Biodegradable and Biocompatible Poly(Ethylene Glycol)-based Hydrogel Films for the Regeneration of Corneal Endothelium

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Corneal endothelial cells (CECs) are responsible for maintaining the transparency of the human cornea. Loss of CECs results in blindness, requiring corneal transplantation. In this study, fabrication of biocompatible and biodegradable poly(ethylene glycol) (PEG)-based hydrogel films (PHFs) for the regeneration and transplantation of CECs is described. The 50-μm thin hydrogel films have similar or greater tensile strengths to human corneal tissue. Light transmission studies reveal that the films are >98% optically transparent, while in vitro degradation studies demonstrate their biodegradation characteristics. Cell culture studies demonstrate the regeneration of sheep corneal endothelium on the PHFs. Although sheep CECs do not regenerate in vivo, these cells proliferate on the films with natural morphology and become 100% confluent within 7 d. Implantation of the PHFs into live sheep corneas demonstrates the robustness of the films for surgical purposes. Regular slit lamp examinations and histology of the cornea after 28 d following surgery reveal minimal inflammatory responses and no toxicity, indicating that the films are benign. The results of this study suggest that PHFs are excellent candidates as platforms for the regeneration and transplantation of CECs as a result of their favorable biocompatibility, degradability, mechanical, and optical properties.

1. Introduction

Annually, thousands of corneal transplantation procedures take place worldwide.[1] The foremost reason for corneal transplantation is the loss of function of corneal endothelial cells (CECs).[1,2] CECs are specialized, polygonally shaped cells that reside on the inner surface of the cornea within the anterior chamber. CECs are responsible for actively pumping fluids to maintain the semi-dehydrated state of the cornea and preserve its transparency.[2] Various factors can lead to the loss of CECs, including aging, trauma, and disease.[2,3] Once the CEC numbers are critically reduced, the cornea loses its optical clarity due to oedema, ultimately leading to blindness.[2] Human CECs do not regenerate in vivo, thus in the case of severe loss of cell numbers, it is necessary to undergo corneal transplantation in order to restore vision.[1,2] As a result of the highly invasive nature of full-thickness corneal transplants, less invasive methods such as Descemet's stripping endothelial keratoplasty (DSEK) have been developed.[4,5] As part of the DSEK procedure, the Descemet's membrane is removed from the diseased cornea via a small incision into the anterior chamber. Subsequently, a donor corneal endothelium supported by a thin layer of stroma is inserted into the anterior chamber and allowed to rest upon the interior surface of the cornea to replace the CECs.[4] The technique itself is less invasive, healing rates are more rapid, and the chances of infection are greatly reduced.[5] However, as a result of using donor tissue, there are also risks associated with rejection and graft

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failure.[5–7] As such, the development of an autologous transplant for the treatment of corneal endothelial dysfunction is exceedingly attractive.

Various studies have investigated the regeneration of CECs as an alternative to the current transplantation methods.[8–10] CEC regeneration in vitro, using primary or immortalized cells, has been studied for CEC monolayer formation, as well as potential transplants.[8–10] Possible application of cultured CEC sheets as transplants has been investigated in rabbit models,[10] but the use of cell sheets alone limits the surgical options, since more invasive procedures are required as a result of CEC sheet fragility. In contrast, other studies have focused on the use of supporting substrates as platforms for CEC culture and transplantations to overcome the issue of cell sheet fragility.[11–13] In these studies, mainly substrates derived from collagen, cross-linked via various different approaches were employed. Although natural collagen of bovine or porcine origin has been demonstrated to be biocompatible and support CEC attachment and regeneration, there still remain issues associated with the effects of harvesting, isolation and purification. Such processing negatively impacts upon the mechanical properties of collagen, which in its processed form is rapidly degraded in vivo due to disassembly of the natural structure and cross-links.[14] Other materials such as hyaluronic acid, amniotic membrane, and silk fibroin have also been investigated as potential substrates for CEC regeneration and transplantation with promising results.[15–17] These materials have desirable tensile properties, support CEC proliferation, and were implanted in vivo; however, there are issues associated with variation in quality, chemical stability, biodegradation, immune responses, and disease transmission, which limit their application.[18–22] In comparison, a synthetic substrate would minimize this variation in quality, eliminate the risk of disease transmission and allow a higher degree of control during fabrication and characterization.[23] Thus, a fully synthetic substrate with controllable and tailorable physical properties would be desirable if it possesses suitable biocompatibility, degradation, and CEC regeneration characteristics.

Poly(ethylene glycol) (PEG) is a water-soluble synthetic polymer that has been widely explored for biomedical applications as a result of its low-toxicity, minimal immunogenicity, and anti-protein fouling properties.[24,25] As a Food and Drug Administration (FDA) approved polymer for human use in drug and cosmetic applications,[24] PEG has also been widely investigated for tissue engineering, including ophthalmic applications.[26] For such applications, PEG is often combined with natural materials such as collagen or cell adhesion ligands such as RGD (Arginine-Glycine-Aspartic amino acid sequence common in proteins such as collagen and fibronectin) for improved cell–surface interactions and attachment.[27,28] Polycaprolactone (PCL), on the other hand, is a hydrophobic polymer that has been utilized for various biomedical and tissue engineering applications as a result of its biocompatible and biodegradable characteristics.[29,30] Studies have shown that when combined with polymers, such as PEG and chitosan, significant improvements in the compressive and tensile properties of the final material were observed.[31,32] Fully synthetic PEG-based films with suitable mechanical properties and without the incorporation of proteins or other natural ligands have not been studied previously for corneal endothelial regeneration and implantation. Hence, the combination of PEG and PCL in a single construct may allow the fabrication of biocompatible and mechanically robust materials that are suitable for such ophthalmic applications.

Previously we had reported the fabrication of epoxy-amine cross-linked chitosan–PEG hydrogel films that demonstrated excellent physico-chemical properties and in vitro responses for applications in regeneration of CECs.[33] Even though our initial studies had shown favorable results in vitro, our subsequent in vivo studies indicated major inflammatory and foreign body responses towards the chitosan-based films (results not published). In order to overcome these unfavorable in vivo responses, in this study, we report the fabrication of fully synthetic, biocompatible and biodegradable PEG-based hydrogel films (PHFs) via a novel synthetic approach, involving the reaction of multifunctional PEG stars with acid chloride derivatives, while PCL was covalently incorporated into the PHFs to provide robust tensile properties. The PHFs were designed for the regeneration of healthy autologous CECs that could be grown in vitro on the films and then transplanted into the damaged cornea via a DSEK-like procedure, allowing full restoration of vision. This would eliminate the need for donor tissue and avoid related complications associated with current corneal transplantation methods.

