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3 **Poly- ϵ -lysine based hydrogels as synthetic substrates for the expansion of corneal endothelial cells**
4 **for transplantation**

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11 Short running title: **Corneal endothelial cells on pEK hydrogels**

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1 **Abstract**

2 Dysfunction of the corneal endothelium (CE) resulting from progressive cell loss leads to corneal
3 oedema and significant visual impairment. Current treatments rely upon donor allogeneic tissue to
4 replace the damaged CE. A donor cornea shortage necessitates the development of biomaterials,
5 enabling *in vitro* expansion of corneal endothelial cells (CECs). This study investigated the use of a
6 synthetic peptide hydrogel using poly- ϵ -lysine (p ϵ K), cross-linked with octanedioic-acid as a potential
7 substrate for CECs expansion and CE grafts. P ϵ K hydrogel properties were optimised to produce a
8 substrate which was thin, transparent, porous and robust. A human corneal endothelial cell line
9 (HCEC-12) attached and grew on p ϵ K hydrogels as confluent monolayers after 7 days, whereas primary
10 porcine CECs (pCECs) detached from the p ϵ K hydrogel. Pre-adsorption of collagen I, collagen IV and
11 fibronectin to the p ϵ K hydrogel increased pCEC adhesion at 24 hours and confluent monolayers
12 formed at 7 days. Minimal cell adhesion was observed with pre-adsorbed laminin, chondroitin
13 sulphate or commercial FNC coating mix (fibronectin, collagen and albumin). Functionalisation of the
14 p ϵ K hydrogel with synthetic cell binding peptide H-Gly-Gly-Arg-Gly-Asp-Gly-Gly-OH (RGD) or α 2 β 1
15 integrin recognition sequence H-Asp-Gly-Glu-Ala-OH (DGEA) resulted in enhanced pCEC adhesion with
16 the RGD peptide only. pCECs grown in culture at 5 weeks on RGD p ϵ K hydrogels showed zonula
17 occludins 1 staining for tight junctions and expression of sodium-potassium adenosine triphosphase,
18 suggesting a functional CE. These results demonstrate the p ϵ K hydrogel can be tailored through
19 covalent binding of RGD to provide a surface for CEC attachment and growth. Thus, providing a
20 synthetic substrate with a therapeutic application for the expansion of allogenic CECs and replacement
21 of damaged CE.

22 **Key words:**

23 Corneal endothelium, transplantation, poly- ϵ -lysine, hydrogel, peptide functionalisation

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1 1. Introduction

2 The corneal endothelium (CE) is the inner most layer of the cornea and is composed of a single
3 monolayer of tightly packed, non-replicative endothelial cells on a thickened basement membrane
4 (Descemet's membrane (DM)). The primary role of the CE is to maintain the transparency of the
5 cornea by regulating its hydration through a leaky barrier and active sodium-potassium adenosine
6 triphosphase ($\text{Na}^+\text{K}^+\text{ATPase}$) pumps present on the membrane of corneal endothelial cells (CECs).[1,
7 2] If CECs are lost, the remaining cells migrate and enlarge to ensure adequate cell coverage to
8 maintain corneal transparency, however, there is a critical number of CECs required to maintain
9 adequate pump function (>500 cells/ mm^2).[3] Acute cell loss due to age, disease (such as Fuchs
10 endothelial corneal dystrophy (FECD)), degenerative changes (bullous keratopathy) and other causes
11 including infection, and physical or surgical trauma can eventually result in corneal oedema and
12 decreased visual acuity.[4]

13 Currently the only therapeutic treatment for corneal endothelial dysfunction is corneal
14 transplantation using donor tissue. This treatment involves the replacement of the CE with donor CECs
15 on their native DM, using most commonly, partial thickness grafts such as Descemet's stripping
16 automated endothelial keratoplasty (DSAEK) or Descemet's membrane endothelial keratoplasty
17 (DMEK). These procedures are not without complications as there is the risk of graft failure (due to
18 rejection or gradual cell loss)[5, 6] and graft survival rate is only 70% at 5 years.[7] At present the ratio
19 of donor tissue to recipient is 1:1 and there is a global shortage of corneas for transplantation,
20 therefore, alternative therapeutic methods using expanded CECs are being developed as they offer
21 the advantage of production of several endothelial grafts from one donor to treat multiple
22 recipients.[5, 8]

23 CECs possess limited replicative capacity but *in vitro* expansion is possible, while still maintaining
24 phenotype and function.[9, 10] Currently, there are two potential modes of delivery of cultured CECs;
25 direct cell injection into the anterior chamber or transplantation of an engineered graft comprising a
26 cell monolayer on a carrier/scaffold.[11-14] Preclinical studies have shown conflicting functional
27 outcomes using injected cells, [11, 15-18] however, a clinical trial of 11 patients with bullous
28 keratopathy did show injected CECs supplemented with Rho kinase (ROCK) inhibitor Y-27632
29 increased the density of CECs.[19] A recent publication directly comparing injected CECs with a tissue
30 engineered graft of CECs in a rabbit model highlighted an important point.[20] When CECs were
31 injected into the eye of a rabbit with DM removed, the CECs failed to improve corneal transparency
32 or decrease corneal thickness and were later found to have failed to attach and form a monolayer. In
33 FECD vision is adversely affected by deposition of focal excrescences, known as guttae, which are

1 present in the central DM. The DM must be removed before delivery of an endothelial graft meaning
2 injected cell therapy will not be suitable for these patients or late stage bullous keratopathy patients
3 with DM scarring. In these cases there is clearly still a requirement for a tissue engineered graft that
4 can also fulfil the role of the DM.

5 Previous studies have demonstrated that CECs can be expanded onto biological substrates, such as
6 amniotic membrane,[21] decellularised endothelium/stroma and lens capsule.[22-25] CECs grew as
7 cell monolayers on these substrates and displayed a uniform shape and size, with cell to cell contacts
8 and expressing typical CEC markers zonula occludens-1 (ZO1), Na⁺/K⁺ATPase and connexin 43.
9 Substrates derived from natural polymers (collagen membranes [23, 24] and sponges [26],
10 compressed collagen I [27, 28], silk fibroin [29] or synthetic polymers (poly(caprolactone)) or a
11 combination of both (chitosan and poly(caprolactone) [30, 31]) have been used as materials to mimic
12 the biological and mechanical properties of DM for culturing CECs. CECs proliferated on these
13 substrates and maintained their typical morphology and phenotype. Natural polymers used as coating
14 on culture dishes, such as collagen I [32] and collagen IV, fibronectin, gelatin, laminin and chondroitin
15 sulphate have also been shown to be beneficial in promoting a cobblestone endothelial-like
16 morphology.[33-37]

17 The properties of synthetic substrates can be tightly controlled to produce a customised material with
18 desired mechanical properties and surface functionalisation to increase e.g. cell adhesion. Synthetic
19 hydrogels are an attractive option for CE replacement as they would allow the diffusion of water and
20 biomolecules into the stroma. Our previous studies have shown that poly-ε-lysine (pεK) cross-linked
21 with octanedioic-acid produces hydrogels that are transparent, are non-toxic to corneal epithelial cells
22 *in vitro* and can be cast into contact lens moulds.[38, 39] In addition, following crosslinking of pεK
23 hydrogels, free amine sites remain for either electrostatic or covalent binding of drugs, proteins or
24 peptides to the pεK hydrogel. The adaptability of this synthetic material means the pεK hydrogel
25 properties, such as stiffness and amine functionality, can be tailored to facilitate production of a stable
26 endothelial monolayer.

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28 The current study aimed to develop the synthetic pεK hydrogel as a substrate for the expansion of
29 CECs. The objectives were to manufacture a thin pεK hydrogel film with optimum transparency and
30 mechanical properties, which maintained sufficient free amines to allow binding of biomolecules (such
31 as extracellular matrix (ECM) proteins and synthetic cell binding peptides (H-Gly-Gly-Arg-Gly-Asp-Gly-
32 Gly-OH (RGD) and H-Asp-Gly-Glu-Ala-OH (DGEA)), to enable production of a confluent endothelial cell
33 monolayer that maintained the characteristic CEC phenotype. We investigated the attachment and
34 growth of both a human corneal endothelial cell line (HCEC-12) and primary porcine endothelial cells

1 (pCEC) onto these pEK hydrogels and the effects of protein and peptide functionalisation of the pEK
2 hydrogels. The results demonstrate, for the first time, that pEK hydrogels tailored to promote cell
3 attachment can be used for the expansion of CECs, which displayed a characteristic phenotype in long
4 term culture.

