Title

A scaffold-free strategy using a PEG-dextran aqueous-two-phase-system (ATPS) for corneal tissue repair

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Abstract

Forming thin tissue constructs with minimal extracellular matrix surrounding them is important for tissue engineering applications. Here, we explore and optimize a strategy that enables rapid fabrication of scaffold-free corneal tissue constructs using the liquid-liquid interface of an aqueous-two-phase system (ATPS) that is based on biocompatible polymers dextran and polyethylene glycol (PEG). Intact tissue-like constructs, made of corneal epithelial or endothelial cells, can be formed on the interface between the two liquid phases of ATPS within hours, and subsequently collected simply by removing the liquid phases. The formed corneal cell constructs express essential physiological markers and have preserved viability and proliferative ability *in vitro*. The corneal epithelial cell constructs are also able to re-

epithelialize the corneal epithelial wound *in vitro*. The results suggest the promise of our reported strategy in corneal repair.

Keywords: Tissue engineering; Ophthalmology; Scaffold free; Wound healing; Cornea

Introduction

Formation of tissue-like structures with scaffolding materials is now a very common strategy for reconstruction of human tissue, especially those with large-sized defects. The applications include bone, tendon, and neurovascular reconstruction and regeneration procedures.[1, 2] However, this strategy has fundamental limitations, which hinders its application in forming thin tissues with minimal extracellular matrix, e.g. various cell layers of cornea in the eye. Firstly, scaffolding materials often have suboptimal long-term biocompatibility and biodegradability, hence may trigger activation of adverse immune responses, resulting in post-transplantation complications.[3] Moreover, if the degradation rate of scaffolding materials does not match the growth rate of extracellular matrices, the transplanted structures may collapse. In contrast, forming tissue-like structures with a scaffold-free strategy may offer several advantages over scaffold-dependent approaches when forming thin tissues. [4, 5] The preservation of cell-cell junctions and membrane proteins during harvesting of cells from the tissue, may promote construction of tissues for purposes of direct regeneration.[6] Furthermore, the potential to use a high initial cell density will allow rapid assembly of intact tissue-like structures, thereby eliminating the need for a scaffold.[7, 8] This strategy is useful in forming individual, thin layers of corneal tissue, as these can mimic the anatomical and physiological organisation of both the corneal endothelium and epithelium layers.

One notable cause of corneal opacity is corneal endothelial dysfunction,[9] of which restoration of vision of these patients requires the replacement of the diseased endothelial layer with a healthy endothelial graft.[10] Dysfunction of the endothelial layer leads to corneal edema, whereby the cornea subsequently loses its transparency and resulting in irreversible vision loss. This cell layer does not have the ability to regenerate *in vivo*.[11, 12] As a result, the replacement of this layer of cells using cadaver donor graft is currently the best approach for treating dysfunctional corneal endothelium.[13] Unlike *in vivo*, human corneal endothelial cells can

proliferate *in vitro* due to absence of contact inhibition.[14] Therefore, corneal endothelial cells can be cultured first and expanded *in vitro*, and subsequently transplanted to the endothelial side of the cornea. However, injecting corneal endothelial cells in the form of a cell suspension is challenging because this approach does not favor large-scale tissue reconstruction.[15] Therefore, a planar tissue-like structure of corneal endothelial cells that can be delivered to the eye, as demonstrated in this study, may be of great clinical value, and help overcome cadaver donor grafts limited availability.[16] Meanwhile, other therapeutics are also being developed, which target the corneal endothelium in hopes of preserving or even rescuing the cellular functions of this layer. [17] Another significant etiology of corneal blindness is corneal epithelial injury, resulting from mechanical or chemical insults. Although current strategies for visual rehabilitation depend on full-thickness corneal transplantation (keratoplasty), these can also, in principle, be treated with transplantation of the corneal epithelial layer alone.[18] Corneal epithelium alone also serves as a selectively permeable barrier and maintain ocular surface integrity.[19] Scaffold-based corneal endothelial transplantation is the mainstream for substituting conventional corneal transplantation. However, concerns about cytotoxicity, inflammation, and *in vivo* biodegradability are raised on the use of scaffold materials.[20] Another issue would be the anatomical and technical considerations in transplanting a scaffold-based corneal endothelial layer onto the posterior cornea. The presence of the scaffold may interrupt the normal anatomy of the corneal layers since such material is typically not present between the corneal endothelial layer and the Descemet's membrane, where these anatomical layers are supposed to be directly adhered to one another. The presence of the scaffold material could also create a physical barrier between the corneal endothelial cells and the anterior chamber, hence affecting its main function of pumping ions out of the corneal stroma into the anterior chamber.[21] Another example would be the transplantation of a scaffold-containing retinal pigment epithelial (RPE) layer into the subretinal space. Since such space is surgically created,

and is already very small, even with the scaffold material made to be very thin, it would still seem like a relatively thick structure as the retinal is normally already very thin, at around 300 μm. This would pose a surgical challenge when inserting the transplanted material into the subretinal space, and more importantly when closing the wound and reattaching the retina since there would be a thick material underneath this layer [22]. Fabrication of both scaffold-less corneal endothelium and epithelium will aid in our reported strategy of corneal repair. Many established strategies in fabricating tissue-like structure without scaffold are based on magnetic-[23-27], electrochemical-[28, 29] and thermo-responsive techniques[18, 30-34] in various applications such as heart and liver tissue repair.[7] However, such methods are currently limited in clinical application owing to the complicated fabrication procedures and high cost.[35, 36] Moreover, the materials involved in these strategies may affect cellular activities and viability.[37-39] Hence, there is a justified need for an alternative technique for cell sheet formation.

Aqueous-two-phase systems (ATPSs), which consist of two immiscible aqueous phases, have been increasingly adopted as an important tool in biomedical research and applications.[40, 41] An abundance of biomaterials and their templates can now be fabricated with ease using the liquid-liquid interface formed by ATPS.[42, 43] In addition, simple scaffold-free tissue structures, such as cell spheroids and cell monolayers, can be formed using such systems.[44, 45] One particular example is the formation of a robust planar cell construct on the interface of a polyethylene glycol (PEG)/dextran (Dex) ATPS after several hours, via suspension of dermal epithelial cells in the PEG phase of this system.[46] The interface, which is characterized by interfacial tension between the two aqueous phases, serves as a liquid substrate where cells can be spatially assembled and cell-cell interactions can be formed. The dermal cell constructs formed by this method were able to attach and incorporate into the dermal matrices, as well as undergo differentiation and stratification to form skin-like tissue. Bilayer constructs were also

produced via this method, and mechanical strength and cellular physiological markers were demonstrated to be suitable for *in vitro* tissue models. In this study, we explore the strategy of forming scaffold-less cell construct at liquid interfaces. We hypothesise that this method of cell construct formation can be potentially translated to the management of eye conditions, with regards to its transplantation purposes on the ocular surface. After modification and optimization of this technique, we demonstrate that cells isolated from the cornea can be fabricated into robust planar cell constructs *in vitro* comprising mostly of viable cells. The cell constructs can adhere onto anatomical and physiological mimics of corneal surfaces and express physiologically relevant markers *in vitro*. Intact planar cell constructs can be formed within several hours, which is much quicker compared with previously documented methods. Furthermore, the technique does not require any specialized instruments such as electron beam irradiation[35, 36] for synthesizing thermo-sensitive scaffold, hence can be easily adopted by general biomedical laboratories. Our results demonstrate that formation of planar cell constructs based on all-aqueous liquid systems may act as a versatile strategy for creating corneal-relevant tissue constructs for both ophthalmic clinical use and research purposes.

Materials and methods

Preparation of aqueous two-phase system (ATPS)

The ATPS involved in this work was PEG/Dex. Solutions of PEG (Mw=35,000) (Sigma, USA) and Dex (Mw=500,000) (Pharmacosmos, Demark) were prepared using Dulbecco's Modified Eagle Medium (DMEM, Gibco) or corneal epithelial cell complete growth medium (ATCC) as the solvents. The range of concentrations of PEG and Dex solutions were 2.5 to 15% (w/w) and 5 to 30% (w/w) respectively. To form the biphasic system, the PEG phase was added directly on top of the Dex phase.