2. Results and Discussion

2.1. Preparation of PEG-Based Hydrogel Films

The PHFs were prepared using glycerol ethoxylate (GE), sebacoyl chloride (SebCl), and various amounts of α,ω-dihydroxy-poly(ε-caprolactone) (PCL) (0, 5, and 10 wt%) dissolved in dichloromethane, which were then cast in glass petri dishes and allowed to cure (**Figure 1**). GE is a hydrophilic star polymer composed of three PEG arms and SebCl is a diacid chloride derived from the naturally occurring sebacic acid. The reaction between the acid chloride groups of SebCl and the hydroxyl end groups of GE leads to the formation of ester bonds, resulting in the evolution of HCl and cross-linking of the GE (Figure 1). Our earlier studies have shown that the covalent incorporation of PCL into PEG hydrogels leads to improvements in mechanical properties.[31] Therefore dihydroxy-PCL was synthesized via ring-opening polymerization (ROP). As part of the precursor formulation, PCL was covalently cross-linked within the hydrogel films via the hydroxyl end groups (Figure 1). As a result of the small thickness of the films and the casting conditions (20 mbar, 60 °C) the volatile solvent dichloromethane and HCl gas generated during the reaction are readily removed during the casting process. Subsequent washing and swelling of the hydrogel film with water was conducted to ensure complete removal of HCl. Complete removal of the HCl is imperative since its highly corrosive nature makes its presence in an implantable material highly undesirable and it could catalyse premature degradation of the films. Theoretically, reaction of two equivalents of GE (tri-functional) with three equivalents of SebCl (di-functional) would generate a fully cross-linked network. However, in this study a 35 mol% excess of SebCl

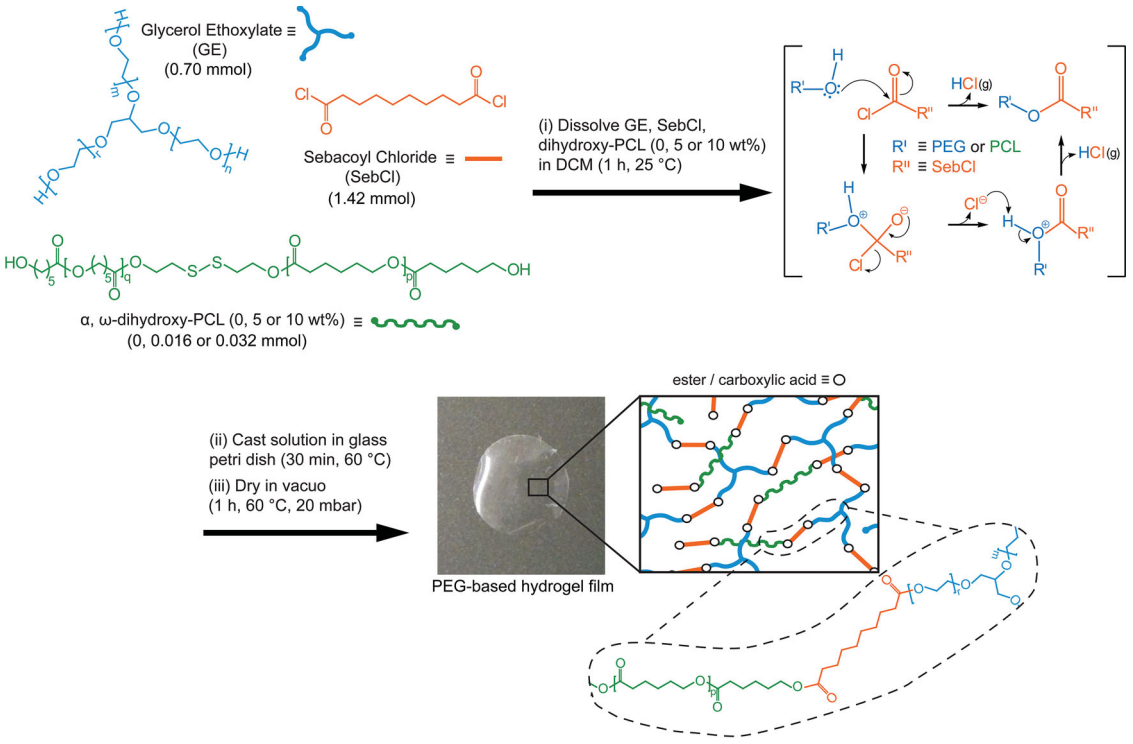

Figure 1. Synthesis of cross-linked PHF via reaction of GE, SebCl, and α,ω-dihydroxy-PCL.

was used (an approximate GE:SebCl ratio of 2:4) to generate cross-linked networks with free acid chloride groups, which were subsequently hydrolysed to carboxylic acid functionalities during washing of the films with water. These carboxylic acid functionalities can be used for future immobilisation of proteins or other molecules depending on the application; however in this study, no post-modification of the films was conducted following cross-linking. Subsequent replacement of the water in the petri dish with tetrahydrofuran (THF) facilitates solvent exchange between water and THF within the film, and the resulting swelling allows the film to easily detach from the glass surface, which in a dry state is not achievable without damaging the cast film. Cross-linking via acid chloride/alcohol chemistry provides rapid and facile access to versatile substrates without the need for additional additives (e.g., coupling reagents) or complex fabrication processes. Surprisingly, acid chloride/alcohol chemistry has not previously been employed to cross-link hydrogel films for tissue engineering purposes. The use of commercially available and cheap precursors also makes this approach particularly appealing and amenable to large-scale production.

2.2. Evaluation of Swelling Characteristics and Contact Angle Measurements

The swelling characteristics of the PHFs were investigated by calculation of the %equilibrium swelling ratio (%ESR) with and without the incorporation of PCL. The films were allowed to completely swell in phosphate buffered saline (PBS) for 24 h. With increasing PCL content (0, 5, and 10 wt%), the %ESR of the PHFs decreased slightly (118%, 109%, and 100%, respectively) (**Table 1**). A similar trend was found in our previous study whereby the water-repelling effect of the hydrophobic PCL component reduced the swelling capabilities of the hydrogels resulting in lower %ESR with higher PCL contents.[31]

Water contact angle measurements were carried out on the surfaces of completely swollen PHFs using a sessile drop method to determine the effect of PCL content on film hydrophilicity. Water droplets were placed on the films and allowed to equilibrate for 1 min. When the PCL content of the films was increased from 0 to 5 and 10 wt%, the contact angles increased from 44° to 58° and 67°, respectively (Table 1). This indicates that as observed with the %ESR, additional PCL leads to an increase in the hydrophobicity of the PHFs.

2.3. Structural Analysis of the PHFs

Attenuated total reflectance infrared (ATR-IR) spectroscopy was employed to confirm the chemical structure of the cross-linked PHFs in the dehydrated state (**Figure 2**). The intense peaks observed in the spectra between 1000 and 1150 cm^{-1} are characteristic of C–O stretches and are attributed to the backbone of the PEG, as the intensity of this peak is not significantly affected by the increase in PCL content. The C–O stretches between 1150 and 1300 cm^{-1}, on the other hand, result from the C–O bonds of the PCL ester groups and the ester groups formed during cross-linking. Figure 2 clearly shows that these C–O stretches increase in intensity as the PCL content is increased, indicating the incorporation of PCL. The sharp C=O stretch observed at ca. 1725 cm^{-1} is indicative of carbonyl

Table 1. Swelling properties and contact angle measurements of the PHFs with comparison of mechanical properties to that of the human cornea and Descemet's membrane at various PCL contents.

Material	PCL content [wt%]	ESR [%]	Contact angle [°]	Ultimate stress [MPa]	Ultimate strain [%]	Tensile modulus [MPa]
PHF	0	118 ± 4	44 ± 0.4	1.5 ± 0.2	33 ± 5	3.3 ± 0.6
	5	109 ± 5	58 ± 0.7	3.7 ± 0.7	71 ± 17	3.8 ± 0.8
	10	100 ± 4	67 ± 0.4	5.2 ± 0.2	61 ± 3	6.3 ± 0.8
Human cornea[a)]	–	–	–	3.3 ± 0.2	60 ± 15	15.9 ± 2.0
Descemet's membrane[b)]	–	–	–	0.3 ± 0.01	1.7 ± 0.2	2.6 ± 0.4

[a)]Values taken from the literature. [14] [b)]Values taken from the literature.[34]

groups and is attributed to the ester groups of the PCL and esters and carboxylic acids formed through reaction between the PEG and PCL with SebCl and the excess SebCl with water, respectively. In addition, an increase in the intensity of the C–O stretches at ca. 1725 cm^{-1} is observed with the increase in PCL content. This corresponds to the increase of esters within the PHF due to the large number of ester groups present in the backbone of the incorporated PCL. The broad stretch observed between 3200 and 3500 cm^{-1} is characteristic of hydrogen-bonded O–H groups and is attributed to either OH groups from unreacted PEG/PCL end groups or carboxylic acids. C–H stretches were also observed within the 3000–2800 cm^{-1} region and are consistent with the alkyl chains of sebacic ester, PCL, and PEG. All of the peaks observed in the ATR-IR spectrum are consistent with the expected structure derived from cross-linking of GE, SebCl, and PCL through ester linkages.