5

6 **2. Materials and Methods**

7 ***2.1. pEK hydrogel synthesis and material testing***

8 The pEK hydrogel was synthesised from pEK (Bainafco- Zhengzhou Bainafco Bioengineering Co. Ltd)
9 cross-linked to 60% with octanedioic-acid (Sigma Aldrich, Dorset, UK) to a polymer density of 0.066
10 g/ml using N-hydroxysulfosuccinimide (NHS, CarboSynth Ltd, Berkshire, UK) and 1-ethyl-3-(3-
11 dimethylaminopropyl) carbodiimide (EDCI, CarboSynth Ltd, Berkshire, UK). Specifically, pEK (0.692 g)
12 was dissolved with dH₂O (2 ml) in a 15 ml polypropylene universal (Starlab Ltd., Blakelands, UK).
13 Octanedioic acid (0.210 g) was dissolved in dH₂O (1 ml) with N-Methylmorpholine (NMM, Sigma, UK;
14 0.442 ml). This was placed on a roller until dissolved and added to the pEK solution. An aliquot of 5%
15 Tween 20 (0.1 ml) was added. NHS (0.232 g) was dissolved in dH₂O (1 ml). EDCI (1.157 g) was
16 separately dissolved in distilled H₂O (dH₂O) (2 ml). The EDCI and NHS were mixed together and
17 immediately added to the pEK/octanedioic acid solution before topping up the solution to a final
18 volume (10 ml) with dH₂O. This was inverted ten times. An aliquot of the polymer solution (40 µl) was
19 pipetted into the condensation rings on the lid of a 48 well plate (Greiner Bio-One GmbH,
20 Kremsmunster, Austria). These were incubated overnight at room temperature until the pEK hydrogel
21 had polymerised, followed by 5 x 5 mins dH₂O washes.

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23 Cast pEK hydrogel thickness was measured in the wet state using a modified Draper micromere gauge,
24 the thickness of a sample of pEK hydrogels used for cell culture (4 for each repeat) were tested and
25 they had a thickness range of 100 - 130 µm and a diameter of 8 mm. For material testing the pEK
26 hydrogel solution was cast into a 90 mm petri dish. To analyse the surface hydrophobicity, contact
27 angle measurements (DSA100, Krüss GmbH, Germany) were performed using a 5 µl drop size, 9 drops
28 were dispensed randomly across the surface of the pEK hydrogel. The percentage water content of 1
29 mm diameter pEK hydrogels was measured. Samples were measured in the wet state and then left in
30 a desiccator overnight to measure dry weight. Mechanical testing was performed until failure using
31 dog bone punched samples with a width of 2.8 mm and gauge length of 10 mm, using the TST350
32 tensile tester (Linkam, UK) with a 20 N load cell at 100 µm/s strain rate. The thickness of the pEK
33 hydrogel sheet was measured prior to punching, thickness values were inputted into the Linkam

1 tensile tester to calculate the stress. For transparency testing a 6 mm punch was used. Samples were
2 placed in a 96 well plate (Griener BioOne) and optical density was recorded across several wavelengths
3 of the visible spectrum (460 nm, 544 nm, 560 nm, 570 nm and 600 nm) using a FLUOstar
4 spectrophotometer (BMG Labtech, UK), a Contaflex 75 contact lens was used as a control (Menicon,
5 UK). All material testing was repeated with 3 synthesised pEK hydrogels to give an n=3. The
6 microstructure of the pEK hydrogel was investigated using Multimode 8 atomic force microscope
7 (AFM) with Nanoscope V controller (Bruker, UK). A silicon nitride cantilever of spring constant 0.4 N/m
8 was used in scanasyst mode. A 2 μm^2 region was scanned at a rate of 1 Hz with 512 samples / line. The
9 scans were subjected to 0th order plane fit, to remove image bow.

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11 **2.2. Functionalisation of pEK hydrogels**

12 The pEK hydrogel was functionalised either by electrostatically binding ECM proteins onto the pEK
13 hydrogel or via covalently binding peptides to the pEK hydrogel. All pEK hydrogels were washed in 70%
14 (v/v) ethanol for 1 hour (hr) and then washed extensively in sterile Phosphate Buffered Saline (PBS,
15 Oxoid, UK).

16 The following proteins were electrostatically bound to pEK hydrogels (all Sigma unless stated
17 otherwise): human fibronectin (50 $\mu\text{g}/\text{ml}$), rat tail collagen I (100 $\mu\text{g}/\text{ml}$), collagen IV from human
18 placenta (100 $\mu\text{g}/\text{ml}$), chondroitin sulphate (20 mg/ml), laminin from human fibroblasts (50 $\mu\text{g}/\text{ml}$)
19 and the commercial FNC coating mix [®] (composed of fibronectin, collagen and albumin) (Enzo
20 Lifesciences) for 1 hr at 37°C, followed by the removal of excess solution.[40]

21 Prior to covalent binding, precast pEK hydrogels were washed 3 times with 10 % NMM in dH₂O solution
22 then washed 3 times in dH₂O. Cell binding peptide H-Gly-Gly-Arg-Gly-Asp-Gly-Gly-OH (RGD, Spheritech
23 ltd, UK), and $\alpha 2\beta 1$ integrin recognition sequence H-Asp-Gly-Glu-Ala-OH (DGEA, Sigma, UK) were
24 covalently bound to precast pEK hydrogels via the available amine sites in a 0.25 M solution of NHS
25 and EDCI dissolved in dH₂O. RGD was bound at 100 % loading capacity to available amine groups
26 whereas DGEA was bound at 5% and 1% loading capacity. Specifically 127 mg of RGD was added to
27 0.072 g NHS and 0.12 g EDCI in 2.5 ml dH₂O and mixed until dissolved. The precast pEK hydrogels were
28 placed in a 24 well plate and 125 μl of the peptide solution was added to each well for 1 ½ hrs at RT
29 on a rocker. For 5% and 1% DGEA either 0.656 mg or 0.131 mg, respectively, was added to 0.029 g
30 NHS and 0.048 g EDCI in 1 ml dH₂O and mixed until dissolved. The precast pEK hydrogels were placed
31 in a 12 well plate and 250 μl of the peptide solution was added to each well for 1 ½ hrs at RT on a
32 rocker. Peptide solution was then removed and 5 x 5 mins dH₂O washes were performed prior to
33 washing in 70% ethanol.

1 **2.3. HCEC-12 cell culture**

2 Immortalised, transformed human corneal endothelial cells HCEC-12 cells (HCEC-12 (RRID:CVCL_2064)
3 ACC 646, DSMZ) were grown in culture medium Hams F12 and Medium 199 (1:1) (Invitrogen, UK) with
4 5% (v/v) fetal bovine serum (FBS) (Biosera, Labtech, UK). HCEC-12 cells were grown to 70-80%
5 confluence and passaged using TrypLE Express (Invitrogen, UK) for 5 mins. Detached cells were
6 collected in culture medium and centrifuged at 1000 rpm for 5 mins. Cell pellets were re-suspended
7 in culture media and diluted to required cell density for use in experiments. HCEC-12 cells were used
8 between passage 8-15 for experiments.