To pre-equilibrate the ATPS, the two immiscible liquids were mixed and vortexed vigorously. After that, the resulting emulsion was centrifuged at 10,000 x *g*. for 10 mins to achieve phase separation of PEG-rich and Dex-rich phases. After allowing the phases to settle for 1 day at 4 ^{o}C, the two immiscible phases, were collected separately for subsequent experiments.

The osmolality of solutions of PEG and Dex, with or without equilibration, was measured by freezing point depression osmometer (Model 3320, Advanced Instruments, MA, US) following the manufacturer's instruction. The osmolality of deionized water was calibrated as 0 mOsm/kg. For each sample, the measurement was repeated three times.

Culture of eye-related cell sources

The Human Corneal Epithelial cell-Transformed (HCE-T) cell line was kindly provided by Dr. Kaoru Araki-Sasaki from Kansai Medical University.[47] The cell line was cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) supplemented with 1% antibiotic-antimycotic and 10% fetal bovine serum. The cell culture reagents were purchased from Gibco, USA.

The primary porcine corneal endothelial cells were isolated from porcine eyes, which were obtained from a local wet market within 12 hours post-mortem with reference to previously described methods.[48] Briefly, the eyes were disinfected with commercially available povidine-iodine solution (Betadine) for 10 minutes at room temperature. The eyeballs were then washed twice with Hank's Balanced Salt Solution (HBSS) supplemented with 100 U/mL Penicillin and 100 µg/mL Streptomycin. The anterior segment of the eye was separated, and 2% dispase in Calcium and Magnesium free HBSS was added onto the endothelial side of the cornea. After incubation for 45 minutes in a humidified cell culture incubator, the endothelial side of the cornea was scratched using a metallic cell scraper. HBSS was used to collect the detached

corneal endothelial cells from the cornea. The collected cell suspension was filtered using a 70 µm cell strainer. The collected endothelial cells, after centrifugation, were cultured in F99 (1:1 mixture of Medium 199 and Ham's F-12 Nutrient Mix) supplemented with 10% newborn calf serum, 1% GlutaMAX, 100 U/mL Penicillin, 100 µg/mL Streptomycin, 0.3 mg/mL chondroitin sulfate and 12.5 µg/ml endothelial cell growth supplement (ECGS). Unless otherwise specified, all primary corneal endothelial cell culture reagents and supplements were purchased from Gibco, USA. Chondroitin sulfate and ECGS were purchased from Sigma Aldrich, USA.

The commercially available primary human corneal epithelial cells (PCS-700-010) were cultured in corneal epithelial cell complete growth medium (ATCC), supplemented with 1% penicillin and streptomycin. Unless otherwise specified, the cell source, as well as all corneal epithelial cell culture reagents and supplements were purchased from ATCC, USA.

The culture medium of all the three cell types was changed every 2 days, and the culture was maintained in a humidified incubator at 37°C with 5% CO_2.

Formation of tissue-like planar constructs

Tissue-like planar constructs were fabricated using pre-equilibrated ATPSs with reference to a previously described method with essential procedural adjustments, as illustrated in Figure 1.[49] Briefly, cells suspended in PEG-rich solution were added to Dex-rich solution in a micro-centrifuge tube. Afterwards, the entire system was centrifuged at 280 x *g* for 4 mins and incubated in an upright position for 3 hours at 37 °C in 5% CO_2 to allow the self-assembly of the tissue-like planar constructs. The formed cell constructs were harvested by pouring the ATPS solutions out of the microcentrifuge tube into a Petri dish containing HBSS. The cell numbers used to make the tissue-like planar constructs were optimized to be 1 million and 0.2

196 million for corneal endothelial in 600 µL micro-centrifuge tubes and corneal epithelial cells in

197 200 µL tubes respectively.

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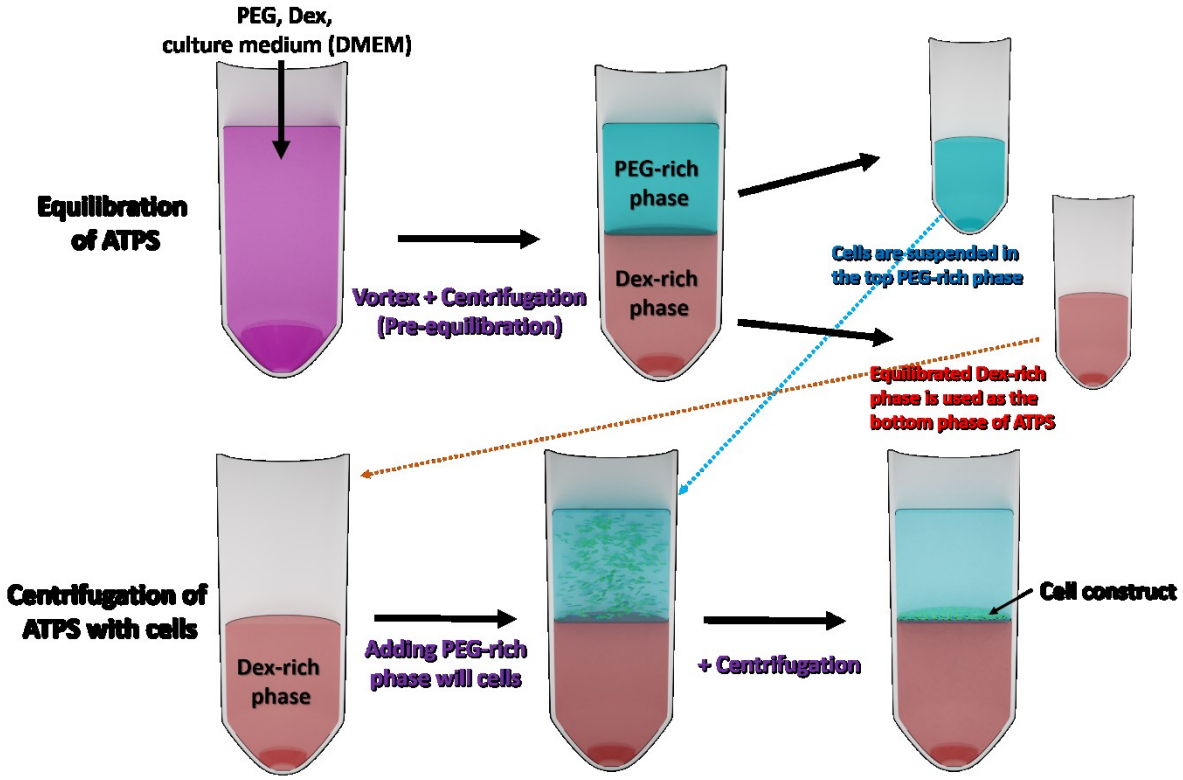

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201 **Figure 1** Schematic illustrations of the cell construct formation process

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To test the roles of ATPS equilibration and centrifugation on cell construct formation, a non-equilibrated ATPS was formed by preparing PEG and Dex solutions separately without pre-mixing of the two solutions. HCE-T cells were used in this study. Cell construct was formed by adding PEG-cell mixture on top of the Dex phase directly without mixing or vortexing. The entire system was centrifuged at 280 x *g* for 4 mins and incubated in an upright position for 2 hours at 37 ºC in 5% CO_2 to allow cell assembly. The cell constructs were harvested as described above.

Characterizations of the tissue-like planar constructs of corneal endothelial cells

Uniformity: To investigate the uniformity of the tissue-like planar constructs formed, primary porcine corneal endothelial cells were pre-stained with CellTracker Green CMFDA (Invitrogen, US) before forming the constructs. The harvested tissue-like planar constructs, with green fluorescence signals, was captured using a digital CMOS camera (Hamamatsu, Japan) on a fluorescence microscope (Olympus, Japan). The fluorescence intensity distribution of each constructs was analyzed using an open-source image processing software ImageJ.[50] Roughness index (RI) was calculated as the percentage standard deviation of the fluorescence intensity distribution from the fluorescence micrograph.

Proliferative ability: The endothelial cell constructs were transferred to a conventional cell culture dish and cultured with the abovementioned corresponding culture medium up to 21 days. The growth in area of the cell constructs over time was measured using ImageJ. Live-dead assay (L3224, Invitrogen) was used to qualitatively assess the cell viability of the constructs.