2.4. Light Transmittance Evaluation of PHFs

The human cornea allows transmission of light in a monotonically increasing manner, whereby UV-light transmission increases from approximately 30% at 310 nm to 75% at 400 nm and the transmission of visible light continues to increase from ≈ 75% at 400 nm to 95% at 700 nm.[35–37] It has been shown that light transmission of the cornea is also dependent on the position that measurements are taken across the corneal surface.[35,36] The PHFs display a similar behavior to the human cornea in regards to the increase and decrease of transmission through the UV and visible light regions. UV–vis spectroscopic analysis of all PHFs revealed that they transmit >98% of visible light at all wavelengths (400–700 nm) regardless of the PCL content (**Figure 3**a). Conversely, UV light transmission significantly reduces from 400 to 290 nm (Figure 3a). Ultimately the potential effect of the PHFs in regards to the corneal transmission would be minimized in two ways. Firstly in DSEK-like procedures only a thin layer of the cornea is removed and replaced leaving the majority of the cornea intact, thus the PHFs would have a negligible effect on corneal transparency when implanted via the same procedure. Secondly as the PHFs are designed to biodegrade, any effect in regards to light transmission would ultimately be eliminated. To prevent hindrance of the patient's vision, it is desired that the PHFs are at least as capable as the human cornea in regards to visible light transmission, and the transmittance profile (Figure 3a) of the PHFs reveals that they are easily capable of achieving this objective throughout the entire visible spectrum with a transmission of >98%.

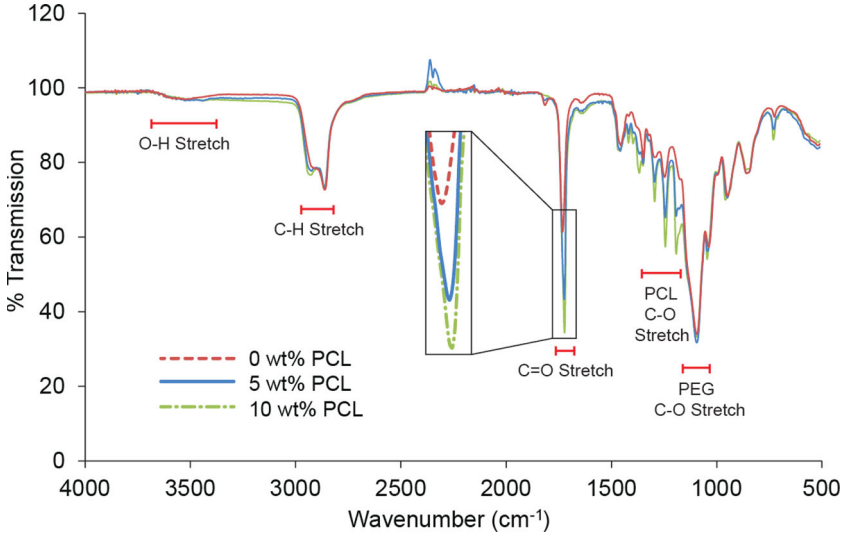

Figure 2. ATR-IR spectra of the PHFs (with 0, 5, and 10 wt% PCL) showing prominent C═O, C–O, C–H, and O–H stretches.

2.5. Thickness Analysis of the PHFs

Environmental scanning electron microscopy (Enviro-SEM) imaging of the PHFs revealed a smooth surface and a uniform cross-sectional structure (Figure 3b,c) with a dehydrated thickness of ≈31 µm. This was also confirmed by spectral reflectance measurements, which provided dehydrated and hydrated thicknesses of 25.3 ± 4.5 and 47.2 ± 1.5 µm, respectively. Grafts with thicknesses ranging from 10 to 150 µm are routinely used in DSEK and similar keratoplasties.[38,39] Hence, with a hydrated thickness of ≈ 50 µm, the PHFs are well suited for the DSEK procedure. The thickness of the films for the purpose of this study was chosen to be 50 µm; however, alteration of

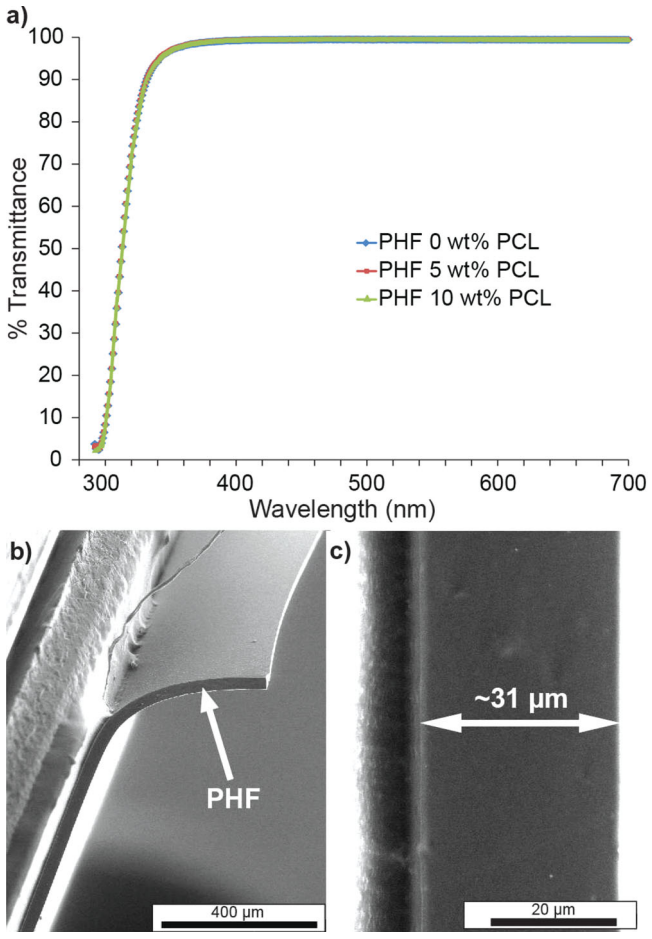

Figure 3. a) Light transmittance of PHFs (with 0, 5, and 10 wt% PCL) across the UV–vis spectrum. b) Side and surface, and c) cross-sectional view of PHF (with 5 wt% PCL) as viewed by SEM.

PHF precursor concentration in the casting solution or the total amount of casting solution would allow the film thickness to be tailored for specific applications. Previous studies using thicker gelatin and hyaluronic acid films (≈650–800 µm) for corneal endothelial tissue engineering require much more invasive implantation procedures such as penetrating keratoplasty.[15,40] The 50 µm PHFs, on the other hand, could easily be inserted into the cornea via minimally invasive small incisions.