9 **2.4. Porcine corneal endothelial cell isolation and expansion**

10 Fresh porcine eyes were obtained from 6-month old pigs within 6 hours of slaughter from a local
11 abattoir. The extra-ocular tissue was removed from eyes followed by a 2 min wash in PBS, (containing
12 1% (v/v) penicillin/streptomycin and amphotericin B (P/S/AmpB) (Sigma, UK)). Eyes were immersed in
13 povidone iodine (3% (v/v) Medisave, UK) diluted in PBS for 2 min and were washed twice in PBS to
14 remove excess povidone iodine. Isolation of CECs was performed as previously reported with minor
15 modifications.[37, 41] Briefly, corneas were excised from whole globes making an incision 3-4 mm
16 from the posterior limbus and the anterior segment was obtained making a circumferential cut along
17 the incision. The lens, iris and ciliary body and trabecular meshwork tissue were carefully removed.
18 The corneas were rinsed 2-3 times in fresh PBS containing 1% (v/v) P/S/AmpB to remove any
19 unwanted cells. Corneas were placed endothelium side up in a sterile Bijou cap in a 12 well plate and
20 incubated with 300 µl TrypLE Express (Invitrogen, UK) for 15 min at 37°C, 5% CO₂. Following
21 incubation, CECs were isolated from both the central and peripheral regions as a single culture. CECs
22 were released into the TrypLE Express solution by gentle scraping using a small inoculation loop to
23 dislodge cells and pipetted into a well of a 6 well plate. A further 300 µl media was added to CE to
24 ensure the capture of all endothelial cells. Isolated cells were re-suspended in culture medium (5 ml
25 DMEM (Sigma, UK) with 10% FBS, 1% (v/v) P/S/AmpB)) and cultured in 6 well plates for 2 weeks and
26 cell media was replaced every 2-3 days until confluence was achieved. Confluent cultures of porcine
27 CECs showing characteristic regular hexagonal morphology were apparent after 2 weeks and were
28 passaged into T25 flasks (passage 2) for further expansion and use for experimental studies at passage
29 3.

30 **2.5. Cell seeding onto pEK hydrogel**

31 HCEC-12 cells and porcine CECs were dissociated from the culture dish using TrypLE Express for 10 min
32 at 37°C, 5% CO₂. Following enzyme dissociation, CECs were re-suspended in 5 ml of media and

1 centrifuged at 1000 rpm for 5 min. pEK hydrogels were transferred into 12 well tissue culture
2 polystyrene (TCPS) plates and excess PBS was allowed to evaporate for approximately 15 min under
3 sterile conditions. CECs were counted and seeded onto 8.5 mm diameter pEK hydrogels at a density
4 of 1800 cells/mm² (100 000 cells per pEK hydrogel) in a concentrated cell solution (50 µl) aliquoted to
5 the centre of the pEK hydrogel and transferred to the incubator for approximately 2 hrs at 37°C 5%
6 CO₂ to allow cell adhesion. After 2 hrs a further 2 ml of media was gently added to the well and the
7 pEK hydrogels were incubated for 24 hrs at 37°C 5% CO₂. After 24 hrs in culture the pEK hydrogels plus
8 attached cells were transferred into fresh 12 well TCPS plates with fresh pCEC culture media, thus
9 excluding any pCECs that may have adhered to the bottom of the original 12 well TCPS plates. Cell
10 medium was replaced every 2-3 days until confluence was achieved.

11 **2.6. Analysis of cell adherence to pEK hydrogel**

12 The number of cells adhered to the pEK hydrogel surface was measured as a longitudinal study at 24
13 hrs, 4 days, 7 days, and 5 weeks, replacing the media every 3 days for long term cultures. Cells which
14 remained rounded were not deemed adhered to the pEK hydrogel. To quantify the number of cells
15 adhered to the pEK hydrogel, 3-5 randomised fields of view were imaged per pEK hydrogel, using
16 phase contrast microscopy on live cells using a Nikon Eclipse Ti-E microscope (Nikon, UK). The same
17 pEK hydrogel with cells was imaged at the specified time points until the experiment was terminated
18 at the final time point. Cells were quantified using ImageJ 1.48v, images were processed to enhance
19 brightness and contrast using the threshold tool to discriminate cell boundaries. The plugin Cell
20 Counter tool on Image J was used to mark and count individual cells.

21 **2.7. Immunocytochemistry**

22 pEK hydrogel/cell constructs were washed briefly in PBS and fixed in 10% (v/v) neutral buffered
23 formalin (Sigma, UK) for 10 min. HCEC-12 and pCECs were permeabilised with 0.1% (v/v) Triton X 100
24 (Sigma, UK) in PBS) at RT for 5 min. Non-specific binding sites were blocked for 30 min at RT with 300
25 µl blocking buffer (10% (v/v) Normal Goat Serum (Sigma, UK) in PBS). Samples were incubated with
26 primary antibody zonula occludens 1 (ZO-1) (Invitrogen Rabbit polyclonal 40-2200) 250 µg/ml, 1:40)
27 and Na⁺/K⁺ATPase (Santa Cruz #sc-58628, Mouse monoclonal IgG₁, 200 µg/ml, 1:20) overnight at 4°C
28 then washed 3 times for 5 mins with PBS (0.05% (v/v) Tween-20 (Sigma, UK)). pEK hydrogels were
29 incubated with secondary antibody (Alexa Fluor 488 Goat anti-Rabbit and Alexa Fluor 488 Goat anti-
30 Mouse (Invitrogen, UK)) and isotype control for 1 hr in the dark at RT, followed by 3 PBS washes for 5
31 mins each. pEK hydrogels were stained with Hoechst 33442 (Thermo Fisher, UK) for 15 min, washed
32 in PBS and imaged using Nikon Ti microscope, using the Z stack option to create a compiled image for

1 brightfield images. A Zeiss confocal LSM800 and Nikon Eclipse Ti-E microscope was used for
2 immunofluorescence images using z stacks to compile a focused image.

3 **2.8. Statistical analyses**

4 Data was presented as the mean and standard deviation (SD) of at least three independent
5 experiments ($n=3$), with each independent experiment being performed on a minimum of 3 pEK
6 hydrogels for each condition. One-way analyses of variance (ANOVA) and post-hoc Tukey's HSD test
7 were performed to evaluate statistical significances between groups of samples. All statistical analyses
8 were performed using Minitab 17.0. Statistical significance is shown using the p -value of $p<0.05$ (*).

9 **3. Results**

10 **3.1 pEK hydrogels are transparent and easy to manipulate**

11 Thin, transparent pEK hydrogel films (Figure 1A) were fabricated with an open porous structure
12 demonstrated by AFM (Figure 1B), an average of 118 μm (SD 16 μm) thickness was observed for gels
13 synthesised for cell culture (Table 1). The pEK hydrogels had an average stiffness and ultimate tensile
14 strength of 0.11 MPa and 0.04 MPa, respectively (Table 1). As expected, contact angle analysis
15 revealed the pEK hydrogel was hydrophilic with an average contact angle of 18.1° (SD 1.26) along with
16 a high water content of 91.25% (SD 1.65) (Table 1). The pEK hydrogels had excellent transparent
17 properties across all wavelengths of the visible spectrum, for example a 99.08% (SD 0.57) light
18 transmission at 560 nm wavelength was observed (Table 1) (560 nm was chosen as a representative
19 wavelength as values did not differ across the various wavelengths). This was not significantly different
20 to the contact lens positive control, 100.4% (SD 1.28) ($p>0.05$). The mechanical properties of the pEK
21 hydrogels meant that they were robust enough to be manipulated with forceps and handled as they
22 would be in a surgical procedure (Figure 1C).

23 **3.2 HCEC-12 cell line, but not pCECs, adhered and remained attached on pEK hydrogels after 7 days**

24 The HCEC-12 cells adhered to the pEK hydrogel at 24 hrs post seeding with an average cell density of
25 281.50 cells/ mm^2 (SD 51.81), comparable to TCPS 288 cells/ mm^2 (SD 17.78) (no significant difference
26 ($p>0.05$)) (Figure 2A,B). By 7 days HCEC-12 cells covered the pEK hydrogel surface to form a cell
27 monolayer with an average cell density of 422.9 cells/ mm^2 (SD 87.88) (Figure 2C,D), which was
28 comparable to TCPS 457.13 cells/ mm^2 (SD 78.32) ($p>0.05$) (Figure 2E). The number of HCEC-12 cells
29 covering the pEK hydrogel surface at 7 days was significantly higher than at 24 hrs post seeding (Figure
30 2E) ($p<0.05$).

1 Primary pCEC adherence on the pEK hydrogel was lower when compared to HCEC-12 cells or pCECs
2 on TCPS at 24 hrs with an average cell density on the pEK hydrogel of 43.86 cells/mm² (SD 13.23)
3 (Figure 2F,G and J), compared to TCPS 1495.64 cells/mm² (SD 403.12), significantly different ($p<0.05$).
4 At 7 days pCECs covered the surface of the pEK hydrogel to achieve an average cell density of 81.82
5 cells/mm² (SD 38.49) (Figure 2H,I and J), significantly different compared to TCPS 1893.27 cells/mm²
6 (SD 170.41) ($p<0.05$). Although there were regions of high pCEC density on the pEK hydrogels, it was
7 evident that pCECs had detached from regions of the pEK hydrogel surface as sheets at 7 days (Figure
8 2I).