Physiologically relevant markers: The corneal endothelial cell construct was cultured in conventional plastic culture dish for a month *in vitro* to allow the reconstruction of

physiological markers of the corneal endothelium found *in vivo*. Immunocytochemistry was performed to visualize the reconstruction of two physiological markers, namely tight junction protein Zonula occludens-1 (ZO-1) and sodium-potassium ATPase (Na^+/K^+ ATPase). After methanol fixation and blocking with 5% normal goat serum, the cell samples were incubated with mouse ZO-1 monoclonal antibody and rabbit ATP1A2 Polyclonal antibody to visualize ZO-1 and Na^+/K^+ ATPase respectively. After primary antibody incubation, Alexa Fluor 488 conjugated anti-mouse secondary antibody and Alexa 594 conjugated anti-rabbit secondary antibody were incubated with the cell samples, following with DAPI nucleus stain. All antibodies and stains for immunocytochemistry were purchased from Invitrogen, USA. The cell area, which is defined as the area bounded by the tight junction expression, was measured using Adobe Photoshop CC 2020. The cell density was calculated by counting the number of nuclei using ImageJ. Cell suspension seeded with 3000 cells/mm^2 was performed as a control experiment.

Functionality assay: The corneal endothelial cell construct was seeded on a 12-well transwell® permeable membrane (#3460, Corning, USA) pre-coated with Geltrex (Gibco, USA). Single cell suspension with the same cell number as the cell construct was seeded as cell suspension-based transplantation. Empty transwell® was set as control group. The cell culture was maintained for 1 month. Osmolality of both the top and bottom compartment culture media was measured by a freezing point depression osmometer (Model 3320, Advanced Instruments, MA, US) following the manufacturer's instruction. Besides the pump function, the barrier function of the cell was also examined by the same Transwell ® system after 1-month *in vitro* culture. FITC-conjugated Dex (Sigma Aldrich, USA) with a concentration of 1 mg/mL in phenol red-free DMEM (Gibco, USA) was added in the bottom phase and the top chamber was filled with phenol-free DMEM. The Transwell ® system was incubated in humidified incubator for 30 min. After incubation, the medium in the top compartment was collected, and the

fluorescence intensity of the medium was measured with multimode microplate reader (Spectramax iD3, Molecular Devices, USA) detected at wavelength of 520 nm with 480 nm excitation light.

Ex vivo porcine eye model for injection of corneal endothelial cell construct

Fresh porcine eyeball was obtained from a local slaughterhouse within 12 hours post-mortem. Saline was continuously perfused to the anterior chamber of the porcine eye to maintain the chamber not to collapse. Then, two incisions of around 4 mm and 1 mm were created at the limbus region. Corneal endothelial cell construct formed using the proposed ATPS approach was pre-stained with 0.4% Brilliant Blue G and loaded into the intraocular (IOL) injector (UNFOLDER Platinum 1 Series Delivery System, Johnson & Johnson). Brilliant blue G is a widely used dye to stain the donor corneal endothelial graft clinically.[51, 52] The IOL injector with the cell construct was inserted into the anterior chamber through the 4 mm incision. Similarly, a syringe connected with a cannula was inserted into the anterior chamber of the porcine eye through the 1 mm incision. The cell construct in the IOL injector was then transported into the anterior chamber through the negative pressure applied by the syringe. After the transportation of the cell construct, a sulphur hexafluoride (SF_6) gas bubble was injected into the anterior chamber to push the cell construct onto the endothelial side of the cornea for 10 minutes. After that, the gas bubble was removed.

Fabrication of the hydrogel bandage vehicle for delivery of corneal epithelium construct

The hydrogel bandage was fabricated as previously described.[53-55] In brief, poly-ε-lysine (Zhengzhou Bainafo Bioengineering Ltd, China) was cross-linked with nonanedoiic acid (azelaic acid) (Sigma-Aldrich), using the activators N-hydroxysuccinimide (NHS) (Sigma-

Aldrich) and 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide HCl (EDCI) (Thermo Scientific) respectively. The solution was then transferred onto a Petri dish and left to polymerize overnight. The gel was subsequently lifted off and sterilized in 70% ethanol for one hour and washed in PBS for 5 times. Circular gels of desired diameters were cut out using an appropriately sized puncher.

In vitro chemical injury model for testing the transplantation of corneal epithelium construct

A monolayer of human corneal epithelial cell was grown on the transwell® permeable membrane pre-coated with 50 μg/mL collagen type I in 0.02M acetic acid. A circular piece of filter paper, soaked in sodium hydroxide (0.1M), was then applied onto the central region of the corneal epithelial layer to create a chemical injury model. Tissue-like epithelial cell constructs were delivered on the *in vitro* chemical wound. Three groups were compared, namely cell construct-only treated group, cell construct transplanted with bandage group, and control group with no cell construct transplantation. The areas of defect remaining on the wounded region were measured over a period of 7 days using ImageJ. In addition, culture medium was extracted from wells each day for analysis of cytokine levels changes over time of healing. These were subsequently run on the LUNARIS^{TM} Human 11-Plex Cytokine Panel by AYOXXA Biosystems GmbH, which covers granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon gamma (IFN-γ), interleukin-1 beta (IL-1b), interleukin-2 (IL-2), interleukin-4 IL-4, interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), interleukin-12, p70 (IL-12p70), tumor necrosis factor alpha (TNF-α).

Ex vivo porcine cornea model for testing the transplantation of corneal epithelium construct

Primary human corneal epithelial cells were pre-labelled using PKH26 Red Fluorescent Cell Linker Kit (Sigma-Aldrich, US) before forming the constructs. The epithelial cell constructs were transferred to bandage and left to attach overnight at 37 ºC in 5% CO_2. The corneal epithelial layer was scraped off using a surgical blade no. 23, including the limbal area. The corneo-scleral button was cut out and a 0.5% agar solution using DMEM solution and agar powder was used to create an agar button to support the shape of the cornea in the 6 well plates. Thereafter, bandages carrying the epithelial cell constructs were applied onto the de-epithelialized *ex-vivo* porcine corneas.

Cell construct formation in oil-water system

To highlight the performance of ATPS compared with oil-water system, cell construct was formed using culture medium and fluorinated oil (FC-40) as an oil-water biphasic system. FC-40 was added into a microcentrifuge tube and culture medium mixed with cells were added on top of FC-40. The biphasic system was centrifuged at 1,500 rpm for 4 min and incubated in a humidified incubator at 37ºC with 5% CO_2.

Statistical analysis

All numeric data obtained were expressed as mean ± standard deviation (S.D.). Data analysis was performed using GraphPad Prism 8. Unpaired *t*-test and one-way ANOVA were performed. A *p*-value less than 0.05 was considered as statistically significant.

Results and Discussion

The use of the biphasic strategy in forming planar tissue-like constructs in ATPS was first reported in 2015.[46] Simply by suspending cells in the top PEG-rich phase of the PEG/Dex biphasic system, the cells were able to gather on the PEG/Dex interface and self-assemble to form an intact tissue-like structure within a day. This strategy opens many possibilities for forming transplanted tissue constructs in a simple, rapid, and robust manner. However, the ability of the planar construct to self-assemble is highly dependent on the initial spatial distribution of the cells within the PEG solution. That is, if there is uneven cell distribution in PEG, the cells will subsequently be dispersed across the PEG/Dex interface in a non-uniform manner. Therefore, cell-cell adhesion will be unequal across the tissue construct. Eventually, the final structure may be coarse and bear significant defects; in extreme cases, only fragments may form. These could act as major drawbacks for this promising strategy to be potentially translated to bedside. To overcome these obstacles, we optimize this strategy by adding two new procedures. These are 1) pre-equilibration of the two immiscible liquid phases, and 2) centrifugation of the system after seeding cells in PEG-rich phase. We used HCE-T cells for proof-of-concept during the development of these modifications. Pre-equilibration helped provide a more stable PEG/Dex interface for cells to self-assemble into constructs. The addition of the centrifugation step enabled cells to be more uniformly spaced on PEG/Dex interfaces, hence yielding cell constructs with more homogenous thickness. We also find the time required to form the cell construct can be shortened by these modifications.