2.6. Tensile Evaluation of PHFs

Mechanical evaluation of PHFs with various PCL contents revealed the significant effect of the PCL on the tensile properties of the films (Table 1). Without any PCL (0 wt%), the PHFs have an ultimate stress of ca. 1.5 MPa, with a 33% strain at break. However, inclusion of 5 wt% PCL led to significant improvements in the ultimate stress and strain, more than doubling these parameters to ≈ 3.65 MPa and 71%, respectively. Further increases in the PCL content to 10 wt% resulted in increases in the ultimate stress and tensile modulus values to ≈ 5.21 and 6.23 MPa, respectively, but a slight decrease in the ultimate strain to 61%. Previous studies have shown that with increasing PCL content the swelling capabilities of hydrogel scaffolds were reduced, whilst improvements in their compressive and tensile properties were noted.[35,41,42] A similar trend is observed for the PHFs, whereby increased PCL contents lead to improved tensile properties and reduced swelling (i.e., %ESR) (Table 1). When there is no PCL incorporated, the PHFs possess the highest %ESR, whereby the cross-linked PEG chains are expected to adopt an extended conformation. When PCL is covalently introduced, swelling is reduced due to the hydrophobic nature of PCL, which would adopt a collapsed conformation to minimize interactions with water. This would increase the capability of the PCL containing films to deform under tensile forces as the PCL chains can be extended, hence increasing the ultimate stress and strain of the PHFs. In addition, the tensile moduli of the PHFs increase with higher PCL contents. This results from the increased cross-linking density caused by the reaction between dihydroxy–PCL and the excess SebCl. The ultimate strain observed with 10 wt% PCL content is well within the standard deviation of the ultimate strain of the PHFs with 5 wt% PCL content and hence the difference is within error of the measurement. The human cornea possesses an ultimate stress of 3.3 MPa.[14] In comparison, the 50 µm thin PHFs can be engineered to have ultimate stresses up to 5.21 MPa with only 10 wt% PCL content, demonstrating that they possess a suitable tensile strength.

The stiffness of substrates can affect the behavior of attached cells,[43] and as such it would be desirable for the PHFs to have a comparable stiffness to that of the Descemet's membrane to provide a mechanically similar surface to the native environment. Previous studies have reported that the Descemet's membrane has a tensile modulus of ≈ 2.6 MPa.[34] Thus, PHFs with 0 and 5 wt% PCL content possess a comparable tensile modulus to that of the Descemet's membrane, which may play a significant role for the proliferation of CECs.

The PHFs possess similar or better tensile properties to that of the human cornea, which is especially important for DSEK-like procedures, whereby demanding physical manipulations are required during surgery. The ability to tailor PHF tensile properties via the alteration of PCL content also makes them amenable for various applications where a range of tensile properties might be required.

2.7. In Vitro Degradation of PHFs

Biodegradation of tissue engineering substrates in a controlled manner without the generation of toxic by-products is vital for allowing the restoration of the target tissue to its native structure, morphology, and function. The use of acid chloride/alcohol chemistry to fabricate hydrogel films leads to the formation of ester cross-links within the network that are susceptible to hydrolytic and enzymatic cleavage.[44] To investigate the degradation rate in vitro, PHFs were stored in PBS solution for 8 weeks at 35 °C (natural temperature of the anterior chamber),[14] to evaluate degradation under physiologically relevant hydrolytic conditions. Calculation of the degree of degradation of PHFs with 0%, 5%, and 10% PCL content revealed mass losses of ≈ 24%, 15%, and 10%, respectively, in 8 weeks (**Figure 4**a). The decreased degradation observed with higher PCL contents

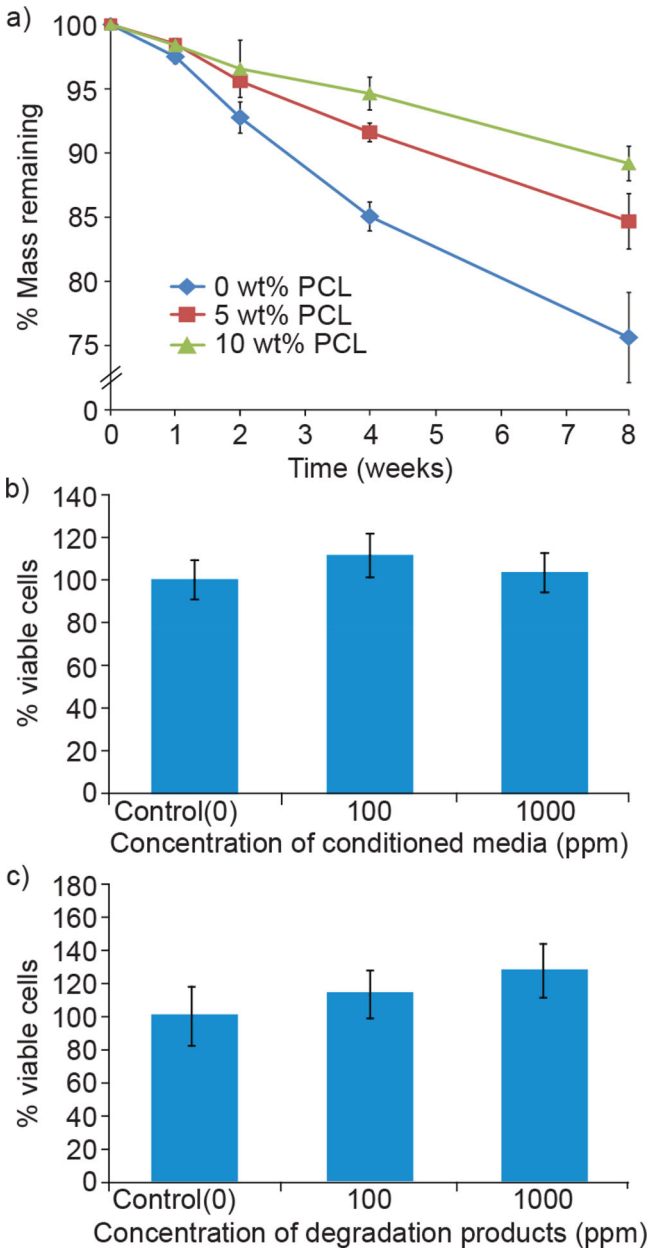

Figure 4. a) In vitro degradation profile of PHFs over 8 weeks (PBS, 35 °C). Cytotoxicity evaluation of b) PHF-conditioned media, and c) PHF degradation products with 3T3-L1 fibroblasts. PHFs with 5 wt% PCL were used for all cytotoxicity studies.

is attributed to the reduced swelling of the films as a result of the hydrophobic nature of PCL. During the synthesis of the PCL component, a disulfide functionality was introduced into the main backbone of the polymer. Disulfides are able to provide biodegradability to biomaterials due to their ability to be cleaved by reducing agents such as glutathione and L-cysteine as shown in our previous study.[31,33] These degradation profiles are only representative of in vitro hydrolytic conditions and may be further affected by the presence of hydrolytic enzymes or disulphide-cleaving agents in vivo. The results of the in vitro degradation study demonstrate the PHFs' characteristics as biodegradable implants, and via variation of the PCL content degradation rates can be readily tailored as required.

