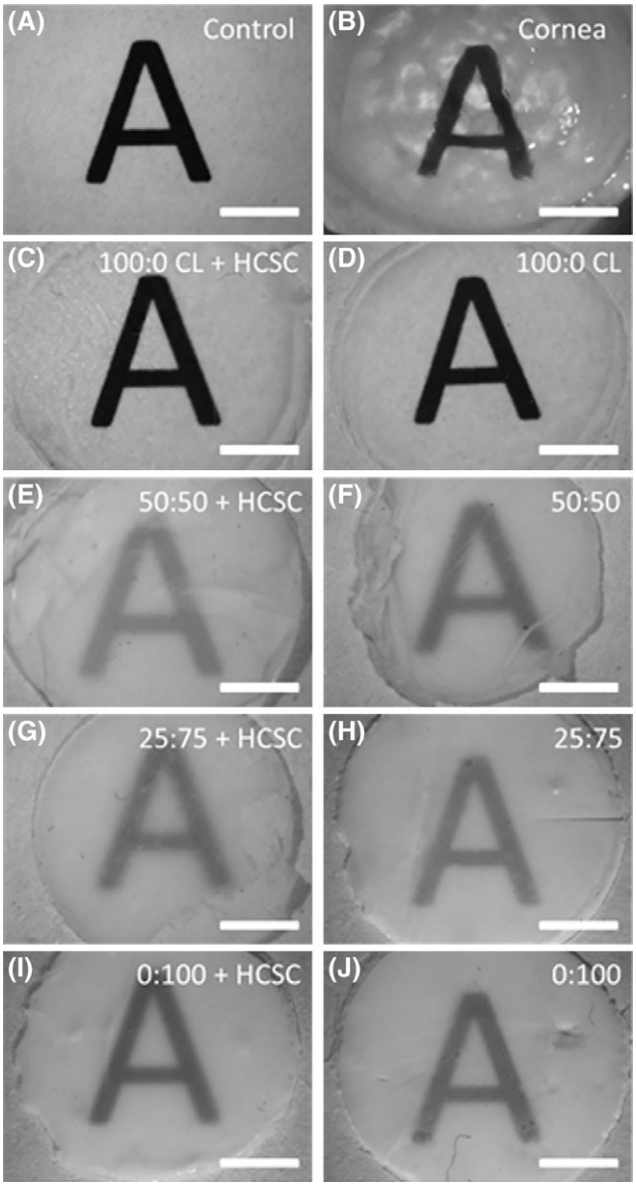


FIGURE 7. Macroscopic images of electrospun scaffolds. (A) Plain letter A covered with acetate sheet; (B) Donor cornea unfit for human transplantation; (C,D) 100:0 CL; (E,F) 50:50; (G,H) 25:75; (I,J) 0:100 electrospun scaffolds after 12 days culture (C, E, G, I) seeded with hCSC and (D, F, H, J) without hCSC. Scale bar is 3 mm.

moduli of the hydrated 50:50 and 100:0 CL scaffolds. As the concentration of PCL increased, the tensile moduli of the scaffolds decreased as seen in other studies where gelatin is included in the blend.[16,17,31] In the hydrated state, this trend was seen to reverse. The moduli of the gelatin scaffolds in both 100:0 CL and 50:50 compositions decreased upon hydration, when brittle fibers swelled to become hydrogel-like.[26] Mechanical testing of gelatin-based electrospun scaffolds in the hydrated state has not been previously reported. The degradation of the scaffolds produced was not studied here (although they were visually intact for up to 21 days) and would be a next step in further evaluating these scaffolds for clinical applications.

For application in corneal stroma repair, it was important to evaluate the biocompatibility of the scaffolds with hCSC. The cells adhered to all scaffolds of various compositions with cell adhesion highest on the gelatin-based scaffolds suggesting that there was insufficient, if any, GA remaining to be detrimental to the cells. Experiments on 2D controls of the same formulations demonstrated that chemistry was the dominant effect on cell adhesion in these studies (rather than fiber morphology). *ALDH3A1*[33,34] and CD34[35–37] are important markers of keratocytes *in vivo* alongside keratocan expression.[38,39] The expression of CD90 (gene name *THY1*) and α-SMA (gene name *ACTA2*) are characteristic of a less desirable myofibroblastic phenotype that leads to scarring.[38] Our data suggested that 50:50 and 25:75 scaffolds offer a substrate more supportive of the keratocyte phenotype, with decreased *α-SMA* and increased CD34 and *ALDH3A1* expression. Previous work published by our laboratories have compared similar isolated cells cultured *in vitro* to those resident in the native cornea. Compared to the native cornea, ALDH3A1, CD34, and ACTA2 were significantly downregulated and THY1 was significantly upregulated.[38] This suggests that when cultured *in vitro*, the whole population of cells do not differentiate to the keratocyte phenotype warranting further optimization of scaffold morphology and *in vitro* culture conditions. In addition, comparison of the expression of these markers from *in vitro* cultured cells with those expressed *in vivo* is further required.

In considering clinical therapies for the cornea, the refractive index of the scaffolds is an important factor.[31,40] Gelatin 100:0 CL scaffolds demonstrated a higher transmittance of visible light and this increased following culture with hCSC suggesting remodeling of the extracellular matrix. Longer culture studies are required to assess matrix remodeling and degradation in the gelatin-PCL scaffolds in relation to transparency. Further optimization of the fiber diameter to allow penetration of the cells throughout the scaffold, while supporting the quiescent (non-scarring) cell phenotype, would also be a focus of future work. Future *in vivo* studies would also be required to ascertain the ability of these scaffolds to support the quiescent (non-scarring) keratocyte phenotype in the longer term and ultimately the restoration of visual acuity. In conclusion, we have demonstrated for the first time that hCSC cultured on blended gelatin-PCL scaffolds adopt a therapeutically desirable quiescent phenotype illustrating their suitability as future corneal repair materials.

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