Above critical concentrations, an aqueous mixture containing two incompatible polymers (e.g. PEG and Dex) will phase-separate.[42] Via the phase separation phenomenon, concentrated PEG and Dex solutions are prepared separately and are used as two immiscible phases to fabricate tissue-like constructs.[46] However, since the contents within the two phases are not pre-equilibrated, all molecules within ATPS, including water, PEG and Dex, will move across the

interface via diffusion and osmosis. The net movement of molecules makes the liquid-liquid interface unstable and not favorable for the assembly of the tissue-like constructs, as illustrated in Fig. 2c. We hypothesized that reducing the osmotic gradient with the equilibration step is crucial in enhancing the success rate in the formation of an intact tissue-like construct, as the net movement of water molecules can be significantly reduced due to this additional step in our methodology. Therefore, cells can interact and self-assemble into tissue-like constructs on a more stable PEG/Dex interface.[56] To test our hypothesis, we fabricated the tissue-like constructs with HCE-T cells using ATPSs with different concentration combinations of PEG and Dex, with or without pre-equilibration. To pre-equilibrate the ATPS, the two phases were first vigorously mixed by vortexing, and then centrifuged at a high speed to facilitate phase separation. Our results show that with pre-equilibration together with centrifugation, intact tissue-like constructs can be formed in more ATPSs (with different concentrations of the two polymers), when compared with those without pre-equilibration or centrifugation (increased from 5 to 9 out of 11 ATPSs) (Fig. 2a, b). We further measured the osmolality of the two phases of ATPSs before and after equilibration using a freezing point osmometer. Our results show that the osmolality difference between the two phases can be reduced by 2.73-times to 37.71-times (within various ATPSs) via equilibration of the ATPS (Table 1). This agrees with our hypothesis that pre-equilibration of ATPS is an essential step in forming intact tissue-like constructs.

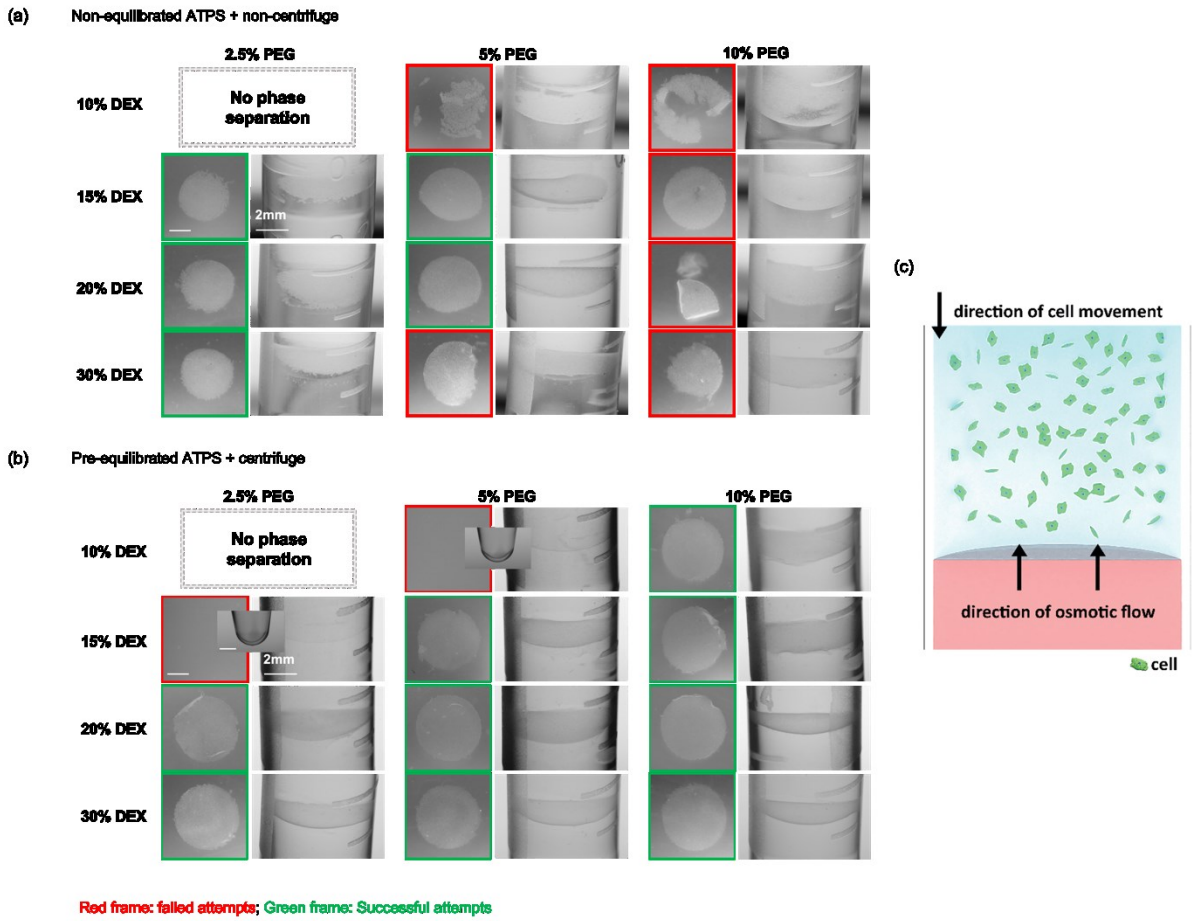

Figure 2 The HCE-T cell construct formed using (a) non-equilibrated ATPS without centrifugation and (b) pre-equilibrated ATPS with centrifugation. Contrast of the photo was adjusted for better visualization of the cell construct. More successful group is observed in pre-equilibrated system with centrifugation compared with non-equilibrated system without centrifugation. Camera photos of the cell construct at the PEG-Dex interface are attached at the side of the microscopic images. Scale bar = 2 mm. (c) Schematic illustrating the cell construct formation with osmotic flow.

Table 1: Osmolality values of PEG and Dex solutions in non-equilibrated and equilibrated aqueous two-phase systems. Red text indicates non-equilibrated system and green text indicates equilibrated system. Unit: mOsm/kg. OD: osmolality difference. (n = 3, *ns* not significant, * p <0.05, **p <0.01, ***p <0.001, ****p <0.0001, unpaired t-test)

DEX conc.	PEG conc. 2.5%	5%	10%
10%	No phase separation	PEG: 390 ± 5 Dex: 360 ± 2 (***) OD: 30 PEG: 381 ± 1 Dex: 370 ± 1 (***) OD: 11	PEG: 505 ± 2 Dex: 360 ± 2 (****) OD: 145 PEG: 424 ± 2 Dex: 415 ± 4 (**) OD: 9
15%	PEG: 357 ± 2 Dex: 391 ± 6 (***) OD: 34 PEG: 373 ± 3 Dex: 363 ± 5 (*) OD: 97	PEG: 390 ± 5 Dex: 391 ± 6 (***) OD: 1 PEG: 392 ± 5 Dex: 384 ± 2 (*ns*) OD: 8	PEG: 505 ± 2 Dex: 391 ± 6 (****) OD: 114 PEG: 453 ± 7 Dex: 446 ± 7 (*ns*) OD: 7
20%	PEG: 357 ± 2 Dex: 454 ± 5 (****) OD: 97 PEG: 394 ± 5 Dex: 375 ± 4 (**) OD: 19	PEG: 390 ± 5 Dex: 454 ± 5 (****) OD: 64 PEG: 416 ± 2 Dex: 407 ± 6 (*ns*) OD: 9	PEG: 505 ± 2 Dex: 454 ± 5 (****) OD: 51 PEG: 488 ± 5 Dex: 472 ± 2 (**) OD: 27
30%	PEG: 357 ± 2 Dex: 769 ± 34 (****) OD: 412 PEG: 450 ± 2 Dex: 428 ± 2(***) OD: 22	PEG: 390 ± 5 Dex: 769 ± 34 (****) OD: 389 PEG: 488 ± 4 Dex: 472 ± 1 (**) OD: 16	PEG: 505 ± 2 Dex: 769 ± 34 (***) OD: 264 PEG: 594 ± 6 Dex: 601 ± 14 (**) OD: 7