2.8. In Vitro Cytotoxicity Assessment of PHFs

Implantable materials and their degradation products need to possess non-toxic properties. As such, preliminary in vitro cytotoxicity studies were conducted to evaluate the cytotoxicity of the PHFs. Conditioned media were prepared by incubation of PHFs in cell culture media for 72 h to determine if harmful products were leaching out from the films. On the other hand, the degradation products were obtained via accelerated acid catalysed hydrolysis of the PHFs. After 72 h of incubation in the presence of various concentrations (100 and 1000 ppm) of PHF-conditioned media and degradation products, minimal toxicity was observed for 3T3-L1 fibroblasts (Figure 4b,c). These results are unsurprising since degradation of the film via hydrolysis of the ester bonds would yield low toxicity compounds such as PEG, sebacic acid, and 6-hydroxyhexanoic acid (from degradation of the PCL). Whereas PEG is approved by the FDA for certain human applications and is considered to be non-toxic,[45,46] sebacic acid is a dicarboxylic acid naturally present in cells as an intermediate in fatty acid oxidation.[47] Sebacic acid also has a very high LD50 (oral, rat) of 14.4 g kg^{-1}, which supports its low toxicity.[48] PCL, on the other hand, is a synthetic polymer that has been widely investigated for tissue engineering applications, and it has been shown to degrade in vivo leading to low-molecular-weight fragments that do not accumulate in the body and can be completely excreted.[49] The results of the cytotoxicity studies demonstrate that ultimately the PHFs and their degradation products have negligible effects on cell proliferation and metabolic activity. To confirm the in vitro response to the PHFs and their behavior in a real biological system, in vivo studies were subsequently conducted.

2.9. In Vitro Permeability Evaluation of PHFs

The permeability of the PHFs for nutrients and biomolecules such as glucose and albumin are crucial, especially within the cornea where the lack of blood vessels means that the tissue relies on diffusion for nutrient transportation. Therefore, it is essential for ophthalmic implants to be permeable to nutrients and other biomolecules.[50] To determine the permeability of the PHFs to glucose and albumin, in vitro permeability studies were conducted and diffusivities of 2.3 (±0.3) × 10^{-6} and 1.0 (±0.2) × 10^{-7} cm^2 s^{-1} were obtained, respectively. In comparison, the human cornea has diffusivities for glucose and albumin of 2.6 (±0.3) × 10^{-6} and 1.0 × 10^{-7} cm^2 s^{-1}, respectively.[14] Thus, the PHFs have almost identical diffusivities to that of the human cornea. The in vitro diffusivity study demonstrates that PHFs are permeable to both large and small molecules that are important for the survival and function of tissue within the cornea.

2.10. In Vitro CEC Attachment and Proliferation on PHF Surfaces

Human CECs do not regenerate in vivo following trauma and disease, thus substrates for CEC regeneration need to support

their proliferation and attachment in vitro. To assess the PHFs for this purpose, in vitro endothelial cell culture studies using primary cells of sheep origin were conducted. Sheep CECs were chosen for this study as they do not proliferate in vivo,[51,52] thus providing more comparable conditions to human corneal endothelium. CECs were first isolated by careful microdissection of the Descemet's membrane; a thick extracellular matrix, which is covered by a monolayer of pure CECs. This isolation technique purifies CECs, removing other cell types. The cells are removed from Descemet's membrane via a series of enzymatic digestions, first with collagenase, and then trypsinized before being resuspended in corneal media. Initial cell cultures on the PHFs (5 wt% PCL) using standard tissue culture plates (TCP) resulted in cell culture times of up to 28 d to reach confluence. This resulted from a large number of cells that were seeded adhering onto the TCP surface during suspension seeding. As a preventative measure, non-cell adherent TCP was utilized, which significantly reduced cell culture times, allowing 100% confluence to be reached within 7 d. The cultured CECs retained their natural in vivo polygonal morphology (**Figure 5**a,b). CAST counting of cells cultured on the PHFs provided a cell density of 3150 ± 459 cell mm^{-2} (std error, $n = 4$). This cell density is identical to that of native sheep CECs in vivo, 3150 ± 88 cells mm^{-2} (std. error, $n = 3$) as determined by phase contrast microscopy. As a result of PEG's low-protein fouling characteristic, previous studies using PEG-based materials for cell attachment and proliferation applications have generally conjugated collagen or other attachment ligands in order to promote cell adherence.[27,28] In comparison, the PHFs demonstrate cell attachment and proliferation properties without any surface modification or ligand conjugation onto their surfaces. These favorable surface properties most likely result from the functional groups present in the PHFs, and the balance between surface hydrophilicity/hydrophobicity resulting from the film precursors. During the synthesis of the PHFs, a slight excess of SebCl was employed, and following solvent casting, the films were soaked in water to hydrolyse the excess acid chloride moieties to carboxylic acids. Previous studies have

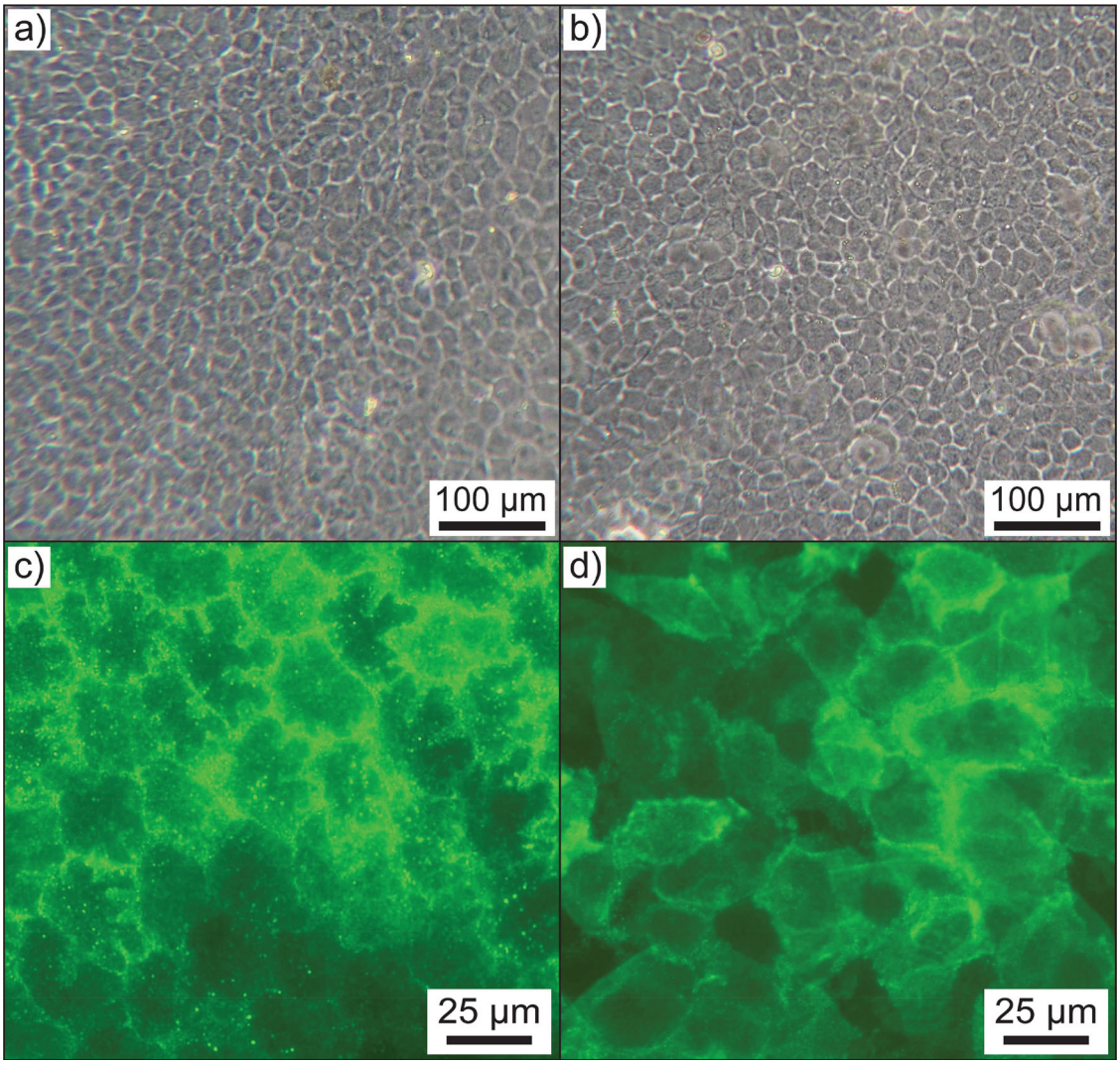

Figure 5. Sheep CECs cultured on a) tissue culture plate and b) PHF (5 wt% PCL); note the polygonal morphology. Na^+–K^+ ATPase immunofluorescent staining on c) CECs on native sheep Descemet's membrane and d) CECs on PHF (5 wt% PCL).