9 **3.3 Pre-adsorbing collagen I, collagen IV and fibronectin onto pEK hydrogels improved the adhesion** 10 **and morphology of pCECs**

11 There was increase in the number of pCECs adhered to the pEK hydrogel surface which were pre
12 adsorbed with either collagen I, collagen IV or fibronectin at 24 hrs, compared to the untreated pEK
13 hydrogels (Figure 3A-H). An average cell density of 1958 cells/mm² (SD 896.31) for collagen I, 1471.6
14 cells/mm² (SD 774.43) collagen IV and 1112.19 cells/mm² (SD 744.55) for fibronectin was achieved
15 compared to 128.43 cells/mm² (SD 129.07) for untreated pEK hydrogels ($p<0.05$) (Figure 3I). There
16 was no significant difference between the numbers of pCECs on the pEK hydrogel surface whether it
17 had been pre-adsorbed with collagen I, IV or fibronectin (Figure 3I) ($p>0.05$). There was variation in
18 the cell number across the pEK hydrogel surface, thus accounting for the large standard deviations at
19 the 24 hr time point. The addition of FNC coat, chondroitin sulfate, laminin or a mix of both chondroitin
20 sulfate and laminin did not significantly improve the cell density on the pEK hydrogel ($p>0.05$),
21 compared to the untreated pEK hydrogel (Figure 3A, E-I).

22 Representative images of pCECs on the pEK hydrogels with pre-adsorbed collagen I, collagen IV and
23 fibronectin at 2 days, 4 days and 7 days, showed that pCECs formed confluent monolayers at 7 days
24 across the pEK hydrogel surfaces (Figure 4A-L). The pCEC monolayers remained attached to the pEK
25 hydrogel surface at 7 days on pEK hydrogels pre-adsorbed with collagen I, collagen IV and fibronectin,
26 in comparison to the untreated pEK hydrogel, which displayed regions of pCECs detachment (Figure
27 4C,F,I,L). Representative images of pCECs show compact monolayers exhibiting the characteristic
28 hexagonal appearance typical of primary pCECs on these surfaces, in comparison to the untreated pEK
29 hydrogel.

30 **3.4 RGD but not DGEA functionalisation of pEK hydrogels increased adhesion of pCECs in long term** 31 **cultures**

1 It was demonstrated by phase contrast microscopy that there were less pCEC coverage on the non
2 functionalised pEK hydrogels (Figure 5A-C), DGEA 1% (Figure 5D-F), DGEA 5% (Figure 5G-I) at 24 hrs, 4
3 days and 7 days, when compared to the RGD (Figure 5J-L) pEK hydrogels. Cell adhesion to the DGEA
4 functionalised pEK hydrogels was low and comparable to the non-functionalised pEK hydrogel.

5 The number of pCECs adhered to the RGD functionalised pEK hydrogel surface was monitored by
6 phase contrast imaging of live pCECs on pEK hydrogels at 24 hrs, 4 days and 7 days, compared to non
7 functionalised pEK hydrogels and TCPS (Figure 6A-I). The average number of cells covering the RGD
8 functionalised pEK hydrogels surface at 24 hrs was 1418 cells/mm² (SD 65.07) and was comparable to
9 TCPS with an average of 1495 cells/mm² (SD 403.12) (Figure 6J). No significant difference was observed
10 between the RGD functionalised pEK hydrogel and TCPS ($p>0.05$), at any time point (Figure 6J). The
11 number of pCECs covering the non functionalised pEK hydrogels surface at 24 hrs was 187 cells/mm²
12 (SD 104.99).

13 By day 4 and 7 the number of pCECs on the RGD functionalised pEK hydrogel remained comparable to
14 TCPS (Figure 6E,F,H,I). On day 4, 1729.12 cells/mm² (SD 279.95) covered the RGD functionalised pEK
15 hydrogel surface, comparable to TCPS 1728.29 cells/mm² (SD 685.54) ($p>0.05$) (Figure 6J). On day 7,
16 2219.19 cells/mm² (SD 171.09) covered the RGD functionalised pEK hydrogel surface, comparable to
17 TCPS 1893.27 cells/mm² (SD 170.41) ($p>0.05$) (Figure 6J). The pCECs on the non-functionalised pEK
18 hydrogel had detached from the surface by 7 days (Figure 6C).

19 pCECs on RGD functionalised pEK hydrogels were maintained in culture for up to 5 weeks post seeding
20 and a monolayer of cells remained adhered to the pEK hydrogel surface without any regions of cell
21 detachment, compared to the non-functionalised pEK hydrogel where no cells were present on the
22 surface (Figure 7A and B). The cell density on these pEK hydrogels at 5 weeks post culture (longest
23 time point monitored) was an average of 2548 cells/mm² (SD 159.48). pCEC monolayers displayed
24 characteristic CEC hexagonal morphology and immunofluorescence staining for ZO-1 was localised at
25 the cell-cell junctions, indicating tight junction formation (Figure 7C-E) To validate the presence of a
26 functional monolayer, immunocytochemistry for Na⁺/K⁺ATPase showed positive membrane staining
27 of CECs, suggesting the presence of a functional Na⁺/K⁺ATPase pump (Figure 7F-H).

28 **4. Discussion**

29 Corneal endothelial injury or dysfunction is currently treated with a corneal transplant with one donor
30 endothelium being used to treat one patient. The opportunity to expand CECs on tissue engineered
31 substrates offers an important alternative treatment to enable multiple recipients to be treated from
32 a single donor. We have demonstrated in this study that we were able to fabricate thin, transparent

1 pEK hydrogels, which can be fine-tuned to control for cell adhesion, enabling the *in vitro* expansion
2 and support of primary CECs whilst maintaining their phenotypic markers. It was determined that pre-
3 adsorption of proteins collagen I, collagen IV and fibronectin increased pCEC adhesion on pEK
4 hydrogels. Functionalisation of the pEK hydrogel surface with the RGD peptide increased cell adhesion
5 and expansion of pCECs and at 5 weeks these cells had formed a monolayer with expression of ZO-1
6 and Na⁺/K⁺ATPase, indicating the formation of tight junctions and presence of functional pumps,
7 respectively.

8 pEK hydrogels used in this study are synthesised from pEK and dicarboxylic acids using carbodiimide
9 chemistry and can be easily and rapidly manufactured from relatively inexpensive reagents, however,
10 we appreciate that there is some variability in our current method of the pEK hydrogel manufacturing
11 process, and parameters such as temperature and humidity would need to be more tightly controlled
12 to upscale the process to a more reproducible method for commercial use. A significant advantage of
13 pEK hydrogels is that we can tailor the mechanical properties of the pEK hydrogel to fit specific
14 requirements. In this study, a pEK hydrogel with polymer density 0.066 g/ml cross-linked to 60% with
15 octanedioic-acid was initially used as previous research had demonstrated that this pEK hydrogel was
16 robust enough to handle easily.[38, 39] The pEK hydrogels had a modulus of 0.11 MPa and ultimate
17 tensile strength of 0.04 MPa. These figures differ from the modulus of human DM, the substrate for
18 the native endothelium, which is 0.05 MPa (range 0.02-0.08) and the ultimate tensile strength, which
19 is 0.3 MPa.[42] The important point to note, however, is that the hydrogel can be easily manipulated,
20 as they would be during DSAEK surgery, without significant loss of cells or substrate integrity. Other
21 materials that have been trialled as a substrate for an engineered endothelial layer such as pre-wet
22 silk fibroin have demonstrated a Young's modulus of 0.022 MPa and ultimate tensile strength of 2.1
23 MPa [43], however these materials have been too fragile to handle. We did not intend to produce a
24 material with mechanical properties approximating the DM, as one of the problems with DMEK
25 surgery is the high level of surgical skill required to handle the DMEK scroll.[44] It is a delicate tissue
26 that can be easily damaged during the stripping and unfolding procedures so we aimed to produce a
27 hydrogel that had improved robustness and was easier to unfold; effectively an intermediate between
28 a DMEK and DSAEK graft combining the advantages of DSAEK tissue in terms of ease of handling and
29 unfolding with the superior visual outcomes of the thinner DMEK tissue. In a recent review [45] the
30 authors discuss the relative mechanical properties of natural materials for corneal tissue engineering
31 and demonstrate that the elastic modulus varies considerably depending on the orientation and depth
32 of the tissue sample (cornea and amniotic membrane) and the method of testing (uniaxial tension,
33 compression or microindentation) but are generally in the range 0.3 to 3.0 MPa. In contrast to the
34 natural tissues, the mechanical properties of purified materials such as collagen 1 and silk fibroin can

1 be tailored by optimising the cross-linking. A similar approach can be taken with the pEK used in this
2 study leading to greater control over the design of the substrate for both cell interactions and surgical
3 handling. In comparison to collagen 1 and silk fibroin the pEK is inexpensive and readily available as a
4 pharmaceutical grade material.