The self-assembly of the planar construct is affected by the initial spatial distribution of the cells in PEG-rich solution. As the cells are randomly suspended in PEG-rich solution, cells will reach the interface at different times resulting in a cell distribution on the PEG/Dex interface that is unpredictable, leading to highly variable tissue-like constructs. In addition, the thickness of the cell constructs formed will be uneven. To overcome this problem, we hypothesized that centrifuging the whole ATPS after seeding cells in the PEG-rich phase will improve the uniformity of the tissue-like structure formed. Centrifugation is a widely applied technique for assisting the assembly of particles and colloidal structures.[57-59] Using centrifugation, all the cells are accelerated to reach and occupy the whole PEG/Dex interface due to the centrifugal force. Hence, this improves the uniformity of the distribution of cells on the interface and speeds up the potential of the formation of cell-cell junctions (Fig. 3a). With the additional centrifugation step, the corneal endothelial cell constructs formed were intact. This is completely different from the fragmented samples that are formed without the centrifugation step (Fig. 3a). From fluorescence intensity measurements, the cell construct formed with centrifugation have a roughness index of 17.70%. This value is significantly lower than that of the cell construct formed without centrifugation (31.46%) (Fig. 3c). Since the fluorescence intensity is related to the tissue thickness, a smaller deviation value indicates a more uniform thickness of the cell construct. We speculate that the low uniformity accounts for the fragmentation of the tissue-like constructs. In the regions of the cell construct that are relatively thinner, the mechanical strength is lower and easily fragmented. With an additional centrifugation step during the fabrication of the cell construct, a more uniform and robust planar tissue-like structure can be formed within ATPS. Moreover, the time for corneal endothelial cell construct formation was shortened with the aid of centrifugation (Fig. 3b). Based on the results obtained after addition of the two procedures, and considering the effect of osmolality to cells, [60, 61] the ATPS components used in the subsequent experiments were chosen to be 10%

PEG / 15% Dex. The incubation time for self-assembly of cells was optimized as 3 hours to minimize the osmolality effect on the cells.

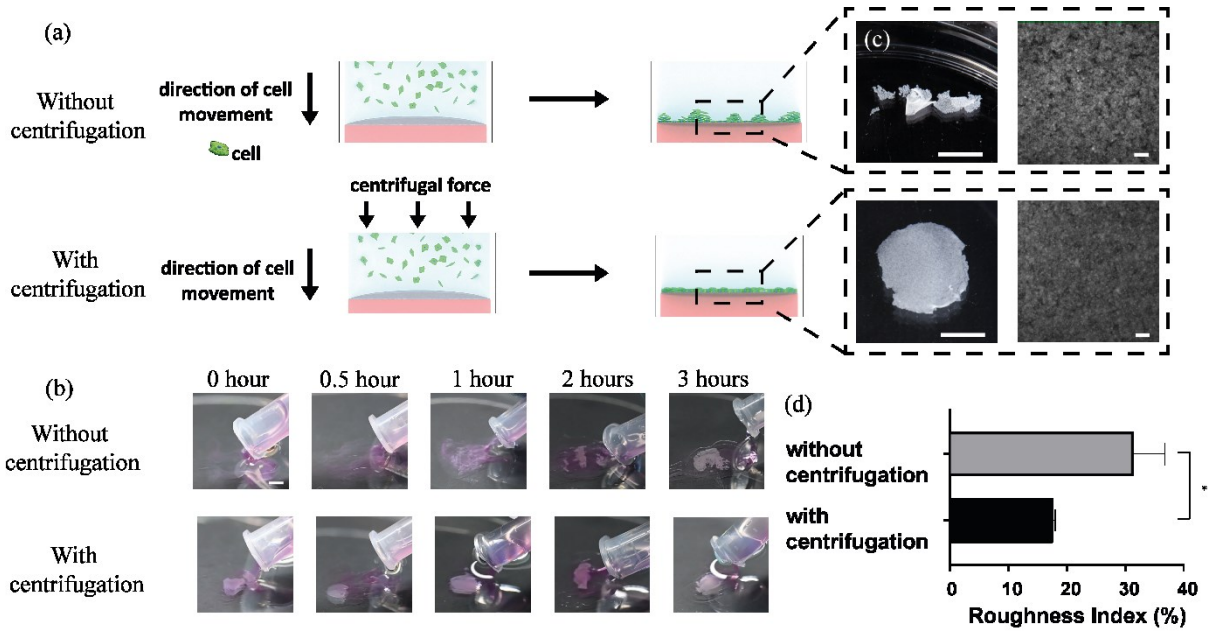

Figure 3 (a) Schematic illustrating the role of centrifugation on cell construct formation. (b) Comparison of the primary porcine corneal endothelial cell construct formation time by the approaches with and without centrifugation. The time indicates the incubation time after construct formation. Scale bar = 3 mm. (c) The camera image (left) and fluorescence micrograph (right) of the primary porcine corneal endothelial cell construct formed with and without centrifugation approach respectively. Scale bars = 3 mm for camera image and 100 μm for fluorescence micrograph. (d) The roughness index of the pre-stained cell construct formed with and without applying centrifugation. (n=3, *p <0.05, unpaired t-test)

The above shows that the ATPS liquid substrate allows for scaffold-free assembly of cell-based constructs, whereas the additional centrifugation procedure improves the compactness and uniformity of the cell constructs formed.. Using the modified and optimized conditions as aforementioned, we fabricated the planar construct using primary porcine corneal endothelial cells. The primary cells obtained are cautiously cultured with appropriate characterizations prior to use. (Supplementary Fig. 1) The majority of the cells in the cell construct were viable, and these are stained in green using live-dead staining (Fig. 4b). In *in vitro* culture, the area of the cell construct doubles after around 3 days, showing the proliferative capacity of the cells in the construct. Moreover, the cultured cell construct expanded to fill up most of the culture space in 6-well plate after 21-day *in vitro* culture (Fig. 4a). The proliferative ability is important because the corneal endothelial cells need to grow and fully cover the endothelial surface of the cornea after removal of the damaged and diseased cells. The coverage of the corneal endothelial cells on the inner corneal surface is critical for restoration of its function, which is to regulate the water content of the corneal tissue effectively. In addition, the cell constructs cultured *in vitro* can express the physiological markers of corneal endothelial cells that express *in vivo*. The cells expressed high levels of both ZO-1 and Na^+/K^+ ATPase, as shown in green and red respectively (Fig. 4c). The reconstruction of these physiological markers is critical in restoring functions of the corneal endothelium. Tight junctions are responsible for resisting the passive diffusion of water,[62] whereas sodium potassium ATPase regulates the water potential of the corneal tissue.[63] These functions are crucial for maintaining the transparency of cornea. Moreover, the endothelial layer formed using the biphasic construct approach has a more compact organization of the cells, when compared with the conventional 2D cell culture method. (Fig. 4c and 4d) The mean cell area of the cells in the planar construct, defined as the area bounded by the ZO-1 expression, is 186.04 μm^2, which is just 40% of that of cells in 2D cell culture (462.14 μm^2) (Table 2). In converting the mean cell area into cell density, a smaller

mean cell area indicates a higher cell density. Since the corneal endothelial cell density is expected to decrease gradually after transplantation, a high initial cell density in the donor graft is more favourable for transplantation purposes.[64] The higher initial cell density also provides promising evidence for restoration of the corneal function in a more physiological representative manner *in vitro*. To prove that the cell construct is physiologically active *in vitro*, a transwell® system is applied to split the *in vitro* environment into top and bottom components. Since corneal endothelial cells are responsible to pump out excess water from the corneal tissue, the corneal endothelial cells cultured *in vitro* can therefore generate an osmotic gradient between the top and bottom phase if the cultured corneal endothelial cells are physiologically active. As shown in figure 4e, after one-month *in vitro* culture, the cell construct can generate a significantly different osmolality value between the top and bottom compartments in the transwell® system. ($p< 0.01$, unpaired *t*-test, n=3). Besides the water transportation, the cell construct was also able to perform the barrier function. From the FITC-Dex diffusion assay (figure 4f), the cell construct transplantation group and cell suspension cultured for 1 month *in vitro* were able to resist the FITC-Dex diffusion compared with blank control, hence indicating we were able to reconstruct its physiological functionality. To demonstrate the possibility of the transplantation of the corneal endothelium construct, we perform a trial to transport the construct into an *ex vivo* porcine eye model with reference to a procedure called Descemet Membrane Endothelial Keratoplasty (DMEK). DMEK is a well-established ophthalmic surgical procedure in which a corneal endothelial donor graft is transplanted onto the recipient's eye.[65] In this trial, the corneal endothelial construct stained with 0.4% brilliant blue G is loaded into the IOL injector. Then, the injector is inserted into the anterior chamber of the porcine eye through an incision, through which the cell construct can be transported into the eye, (Figure 4g). The success of this trial suggests that the ATPS approach in fabricating corneal endothelial construct possesses the translational potential into clinical application.