shown that carboxylic acid groups play an important role in the attachment and migration of various cell types.[53] In addition, a balance between hydrophilicity and hydrophobicity is required for optimal cell–surface interactions.[54] Arima and Iwata reported that surface wettability in addition to the functional groups (e.g., carboxylic acid and amine groups) plays a significant role in the adherence of cells. For example, the highest adherence of HUVECs and HeLa cells was observed on alkanethiol self-assembled monolayers with high densities of carboxylic acid groups and contact angles between ca. 40° to 60°, whereas higher contact angles led to reduced cell adhesion.[55] The surface contact angles of the PHFs also ranged between 44° and 67°, which is also expected to contribute to the observed attachment of CECs. We had also previously studied CEC attachment on the films without any PCL (not presented in the manuscript) and also observed the same attachment and proliferation of CECs on film surfaces. PCL is able to significantly improve PHF tensile properties but it is not an essential component for cell attachment and proliferation on the PHFs.

Immunofluorescence staining of the cells on the sheep Descemet's membrane and the PHFs demonstrated that the cultured cells are positive to Na^+–K^+ ATPase (Figure 5c,d). Na^+–K^+ ATPase is a regulator of pump function and has been used as a marker for CECs previously.[56–59] The presence of Na^+–K^+ ATPase at the lateral periphery is indicative of intact pump function,[57] as observed for both the native and cultured CECs (Figure **5**c,d). Although sheep CECs are delicate cells that do not have a replicative capability in vivo,[51,52] they readily proliferated on the PHFs.

In summary, the fully synthetic PHFs were capable of supporting CEC attachment and proliferation without surface modification or the conjugation of any adhesion ligands such as collagen or RGD. This demonstrates that the PHFs inherently possess the appropriate surface characteristics for CEC regeneration.

2.11. In Vivo Evaluation of PHFs

PHFs without any seeded cells were implanted into live ovine corneas via a DSEK-like procedure to evaluate toxicity and inflammatory responses. Following anaesthesia, a small incision was made into the cornea with a slit knife to provide access to the anterior chamber. Using fine forceps, the PHFs were placed into the anterior chamber directly (**Figure 6**a,b).

PHFs with 5 wt% PCL content were used for this study and were found to maintain their integrity during the procedure with their elastic properties, allowing facile handling throughout the surgery. Over the 28-d slit-lamp observation period, the test corneas maintained their optical transparency and no significant differences were noted visually in comparison to control corneas (Figure 6c–h). No evidence of oedema or opacity was observed.

Histological analysis of the control and test corneas following harvesting at 28 d revealed the natural morphology of the stroma, Descemet's membrane and the corneal endothelium (Figure 6i,j). It was also observed that the PHFs had adhered onto the interior surface of the cornea in some regions, with no toxicity or inflammatory response towards the film being observed (Figure 6j). The adherence of the PHFs on top of the CECs did not compromise native CEC function as the corneas maintained their optical transparency throughout the study. In addition, the thicknesses of the PHFs observed from the histology were ≈10 μm, which is less than that of the dehydrated thickness of the films prior to implantation. This reduction in thickness is an indication of PHF degradation. Despite this evidence of degradation, no blockage of the trabecular meshwork was observed. This preliminary in vivo study confirms the results of our in vitro CEC cytotoxicity study, demonstrating the benign nature of the films, and that they do not incite negative inflammatory responses. The ability of the PHFs to adhere onto the interior surface of the cornea also makes them well suited for the target application, as the adherence of the film is important in DSEK and similar lamellar keratoplasties. Further in vivo work is under way by using allogeneic and autologous cell transplantation and their outcomes will be reported in due course.

3. Conclusions

PEG-based hydrogel films for CEC regeneration and implantation were successfully prepared via a facile synthetic approach using acid chloride/alcohol chemistry. This approach allows the fabrication of biocompatible and biodegradable PHFs from cheap and commercially available precursors via a simple fabrication method, which is highly amenable to large-scale production. PHFs produced via the cross-linking of PEG stars with SebCl were found to possess significantly enhanced mechanical properties when PCL at various weight percentages was covalently incorporated into the film. Transmission of visible light through the PHFs was determined to be >98%, which makes them well suited for ophthalmic applications. Permeability of the PHFs for glucose and albumin was found to be almost identical to that of the native human cornea, which is highly desirable due to the diffusion needs of the cornea. Hydrolytic degradation of the PHFs was confirmed over an 8-week period, with the acid-catalyzed degradation products displaying no toxicity in cell viability assays. Furthermore, sheep CECs, which show poor proliferation in vivo, were successfully grown to confluence on the PHF surfaces complete with natural morphology and cell densities. The robust mechanical properties, ease of handling, and hydrated film thickness of only 50 μm make the PHFs promising candidates for minimally invasive surgical procedures, such as DSEK, as demonstrated by in vivo sheep ocular implantation. In addition, in vivo studies revealed the benign and non-inflammatory nature of the PHFs, which is essential for tissue engineering constructs. The reported PHFs possess desirable mechanical, optical, biodegradable and biocompatible properties that make them attractive candidates as substrates for the attachment, proliferation, and implantation of CECs to treat corneal endothelial dysfunction.

4. Experimental Section

Materials: GE ($\bar{M}_n \approx 1$ kDa), SebCl (≥95%), PBS tablets, ε-caprolactone (97%), 2,2′-dithiodiethanol (90%), stannous octoate (≈95%), toluene (anhydrous, 99.8%), sodium trifluoroacetate (NaTFA) (99.999%) Costar Ultra low attachment plates, insulin, transferrin, selenium, 4′,6-diamidino-2-phenylindole (DAPI) fluorescent stain, Triton X-100, dextran $M_r \approx 500$ kDa, glucose assay kit (Sigma GAGO-20: glucose