5 Substrates for the replacement of CE must be porous, thin and transparent, to allow the passage of
6 fluid and nutrients from the aqueous humour into the stroma, as well as allowing the passage of light.
7 We have demonstrated in this current study that our pEK hydrogel has an open porous network,
8 observed via AFM, and that it is possible to cast thin pEK hydrogels (~100µm). The pEK hydrogel was
9 hydrophilic (contact angle 18.1°), most likely due to the presence of amines on the surface and the
10 high water content within the pEK hydrogel (water content of 91.25%), had excellent transparency
11 allowing the passage of light across all wavelengths of the visible spectrum (99.08% light transmission),
12 and excellent cytocompatibility for other ocular applications. One reported example is the potential
13 use of these pEK hydrogels as antimicrobial contact lenses, demonstrating that the pEK hydrogels
14 showed good cytocompatibility with corneal epithelial cells.[38]

15 The pEK hydrogel provides a platform that can be modified and tailored to specific cell requirements
16 to control for cell attachment. As the crosslinking density of the pEK hydrogel increases, the number
17 of free amine functional groups decreases and the stiffness of the pEK hydrogels increases. Both these
18 properties can influence the interaction of cells with surfaces, thus altering cell activity and
19 attachment. Our synthesised pEK hydrogel theoretically has 40% of the amine groups unbound and
20 free. The amine surface of the pEK hydrogel not only promotes cell interactions but provides useful
21 binding sites for biomolecules allowing specific functionalisation of the surface, either by pre-
22 adsorption of ECM proteins or covalent binding of additional peptides. The non-functionalised pEK
23 hydrogel seeded with the HCEC-12 cell line showed promising results, forming a confluent monolayer
24 of cells with characteristic CEC hexagonal morphology and the numbers of cells present were
25 comparable to cells on TCPS. However, primary pCEC attachment was significantly lower and although
26 confluent monolayers were achieved, by 7 days, detachment of cell monolayers from the pEK hydrogel
27 was observed. The differences between cell line and primary cells, with the primary pCECs detaching
28 away from the pEK hydrogel surface as sheets, suggests the primary cells had a stronger attachment
29 to one another rather than the material surface perhaps due to less deposition of a basement
30 membrane (BM), although this would require confirmation by characterising the deposited ECM. The
31 HCEC-12 cell line may have a lower requirement for cell adhesion or secrete more BM proteins in a
32 short period of time compared to the pCECs to enable maintenance of a cell monolayer. We and others
33 have reported that primary human corneal epithelial cells require long term culture (>3 weeks) to
34 secrete substantial amounts of basement membrane proteins [46] and that the presence of

1 neighbouring cells (stromal[46] or corneal endothelial cells[47]) in organotypic 3D cultures leads to
2 the synthesis of a nearly continuous basement membrane. One mechanism that could explain this
3 effect is that the neighbouring cells may produce a cytokine or matrix component that stimulates the
4 epithelial cells to differentiate and assemble a basement membrane. It is likely that the stromal or
5 epithelial cells also signal to the endothelial cells in a feedback loop. In the present study, primary
6 endothelial cells were cultured in isolation so are not exposed to any such signalling, consequently,
7 production of their own matrix may be substantially delayed. In order to ameliorate this, addition of
8 exogenous ECM proteins on culture surfaces or biomaterials is a viable option. Modification of the PeK
9 hydrogel with peptides/proteins clearly elicits different cellular responses, however, in this study we
10 have not quantified the amount of bound peptide/proteins as it is challenging in this setting. The
11 peptide structure of the PeK hydrogel masks the spectroscopic peaks of the attached peptides if using
12 Fourier transform infrared spectroscopy (FTIR) and reagent absorption by the hydrogel creates
13 considerable variability in background readings in ELISA based assays.

14 The DM is composed predominantly of collagen IV, collagen VIII, laminin and fibronectin.[48] Others
15 have shown that mimicking the structure of DM, for example, with a dense layer of collagen IV and
16 laminin (bovine cells) or collagen IV alone (human cells) encouraged endothelial cells to form high
17 density monolayers that adhered to an underlying collagen I substrate.[49, 50] ECM proteins and
18 substrates that mimic mechanical and biological properties of DM have been shown to be more
19 favourable, increasing cell attachment and maintaining characteristic endothelial cell morphology.[33,
20 51, 52] In this present study we showed that electrostatic binding of ECM proteins (collagen I, collagen
21 IV and fibronectin) to the surface of our pEK hydrogels improved attachment, maintenance of a cell
22 monolayer and sustained the cell sheet adhesion at 7 days. Another study demonstrated that human
23 CECs cultured on collagen I, collagen IV, fibronectin and commercial FNC coat attached well and spread
24 but were only weakly attached to laminin and TCPS control plates.[53] This agrees with the findings
25 of our study that the laminin did not increase adhesion of the primary porcine cells. Others have also
26 modified the surface of their materials to increase cell attachment. Silk fibroin lacks ECM proteins and
27 studies have shown that modification of the substrate via the incorporation of pre-adsorbed ECM
28 proteins (collagen IV) onto the surface, promoted attachment and proliferation of human CECs.[29]
29 Palchesko et al. also demonstrated that bovine CECs could be cultured on a poly(dimethylsiloxane)
30 surface and the adhesion and morphology of CECs was improved with collagen IV proteins.[40]

31 Although coating with natural ECM molecules appears to be beneficial to CEC adhesion and growth,
32 they do have some disadvantages. An interesting solution to this problem was described by Rizwan et
33 al. in 2017 using gelatin methacrylate (GelMA) as the base for a tissue engineered graft.[54] Instead
34 of coating the surface of the hydrogel with ECM proteins themselves they nano-patterned the surface

1 to mimic the nanoscale topographical cues contained on *in vivo* extracellular matrix. They showed that
2 the topographical cues were able to improve the cell functions and phenotype of a CEC monolayer. It
3 is preferable to use chemically and physically defined ECM mimics that can be reliably reproduced so
4 this approach could be useful if direct functionalisation of the surface with synthetic peptides is not
5 possible. We have successfully shown that we are able to functionalise the pEK hydrogel through
6 covalent binding of synthetic ECM adhesive peptides, DGEA and RGD. Integrins form an important and
7 widespread group of adhesion receptors as they interact with proteins of the extracellular matrix or
8 with the receptors expressed on cells as shown in the binding of the leukocyte integrins to the
9 intercellular adhesion molecules (ICAMs).[55] DGEA (Asp-Gly-Glu-Ala), corresponding to residues 425-
10 438 of the Type 1 collagen sequence, has been identified as a recognition motif used by the type I
11 collagen to bind to $\alpha 2\beta 1$ integrin.[56] The RGD peptide (Arg-Gly-Asp) is the most widely studied
12 adhesive peptide and is the principal integrin binding domain present within ECM proteins such as
13 fibronectin, vitronectin, fibrinogen and others. pEK hydrogels functionalised with RGD did facilitate
14 pCEC adhesion, however, pCECs were unable to adhere to the surface of pEK hydrogels functionalised
15 with DGEA. DGEA therefore did not appear to serve as an adhesion ligand for pCECs in this context.
16 These results are consistent with a previously reported study describing rat calvarial osteoblasts and
17 MC3TT3-E1 osteoblasts demonstrating limited adhesion on hydrogel surfaces presenting the DGEA
18 peptide, which was described as 'weakly adhesive'.[57, 58]. The glycine-phenylalanine-
19 hydroxyproline-glycine-glutamate-arginine (GFOGER) peptide could be an alternative collagen I
20 peptide to trial in the future. It known to provide a significant basal level of adhesivity along with
21 RGD[59] and the integrin $\alpha 2\beta 1$ recognizes the GFOGER motif in residues 502–507 of the $\alpha 1$ chain of
22 type I collagen. Not only has the $\alpha 2\beta 1$ integrin been identified on corneal endothelial cells [60] but
23 the expression of $\beta 1$ integrin has also been shown to be upregulated in cultured corneal endothelial
24 cells when compared with native tissue, so this fact can be exploited with designing peptide
25 functionalisation for tissue engineered endothelial grafts.[61]