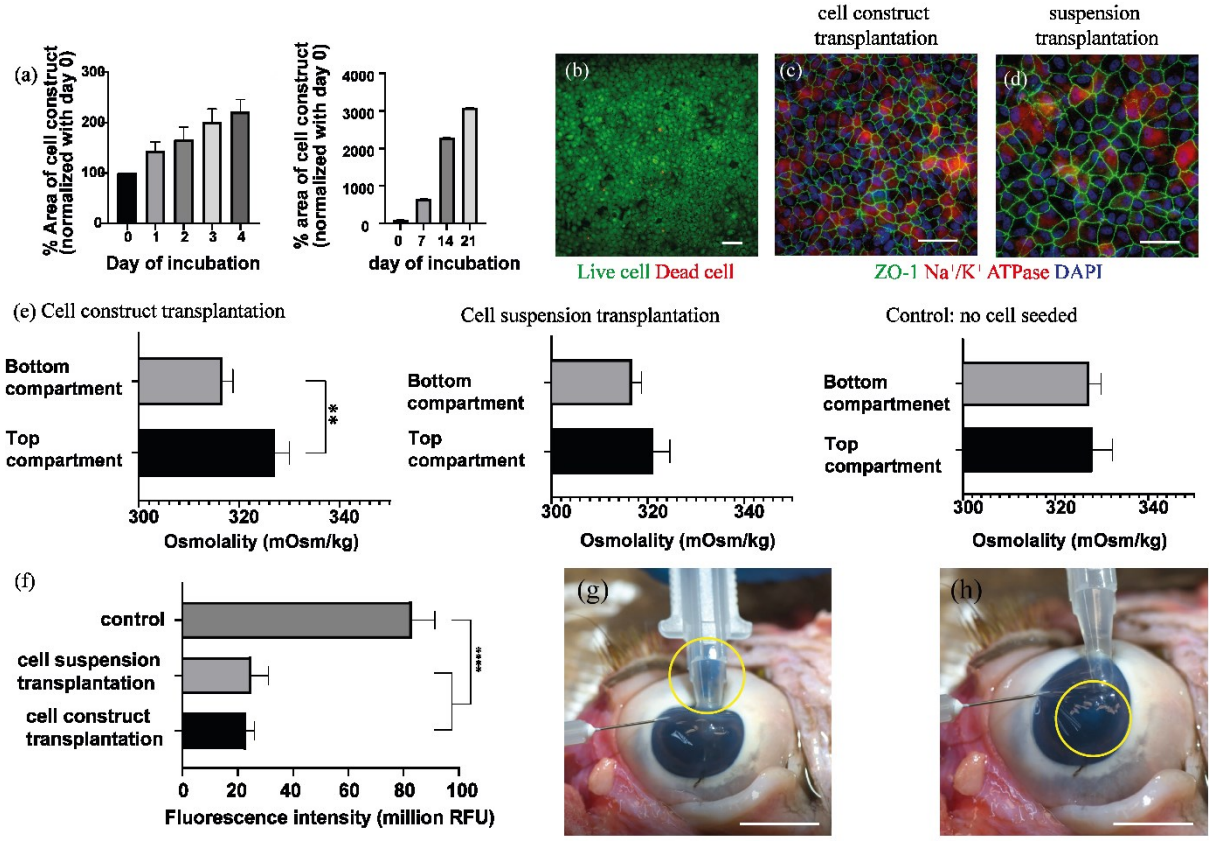

Figure 4 (a) The primary porcine corneal endothelial cell construct is able to proliferate *in vitro*. (b) Majority of the live cells are observed in the cell construct cultured *in vitro* for 4 days. Green and red expressions are representing live cells and dead cells respectively. Scale bar = 100 µm. The primary porcine corneal endothelial cell construct can express proteins that are related to its physiological function *in vitro*. Sodium-potassium ATPase (Na^+/K^+ ATPase) and Zonula occludens-1 (ZO-1), which is stained in red and green respectively, are both expressed in (c) cell construct transplantation and (d) cell suspension-based transplantation after 1-month *in vitro* culture. Scale bar = 50 µm. *ex vivo* injection of the primary porcine corneal endothelial cell construct into isolated porcine eyeball. (e) The osmolality value of the top and bottom phases of the transwell® system cultured with cell construct and cell seeded as single cell suspension. (** $p< 0.01$, unpaired *t*-test, n=3) (f) The fluorescence intensity of the top compartment medium in the Transwell® system cultured with cell construct and cell seeded as single cell suspension in FITC-Dex diffusion functionality assay. (**** $p< 0.0001$, one-way ANOVA) (g) The cell construct pre-stained with 0.4% Brilliant Blue G is loaded into an intraocular injector. The nozzle inserted in the eyeball is connected to the saline perfusion system. (h) After injection, the cell construct is injected into the anterior chamber of the porcine eyeball. Scale bar = 1 cm.

493 Table 2: Mean cell area and cell density for primary porcine corneal endothelial cell construct

	Mean cell area (μm^2)	p-value with respect to cell construct	Translated cell density (cells/mm^2)
Cell construct	186.04 ± 76.62	/	5375.19
Experimental control seeded as cell suspension	462.14 ± 169.25	< 0.0001 (****)	2163.57

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Besides reconstruction of corneal endothelium, regeneration of corneal epithelium also has a high clinical value.[18, 66] Under corneal epithelial injury, the loss of the epithelial layer can lead to corneal ulcer, as the inner structure of cornea is exposed. Stromal complications such as scarring, hazing, edema, and blindness as an end-stage sequelae may therefore occur.[67] First-line treatment options for corneal epithelial defects, including drugs, bandage contact lens, and amniotic membrane or corneal donor draft transplantation for severe defects, are available.[68] However, most come with complications and may compromise the long-term efficacy of the respective management strategies.[69] We believe the rapid formation of corneal epithelial layer through this biphasic strategy may provide an alternative treatment for corneal epithelial defects. To demonstrate such translational potential, we have developed an *in vitro* chemical corneal injury model on a typical transwell® culture system. A monolayer of human corneal epithelial cell is firstly grown on the transwell® permeable membrane. Sodium hydroxide solution, as absorbed by a filter paper, is then applied onto the central region of the corneal epithelial layer to create a chemical injury. Tissue-like corneal epithelial cell constructs are successfully fabricated using the biphasic strategy, and delivered onto the *in vitro* chemical wound. Proliferation of cells from both the cell construct and the edges of the chemical wound is observed from time-lapse images. The percentage areas of defect of the chemical wound, after transplantation of epithelial cell construct, significantly decrease over time. A complete recovery, defined as when the cells re-epithelize to fully cover the wounded area, is achieved after day 7 on the *in vitro* chemical injury model (Fig. 5a). As the epithelial cell construct is not monolayered, the cells which are not adhered to the membrane eventually detach from the cell construct on day 4 (Fig 5b). An intact layer of epithelial cells remains underneath to cover the wound area. The layer successful integrates with the peripheral cells on the transwell® membrane after chemical injury and closes the wound eventually on Day 4. Cytokine studies reveals significant changes in levels in GM-CSF, IL-1b, IL-6, IL-8, and TNF-α. A rise in these

levels is observed during the initial period of cell construct transplantation in both the cell construct-only group and the group with cell construct transplantation using the bandage, compared with control group ($p < 0.05$) (Fig 5c). Levels peak generally at either day 2 to 3 for the cytokines, and gradually diminish to reach baseline level by day 7, matching that of control groups. The initial surge in cytokines during day 1 to 3 correlates with an increased rate of proliferation of the cell construct during the same period, hence a greater initial drop in percentage area of defect on the Transwell® chemical injury model. This is expected since GM-CSF, IL-1, IL-6, TNF-α are recognised for aiding corneal epithelial cell migration after injury.[70] GM-CSF has been demonstrated to improve the rate of corneal epithelial wound repair by increased cell migration both in vitro and in vivo, but without significant effect on corneal epithelial cell proliferation.[71] IL-1 has been postulated to have additive effect on corneal wound closure with epidermal growth factor (EGF), via increasing the receptor binding ability of EGF up to 110%.[72,73] It is also believed that IL-1 could induce keratocyte apoptosis, hence regulating the epithelial-stromal interactions by maintaining tissue organisation hence plays a role in corneal wound healing.[74] In previous studies, elevated TNF-α levels was found after corneal chemical injury, since it is a major proinflammatory cytokine.[75] Its interaction with other cytokines such as TGF-β and IL-1β may play a role in balancing of signalling pathways in corneal wound healing.[75, 76] IL-6, on the other hand, is involved in the initial phases of epithelial wound closure by stimulating the epithelial cells. [77, 78] Lastly, IL-8 is positively regulated by macrophage colony-stimulating factors (M-CSF) during active inflammatory processes, as in the chemical insult demonstrated in this *in vitro* model.[79]