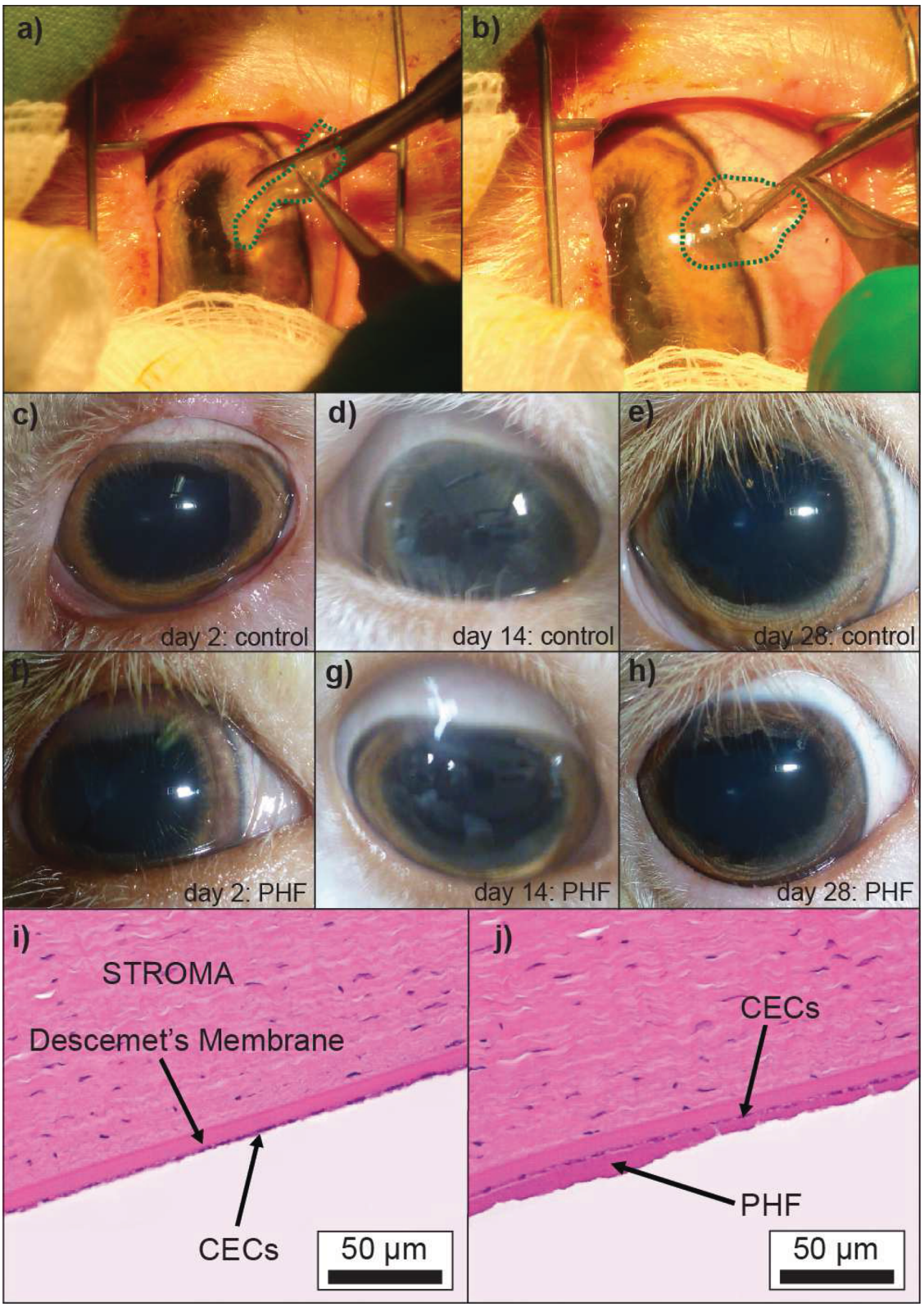

Figure 6. DSEK-like procedure performed on live sheep corneas a) before and b) after insertion of the PHF into anterior chamber. Images of c–e) control and f–h) implanted (test) eyes after 2, 14, and 28 d. H&E stained section of i) control and j) PHF implanted sheep cornea after 28 d implantation. PHFs with 5 wt% PCL were used for all in vivo studies.

oxidase/peroxidase reagent and O-dianisidine dihydrochloride), *trans*-2-[3-(4-tert-butylphenyl)-2-methyl-2-propenylidene]malononitrile (DCTB) (≥99.0), and albumin–fluorescein isothiocyanate conjugate from bovine (albumin-FITC) were obtained from Sigma–Aldrich and used as received. Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), L-glutamine, trypsin-EDTA (0.05%), trypan blue (0.4%), and penicillin–streptomycin were obtained from GIBCO. DMEM was supplemented with 10% v/v FBS, 1% v/v L-glutamine, and 1% v/v penicillin–streptomycin prior to use for the cell viability assays. NUNC T225 canted neck flasks were obtained from Thermo Fisher Scientific. Thermanox tissue culture plastic (TCP) coverslips were obtained from NUNC. DMEM:nutrient mixture F12 (DMEM:F12), antibiotic–antimycotic, epidermal growth factor (EGF), fetal calf serum (FCS), Alexa Fluor 488 goat anti-mouse IgG, trypsin, and EDTA were obtained from Invitrogen. Anti-Na^+–K^+ ATPase (β2-subunit) monoclonal IgG produced in mouse clone M17-P5-F11 was obtained from Santa Cruz Biotechnology and used as received. THF (Honeywell, 99.99%), D-glucose (≥99.5%), and dichloromethane (99.5%) were obtained Chem-Supply and used as received. CelltiterAqueousOne solution for cell viability assays was obtained from Promega and used as received. Haemotoxylin & Eosin dyes were purchased from ProSciTech, Queensland, Australia.

Instrumentation: Enviro-scanning electron microscopy (E-SEM) was carried out using a FEI Quanta FEG 200 Enviro-SEM with the samples mounted on carbon tabs at the Advanced Microscopy Facility, Bio21. Immunofluorescence images were obtained using an Olympus BX61. UV–vis light transmittance measurements were carried out using a Shimadzu UV-1800 UV–vis Scanning Spectrophotometer. A side-bi-side diffusion cell with magnetic stirrers was obtained from PermeGear Inc. Mechanical testing was conducted using an Instron Microtester 5848 equipped with Bluehill material testing software. Spectral reflectance measurements were conducted in air using a Filmetrics F20 Thin-Film Measurement System. ^{1}H NMR spectroscopy was performed using a Varian Unity400 spectrometer operating at 400 MHz (using the deuterated solvent as lock). ATR-IR spectroscopy was carried out using a Bruker Tensor 27 FTIR, with GladiATR ATR attachment obtained from Pike Technologies. The FTIR was equipped with OPUS 6.5 spectroscopy software from Bruker Optik GmBH. Contact angle measurements were carried out using a DataPhysics OCA 20 Tensiometer, measurements were recorded with the OCA software. Matrix-assisted laser desorption/ionization–time-of-flight mass spectroscopy (MALDI–ToF-MS) was performed on a Bruker Autoflex III mass spectrometer operating in positive/linear mode. The matrix (DCTB) and cationization agent (NaTFA) were dissolved in methanol (10 mg mL^{-1} and 1 mg mL^{-1}, respectively) and then mixed with the analyte in a ratio of 10:1:1. 0.3 µL of this solution was then spotted onto a ground steel target plate and the solvent allowed to evaporate prior to analysis. FlexAnalysis (Bruker) was used to analyse the data. The values quoted correspond to the molecular weight of the species. Gamma sterilization was carried out at Steritech, Victoria, Australia.

Synthesis of α, ω-Dihydroxy PCL: α, ω-Dihydroxy PCL was prepared via ROP. ε-Caprolactone (20.0 g, 175 mmol), 2,2′-dithiodiethanol (1.08 g, 7.00 mmol) and stannous octoate (0.95 g, 2.34 mmol) were dissolved in anhydrous toluene (45 mL) and heated at 110 °C under argon for 24 h. The mixture was cooled to room temperature, diluted with THF (50 mL), and precipitated into cold methanol (–18 °C, 1 L). The precipitate was collected by filtration and dried in vacuo (0.1 mbar) to afford α,ω-dihydroxyl PCL as a white powder, 18.8 g (94 %); $\bar{M}_n$ (NMR) = 3.2 kDa, $\bar{M}_n$ (MALDI ToF) = 3.3 kDa; PDI = 1.07.