26 The biomimetic RGD domain is able to promote cell adherence to the pEK hydrogel surface and
27 improve the cell/material interactions for many different applications.[62, 63] The advantage of using
28 an RGD peptide instead of a natural protein is a reduction in immune reactivity or pathogen transfer,
29 synthesis is easy and inexpensive and it can be performed in a controlled and defined manner.[64] For
30 example, the coupling of the RGD peptide to silk was beneficial in enhancing the cell attachment and
31 proliferation of corneal stromal cells in tissue engineering of the corneal stromal lamellae, which when
32 implanted into an animal model, maintained their functionality.[65, 66]

33 This study has demonstrated that pEK hydrogels offer a compatible substrate to support the expansion
34 and growth of a human corneal endothelial cell line and primary pCECs *in vitro*. We found that the

1 addition of pre-adsorbed collagen I, collagen IV and fibronectin increased the cell adhesion onto the
2 pεK hydrogel and the cells remained attached at 7 days. The free amine sites on the pεK hydrogel
3 enabled the functionalisation of the pεK hydrogel surfaces with RGD and DGEA cell binding peptides.
4 The results demonstrated that bound RGD increased cell adhesion compared to non-functionalised
5 pεK hydrogels and maintained cell attachment up to 5 weeks in culture. Long term cultures exhibited
6 a characteristic CEC phenotype which showed the formation of tight junctions at the cell boundary
7 and expression of Na⁺/K⁺ATPase demonstrating the functionality of the cell monolayer.

8 **5. Conclusions**

9 Tissue engineered corneal endothelial grafts are being developed by a number of research groups in
10 order to alleviate pressure on the demand for transplant tissue due to a worldwide donor cornea
11 shortage. Our pεK hydrogel has great potential as a clinically viable option as the peptide is readily
12 available in a purified state, its mechanical properties are customisable by design of the cross-linking
13 density and it can be surface functionalised via free amine groups for any particular application. We
14 have tailored the mechanical properties to enable easy handling of the graft by surgeons and
15 functionalised the surface to improve corneal endothelial cell attachment. Successful development
16 along the translational pipeline could see this technology being delivered to patients to treat
17 endothelial dysfunction.

18 **Conflicts of interest**

19 AGG is an employee of Spheritech and DAW is the CEO of Spheritech and holder of the specific
20 patent for the polymer (patent No: 9938378). All other authors have no conflict to declare.

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25

26 **Figure captions**

27 **Table 1.** Physical properties of a pεK hydrogel cross-linked to 60% with octanedioic-acid to a polymer
28 density of 0.066 g/ml n=3.

29 **Figure 1.** (A) Representative photograph of a pεK hydrogel cross-linked to 60% with octanedioic-acid
30 to a polymer density of 0.066 g/ml demonstrating a thin transparent hydrogel, (B) Atomic force
31 micrograph detailing the microporous structure of the same pεK hydrogel. (C) Image to show how
32 the hydrogels can be manipulated easily using forceps.

1 **Figure 2.** HCEC-12 and pCEC expansion on a pEK hydrogel cross-linked to 60% with octanedioic-acid
2 to a polymer density of 0.066 g/ml. HCEC-12 cells and pCECs were seeded onto pEK hydrogels at a
3 density of 1×10^5 cells per pEK hydrogel (1800 cells/mm^2). Representative phase contrast images of
4 HCEC-12 cells expanded 24 hrs on (A) TCPS and (B) pEK hydrogel or at 7 days and on (C) TCPS (D) pEK
5 hydrogel. (E) Quantification of cell density (cells/mm^2) at 24 hrs and 7 days post seeding of HCEC-12
6 cells on hydrogels. Representative images of pCECs seeded at 24 hrs on (F) TCPS and (G) pEK
7 hydrogel or at 7 days on (H) TCPS and (I) pEK hydrogel. (J) Quantification of cell density (cells/mm^2)
8 at 24 hrs and 7 days post seeding of pCECs on hydrogels. Scale bar $100 \mu\text{m}$, $n = 4$ for HCEC-12 pEK
9 hydrogels, $n = 3$ for pCECs. Error bars show SD. Symbol * represents significant difference ($p < 0.05$)
10 using one way ANOVA and post-hoc Tukey's HSD analysis across groups.

11

12 **Figure 3.** Representative phase contrast images of adhesion of pCECs on a pEK hydrogel cross-linked
13 to 60% with octanedioic-acid to a polymer density of 0.066 g/ml pre adsorbed with ECM proteins.
14 pEK hydrogels were uncoated (A) or electrostatically bound for 1 hr with proteins (B) collagen I 100
15 $\mu\text{g/ml}$, (C) collagen IV $50 \mu\text{g/ml}$, (D) fibronectin $50 \mu\text{g/ml}$, (E) chondroitin sulfate 20 mg/ml , (F)
16 Laminin and chondroitin sulfate (G) laminin $50 \mu\text{g/ml}$, and (H) FNC coat. pCECs were seeded onto
17 hydrogels at a cell density of 1800 cells/mm^2 . pEK hydrogels were imaged 24 hrs post seeding. Scale
18 bar $100 \mu\text{m}$, $n = 3$. (I) Quantification of cell density (cells/mm^2). Symbol * denotes significantly
19 different $p < 0.05$ compared to pEK hydrogel only using ANOVA and post-hoc Tukey's HSD analysis,
20 Error bars show SD.

21 **Figure 4.** Adhesion and expansion of pCECs on a pEK hydrogel cross-linked to 60% with octanedioic-
22 acid to a polymer density of 0.066 g/ml pre-adsorbed with ECM proteins. pEK hydrogels (A-C) were
23 electrostatically bound with proteins for 1 hr with (D-F) collagen I $100 \mu\text{g/ml}$, (G-I) collagen IV 50
24 $\mu\text{g/ml}$ and (J-L) fibronectin $50 \mu\text{g/ml}$. pCECs were seeded onto pEK hydrogels at a cell density of 1800
25 cells/mm^2 . pEK hydrogels were imaged 2 days, 4 days and 7 days post seeding. Scale bar $100 \mu\text{m}$.

26 **Figure 5.** Primary pCECs seeded on pEK hydrogels cross-linked to 60% with octanedioic-acid to a
27 polymer density of 0.066 g/ml and non-functionalised or functionalised with DGEA and RGD.
28 Representative phase contrast images of pCECs expanded for 24 hrs, 4 and 7 days respectively on (A-
29 C) non-functionalised pEK hydrogels, (D-F) DGEA 1% functionalised pEK hydrogels, (G-I) DGEA 5%
30 functionalised pEK hydrogels and (J-L) RGD functionalised pEK hydrogels. Scale bar $100 \mu\text{m}$.

31 **Figure 6.** Primary pCECs seeded onto a pEK hydrogel cross-linked to 60% with octanedioic-acid to a
32 polymer density of 0.066 g/ml with/without RGD functionalisation. pEK hydrogels were imaged at 24
33 hrs, 4 days and 7 days. Representative images of cells expanded on (A-C) non-functionalised or (D-F)
34 RGD functionalised pEK hydrogels or (G-I) TCPS. (J) Quantification of cell density (mm^2) on pEK
35 hydrogels at specified time points. pCECs were seeded onto pEK hydrogels at a cell density of 1800
36 cells/mm^2 . Scale bar $100 \mu\text{m}$, $n = 3$. Error bars show SD. Symbol * represents significant difference
37 ($p < 0.05$) using one way ANOVA and post-hoc Tukey's HSD analysis across groups.