Since the corneal epithelial cell construct will ultimately be transplanted onto the corneal wound to facilitate repair of the epithelial defect, a hydrogel bandage is needed to act as a vehicle to transport and stabilize the construct on the wound. The bandage will also be able to protect the cell construct from detaching off of the ocular surface after transplantation onto real

patients, since it will act as a barrier between friction generation from the ocular surface and the eyelids during blinking action. The corneal epithelial construct can attach well on our antimicrobial peptide hydrogel bandage. [53] On the *in vitro* corneal injury model, the corneal epithelial construct on the bandage exhibits similar proliferative capacity to the construct without the bandage vehicle (Fig. 5a and Fig. 5b). The results suggest that the vehicle does not hinder the proliferation of the cells on the wound, and therefore does not affect the potential wound healing capacity of the construct. To further demonstrate its clinical translation potential, the corneal epithelial construct on the bandage is first transported onto a de-epithelialized *ex vivo* porcine cornea. The bandage is removed 3 days after transplantation, and the cell construct is successfully retained and attached onto the denuded porcine cornea. (Fig. 6a) Only a small amount of corneal epithelial cells remains on the bandage. The area covered by corneal epithelial cells increases from 14.8 ± 3.5 mm^2 on day 3 to 18.9 ± 3.4 mm^2 on day 7 ($n = 6$) (Fig. 6a). In comparison the conventional cell-seeding, which involves seeding of corneal epithelial cells onto the bandage directly, allowing them to adhere on the hydrogel surface, then gently placing them onto the denuded corneal surface with cell-side facing down, we observe a reduced transfer of cells from the bandage onto the porcine cornea, with increased retention of cells on the bandage material (Fig. 6b). A thinner layer of epithelial cells is also observed in the conventional cell-seeding group through H&E-stained corneal tissue sections, when compared with ATPS-construct group (Fig. 6c). These results highlight the higher cell transferal efficiency with the ATPS-construct approach for delivering cells onto the corneal epithelial wound. In addition, the multi-layered nature of the epithelium, as demonstrated by the ATPS-construct group, bears greater resemblance to the normal architecture of the corneal epithelium when compared with the transplanted epithelium derived from the conventional cell-seeding group. This is important since the epithelial layer has to be thick enough to act as

a protective barrier for the eye, and also thin and hence transparent enough to allow light to reach the retina.

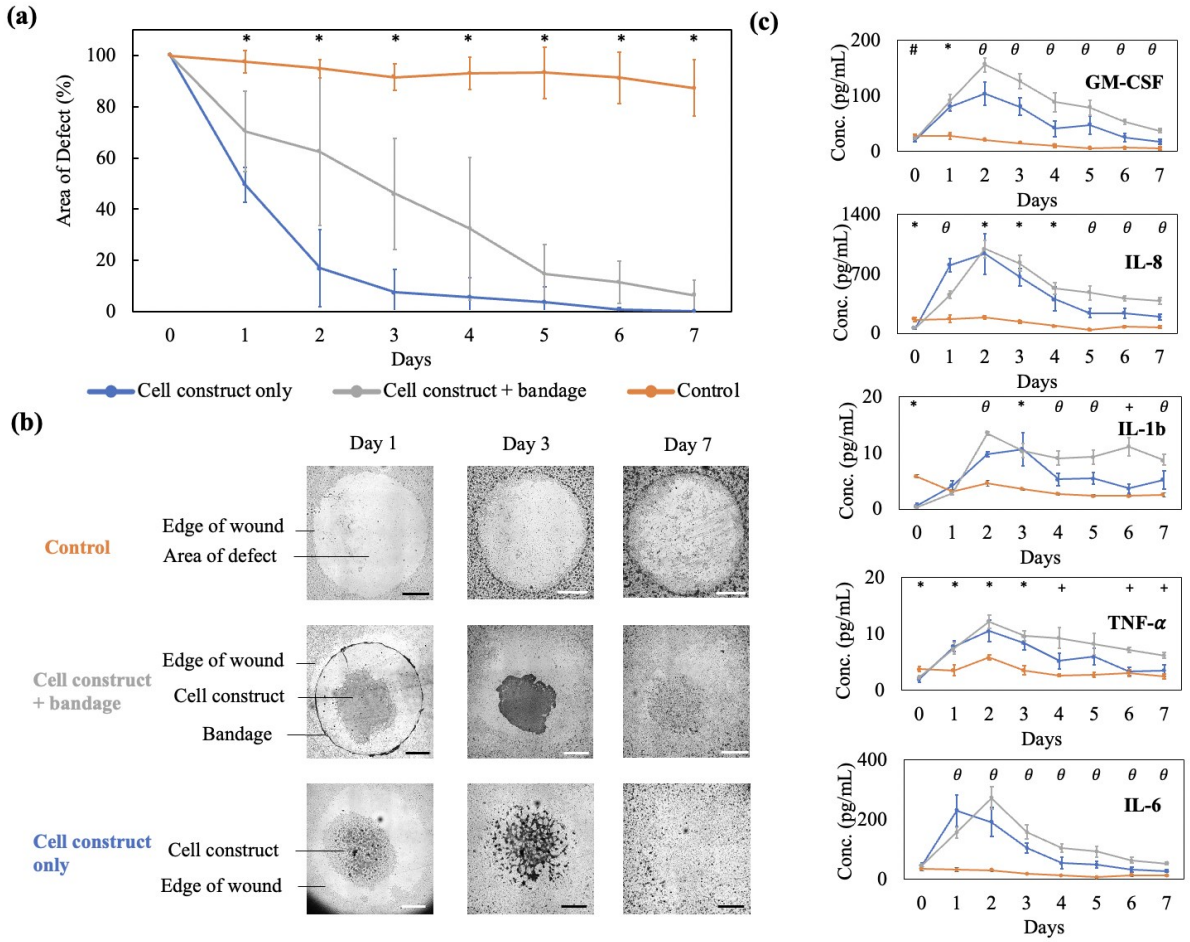
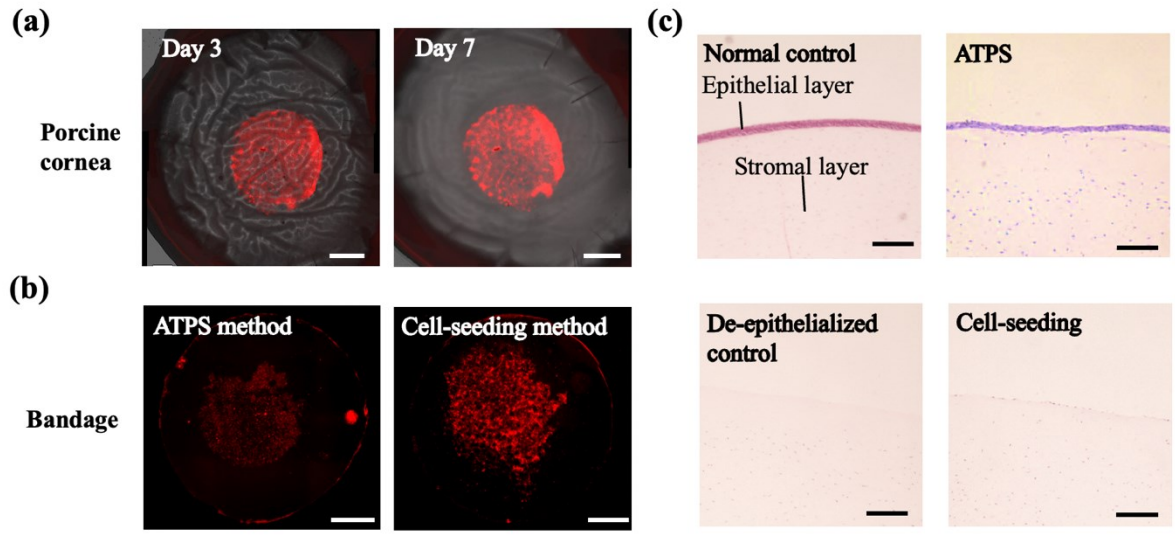