38

39 **Figure 7.** Long term culture of pCECs expanded on hydrogels. (A) non-functionalised hydrogel (B)
40 RGD functionalised hydrogel 5 weeks post seeding of pCECs. Scale bars $100 \mu\text{m}$. (C) ICC of ZO-1
41 (green) and Hoechst 33342 (blue). Monocoloured images (D) ZO1 and (E) Hoechst 33342. (F)
42 $\text{Na}^+/\text{K}^+\text{ATPase}$ (green) and Hoechst 33342 (blue) expression. Monocolour images (G) $\text{Na}^+/\text{K}^+\text{ATPase}$
43 and (H) Hoechst 33342. Scale bars $20 \mu\text{m}$.

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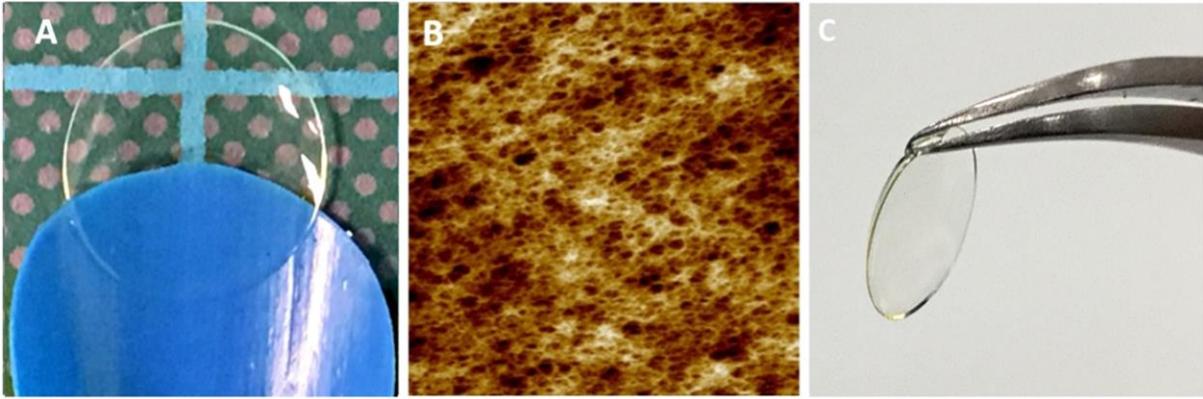
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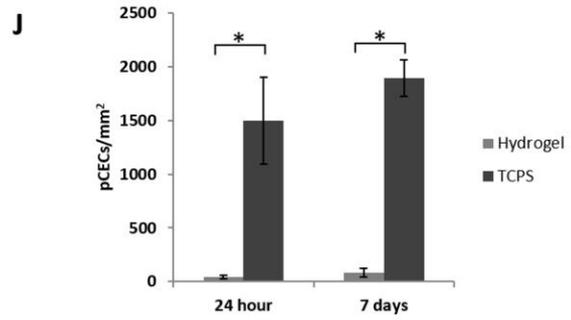
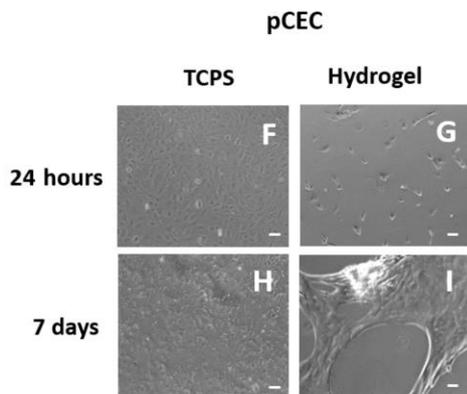
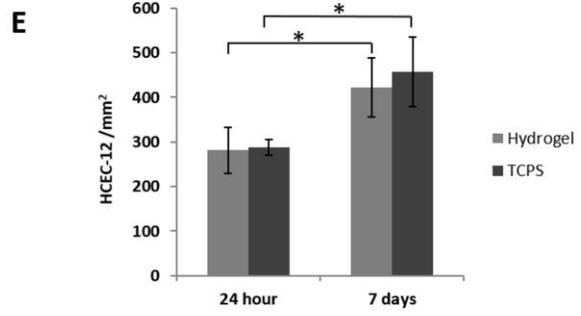
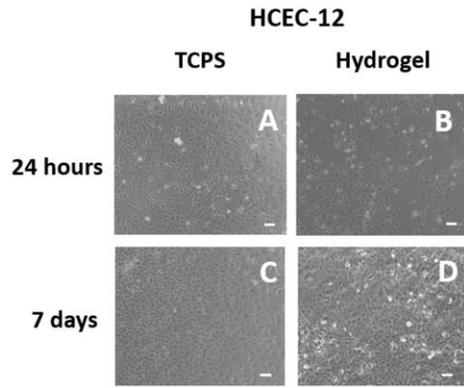
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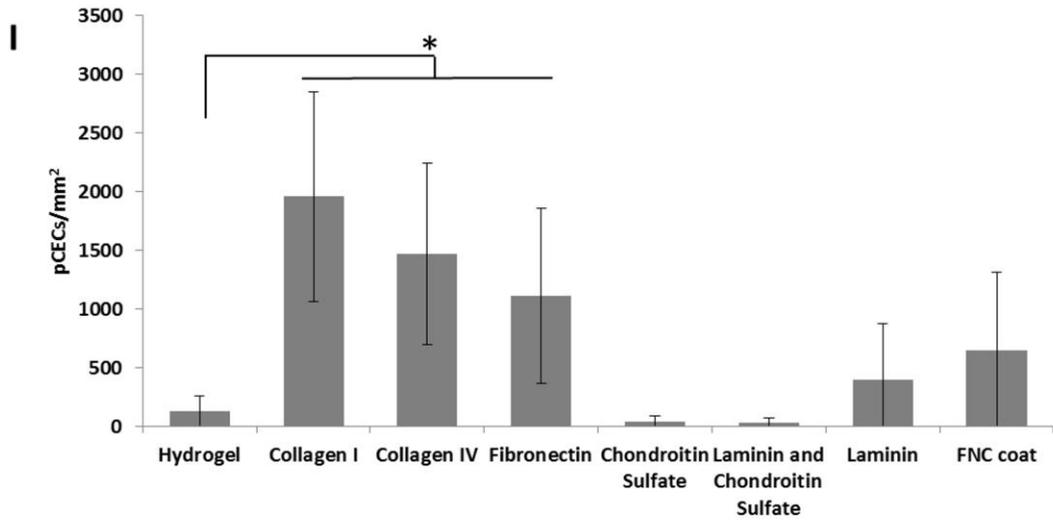
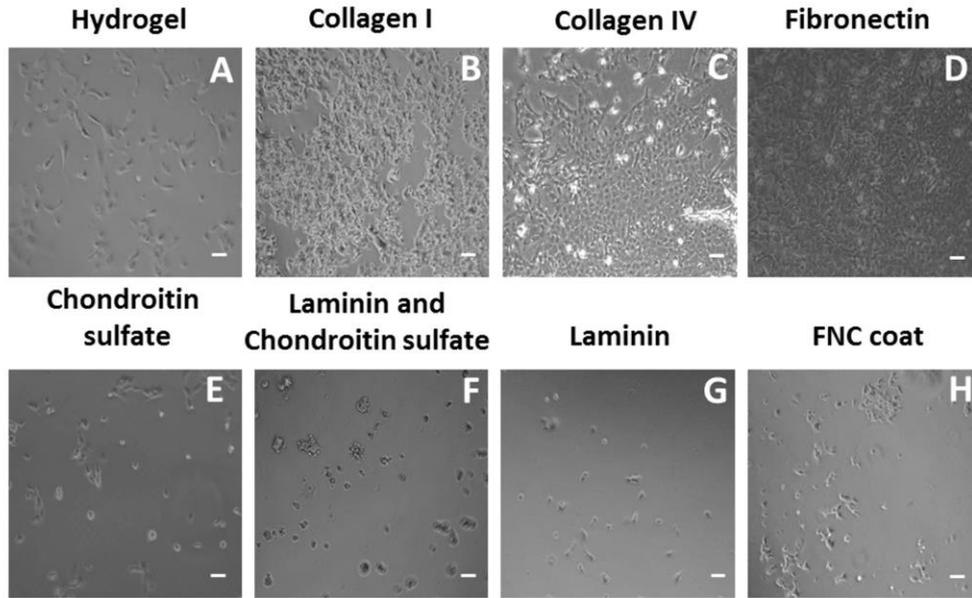
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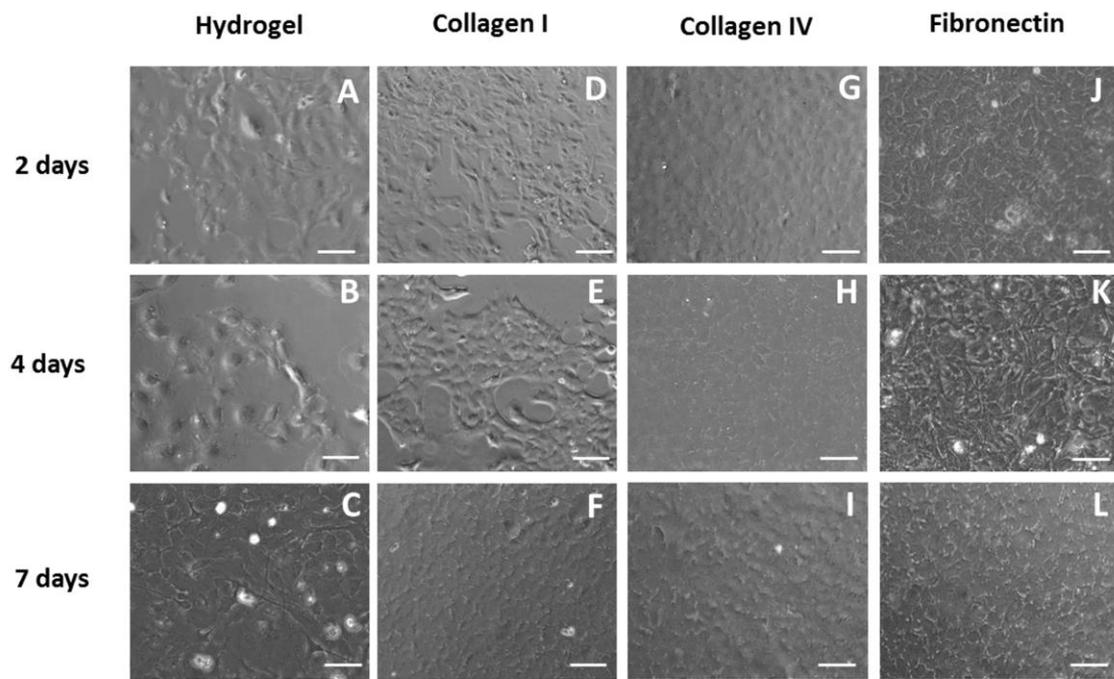
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2 **Figure 1**



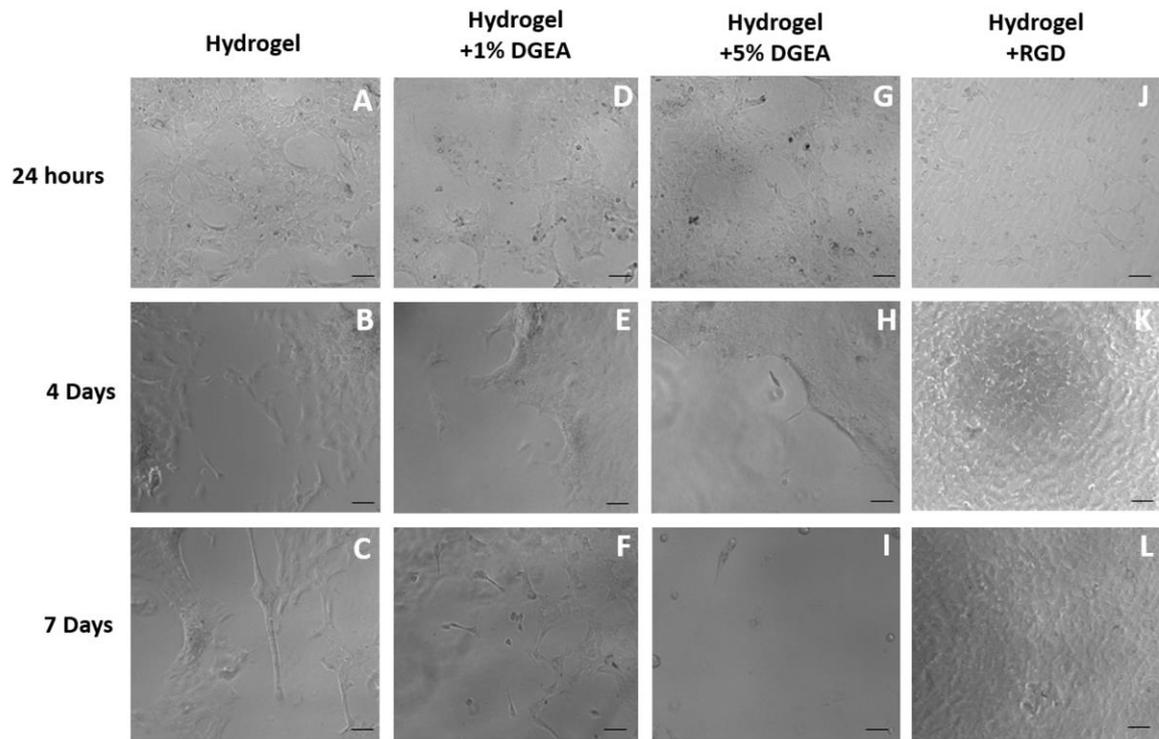
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2 **Figure 2**



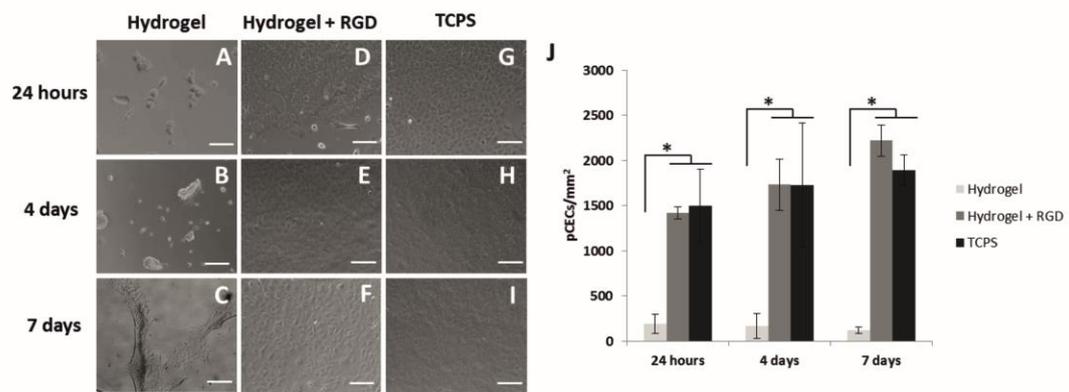
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2 **Figure 3**



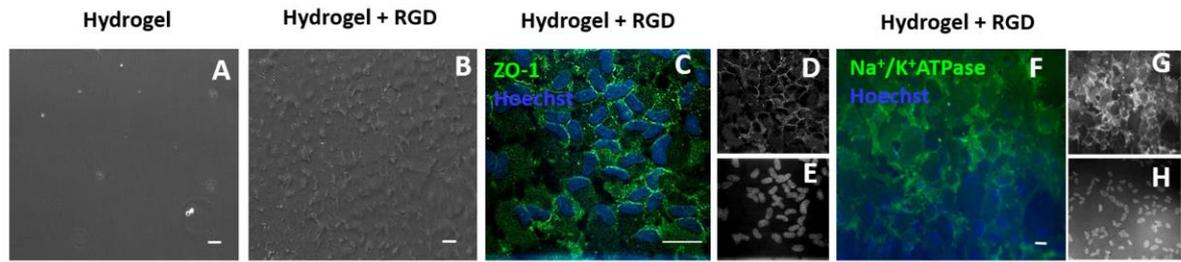
1
2 **Figure 4**



1
2 **Figure 5**



1
2 **Figure 6**



1
2 **Figure 7**

60% cross-linked octanedioic-acid with 0.066g/ml pεK	Average	Standard deviation ±
Thin film thickness (μm)	118	16
Young's Modulus (MPa)	0.11	0.01
Ultimate Tensile Strength (MPa)	0.04	0.004
Contact Angle °	18.1	1.26
% Water Content	91.25	1.65
% Transparency (560nm)	99.08	0.57

1
2 **Table 1**