Figure 5 Results of experiments on the *in vitro* Transwell® chemical injury model. (a) The area of defect measured in percentage of initial area of injury on the *in vitro* Transwell® chemical injury model. (b) Wound and cell construct proliferation observed under light microscopy. Control group demonstrated minimal proliferation of wound edges up until Day 7 post-transplantation. Cell construct proliferation was demonstrable in both cell sheet only group and cell construct with bandage groups, but the former had much more significant growth rates. Scale bar = 2 mm. (c) Changes in concentrations of cytokines with cell construct growth across a 7-day observation period. (* $p < 0.005$ for both cell construct only vs control, and cell construct with bandage vs control; # $p < 0.005$ only for cell construct vs control; + $p < 0.005$ only for cell construct with bandage vs control; θ $p < 0.005$ for all of cell construct only vs control, cell construct with bandage vs control, and cell construct vs cell construct with bandage)



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586 **Figure 6** Results of experiments on the *ex vivo* porcine cornea model. (a) Cell construct (in red)
587 on porcine cornea observed microscopically on Day 3 and Day 7 after removal of bandage.
588 Scale bar = 2 mm. (b) Remaining cells (in red) adhered on bandage after its removal from
589 porcine cornea at Day 3, whilst the rest of the cells are transferred onto the porcine cornea
590 model. Bandage from both ATPS method and cell-seeding method are demonstrated. Scale bar
591 = 2 mm. (c) H&E staining of corneal sections which underwent cell construct transplantation
592 via ATPS and conventional cell-seeding method, compared with normal and de-epithelialized
593 controls Scale bar = 200 μm.

594

595

The use of a liquid-liquid interface as a liquid substrate allows individual cells to gather and assemble to form scaffold-free tissue-like constructs. However, only polymer-polymer ATPS, not other biphasic systems, such as oil-water and oil-oil system, can be used. FC-40, a fluorinated oil, is regarded as a relatively biocompatible oil. We repeated the same experiment using a FC-40/culture medium biphasic system. However, unlike in a PEG/Dex system, the cell constructs could not be formed in FC-40/culture medium system (Supplementary Figure 2). Although the oil-water system is extensively used in the fabrication of emulsion-based biomaterials,[80, 81] the oil phase can lead to protein denaturation and lipid oxidation at the oil-water interface.[82, 83] We also find that cell construct cannot be formed in polymer-salt ATPS (Supplementary Figure 3). We repeated the cell construct formation experiment with PEG/sodium citrate polymer-salt ATPS and no cell construct formation is observed. The reason could be due to the loss in cell viability in hypertonic environment. Since a significant high salt concentration (at least 5 wt%) is required to establish a salt-polymer ATPS [84], such high salt concentration environment is undoubtedly not favourable for biological cells. The results highlight the superiority in using polymer-polymer ATPS to form scaffold-less tissue-like constructs using the biphasic strategy. We anticipate this approach to be extended to the fabrication of transplantable cell layers in other areas of the eye, including the retinal pigment epithelium,[25, 31, 85] which is associated with aged-related macular degradation.[86, 87] Moreover, the proposed strategy should, in principle, allow layer-by-layer assembly of different cell types. Such assembly can be achieved simply by adding another layer of PEG-cell mixture after forming the first tissue layer.[46] This strategy may therefore pave the way towards the reconstruction of complex multicellular tissues such as the retina.

Conclusion

The described ATPS interfacial strategy for fabricating corneal-relevant tissue constructs entails modification of the previously described protocol, yet with demonstrable improved success rates and robustness of formed tissue-like constructs. This strategy can be easily implemented in general biomedical laboratories, as no specialized instruments are required. The corneal endothelial cell construct fabricated using this strategy demonstrated proliferative capacity and expression of physiologically relevant markers *in vitro*. The corneal endothelial cell construct could also be able to implant into the anterior chamber of *ex vivo* porcine eyeball using standard surgical tools. Similarly, the corneal epithelial construct expressed its potential to facilitate corneal wound healing *in vitro*. These results demonstrate that formation of planar cell constructs based on all-aqueous liquid systems may act as a versatile strategy for creating corneal-relevant tissue constructs for both potential transplantation purposes.

635 **Supporting Information**

636 Additional details of experiments including images of experimental results.

637

638 **Figure SI-S1.** Characterization of the isolated porcine corneal endothelial cell. The porcine
639 corneal endothelial cell isolated is physiologically representative with high purity.
640 Physiological markers are expressed after *in vitro* culture. Immunocytochemistry is
641 performed after 1-month culture of the corneal endothelial cells. Green and red expressions
642 are representing Zonula occludens-1 (ZO-1) and N-cadherin (N-cad) expression respectively.
643 Scale bar = 100 μm.

644 **Figure SI-S2.** Formation of intact cell construct in ATPS but not oil-water systems. (a) Cell
645 construct is formed in ATPS. Black arrow indicates the position of the cell construct. (b) The
646 cell construct formed is intact. Scale bar = 1 mm. (c) Cells in the cell construct are tightly
647 packed. Scale bar = 100 μm. (d) However, cell construct cannot be formed in oil-water system.
648 (e, f) Only cell clumps are observed under inverted light microscope. Scale bars = 1 mm for (e)
649 and 100 μm for (f).

650 **Figure SI-S3.** Result of cell construct formation experiment in PEG/sodium citrate polymer-
651 salt system. (a) Cell construct cannot be formed in polymer-salt system with all concentration
652 combination of PEG and sodium citrate. (b) MTS cell viability assay of porcine corneal
653 endothelial cell incubated in sodium citrate for 3 hours. (n=5, ****p<0.0001, one-way
654 ANOVA followed with Dunnett's test)

655

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679 Data availability statement: Data can be made available upon reasonable request from the
680 corresponding author.

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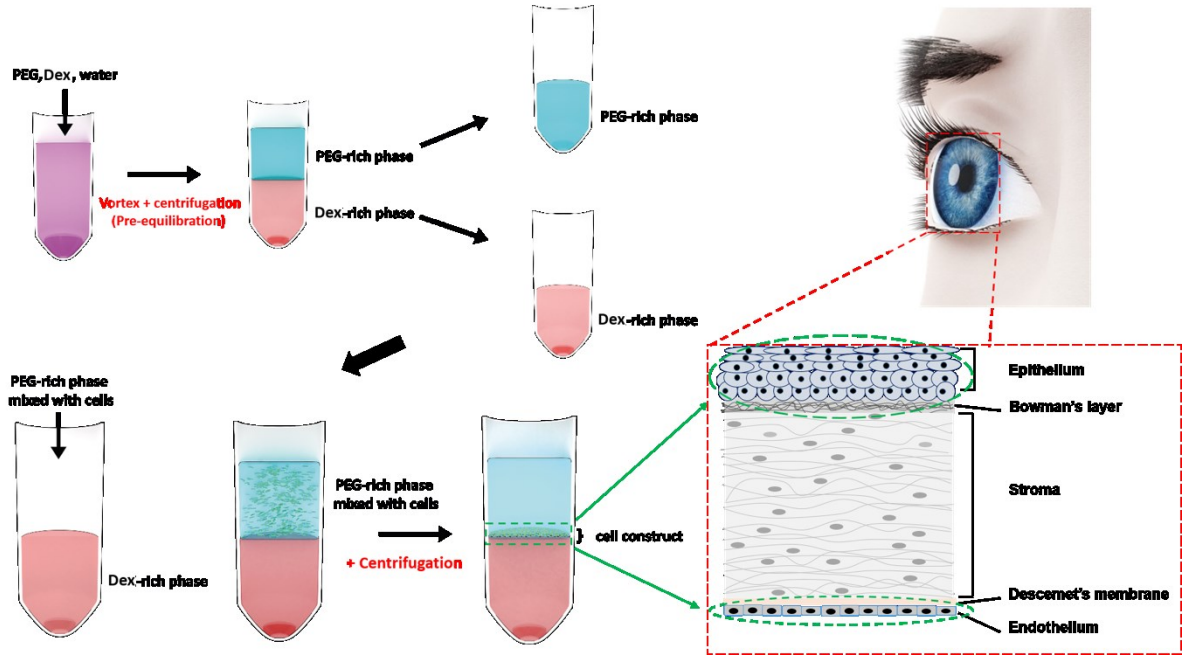

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