Casting of PHFs: Dihydroxy PCL (0.052 or 0.104 g for 5 or 10 wt% PCL content, respectively) was dissolved in DCM (15 mL). GE (0.62 mL, 0.70 mmol) and SebCl (0.30 mL, 1.42 mmol) were added to the dihydroxyl PCL solution, which was mixed thoroughly and allowed to stand at room temperature for 1 h with occasional agitation. Samples with no PCL were also prepared. 7.5 mL of the solution was then pipetted onto a glass petri dish (diameter 10 cm), which was placed in a vacuum oven at 60 °C for 30 min prior to the application of vacuum (20 mbar). After a further 1 h, the dish was removed and allowed to cool to room temperature. The circumference of the film was scored with a scalpel and the petri dish was filled with deionized water (20 mL). After 15 min the water was removed and 1:1 THF:deionized water (40 mL) was added. The film detached from the petri dish surface and was placed in water (250 mL). The water was changed with fresh water every 15 min (3 × 250 mL) and the films were dried under vacuum for 24 h (20 mbar, 30 °C) and stored in a desiccator until further use.

Equilibrium Swelling Ratio and Contact Angle Measurements: To observe the swelling characteristics, dehydrated PHFs were allowed to swell in PBS for 24 h. The %ESR of the films with various PCL contents was calculated using the equation $\%ESR = ((W_s - W_d)/W_d) \times 100$ %, where W_s and W_d refer to the swollen and dried weights, respectively. The analysis was conducted in triplicate for each type of PHFs and the results averaged. Water contact angle measurements were carried out using the sessile drop technique (10 µL droplet) on completely swollen films. PHFs with 0, 5, and 10 wt% PCL contents were used. Measurements were taken 60 s after the water droplets were placed onto the PHF surfaces.

Attenuated Total Reflectance Infrared Spectroscopy: Hydrated PHFs were cut into 2 cm × 2 cm squares, dehydrated in 100 % ethanol (50 mL, 15 min) and dried in vacuo for 24 h (20 mbar, 30 °C). Subsequently, the PHFs were subjected to ATR-IR measurements.

Light Transmittance Evaluation of PHFs: Hydrated PHFs were placed in PBS solution for 1 h and then subjected to UV–vis evaluation. The transmittance of the films was recorded at 25 °C over the UV and visible spectrum (290–750 nm).

Thickness Analysis of PHFs: Environmental scanning electron microscopy (Enviro-SEM) and spectral reflectance analysis were used to determine the thickness of the cross-linked films. Films mounted on carbon tabs were analysed under low vacuum conditions to observe the dehydrated thickness of the films. Using the tilt adjustment of the sample holder, the cross sections of the PHFs were observed. Subsequently, ImageJ (National Institute of Health, USA) software was used to determine the average thickness of dried films. Spectral reflectance measurements of the dried and hydrated PHFs were conducted in air with a film refractive index of 1.55, at various locations on the films. Thickness values were obtained from an average of three measurements between the wavelengths of 200 and 400 nm using the Filmetrics F20 configuration.

Evaluation of Tensile Properties of PHFs: Dehydrated PHFs were swollen in PBS and cut into dog-bone shapes with 2 cm × 2 cm gage area and 2 cm tabs for the evaluation of their tensile properties. The films were clamped between wooden tabs within the metal clamps of the Instron Microtester to prevent slippage and jaw tearing of the films. The PHFs were not stress preconditioned prior to tensile testing. Tensile evaluation of the PHFs was carried out in the temperature controlled water bath of the microtester in PBS solution at 35 °C. A minimum of three repeats of each type of film (with 0, 5, or 10 wt% PCL) were tested. The clamped samples were stretched at a rate of 0.1 mm s^{-1} with a 50-N load cell, until breakage of the films in the gage area occurred. Any film that did not break within the gage area was disregarded for compilation of raw data. Data obtained from the Bluehill software was exported into OriginPro 7.5 software for graphing and determination of key parameters such as ultimate tensile stress/strain and tensile modulus. A Poisson ratio of 0.5 was assumed for all calculations.

In Vitro Degradation Studies: Hydrated PHFs were cut into 2 cm × 2 cm squares. After three washes with water (20 mL) at 5 min intervals, the films were dried in vacuo (20 mbar) at 40 °C for 24 h. The dried PHFs were weighed, transferred to vials (28 mL), and PBS (20 mL) was added. Subsequently, the containers were placed in a temperature-controlled orbital shaker at 35 °C, the natural temperature of the corneal anterior chamber.[14] Three containers with films were removed from the orbital shaker at time points of 1, 2, 4, and 8 weeks. The removed films were washed with water (3 × 20 mL) for 15 min with occasional gentle agitation and were dried in vacuo (20 mbar) for 24 h at 40 °C. The dried films were weighed and the masses were plotted against time to obtain a degradation profile of the films.

In Vitro Permeability Evaluation of PHFs: Permeability studies were adapted from a previously described method.[14] Glucose and albumin

diffusivity measurements were carried out in a PermeGear side-bi-side diffusion cell with 5 mL cell volumes. The PHFs were placed between the diffusion cells and tightly clamped to prevent leakage. For glucose measurements one cell (source) was filled with glucose in PBS (0.05 g mL^{-1}, 4 mL) and the other cell (target) with PBS buffer only (4 mL). For albumin measurements, albumin-fluorescein isothiocyanate conjugate (albumin-FITC) in PBS (50×10^{-6} M, 4 mL) was placed in the source cell and PBS buffer (4 mL) was placed in the target cell. For both glucose and albumin measurements, the chambers were maintained at 35 °C with magnetic stirring in each cell. At set times (30, 45, 60, 80, and 100 min), aliquots (2 mL) were removed from the target cell and replaced with fresh PBS (2 mL). For glucose measurements, samples were prepared for spectrophotometric analysis using a glucose assay kit and analysed using a Shimadzu UV-1800 spectrophotometer at 540 nm. For albumin measurements, the absorbance of the albumin-FITC at 495 nm was measured using a Shimadzu UV-1800 spectrophotometer.

In Vitro Cytotoxicity Evaluation of PHFs: To assess the cytotoxicity of the films, the dehydrated PHFs (5 wt% PCL, 100 mg) were placed in 80% v/v ethanol solution for 30 min. The films were then washed and rehydrated with sterile PBS (3×20 mL) at intervals of 10 min. The rehydrated films were subsequently placed in sterile DMEM (2 mL) and incubated at 37 °C for 72 h. The films were removed from the solution and the conditioned media were used in the cell viability assay. To investigate the effects of film degradation products on cell viability, dehydrated PHFs (500 mg) were degraded in 1 M HCl (5 mL) over 30 min. The solution was concentrated in vacuo and the residue was azeotroped with water (5×20 mL) and then dried in vacuo (0.1 mbar). The degradation products (100 mg) were dissolved in sterile DMEM (2 mL). The solutions were then sterilized under UV for 30 min and filtered through 0.22 µm nylon filters prior to use in the toxicity studies. NIH 3T3-L1 cells were grown to confluence in T225 flasks in supplemented DMEM at 37 °C in 5% CO_2 atmosphere with 95–100% humidity. Cells were trypsinized using 0.05% Trypsin–EDTA, counted manually using trypan blue as live/dead stain, diluted with fresh medium to afford a seeding density of 1.25×10^5 cells mL^{-1} and plated onto 96 well plates (80 µL $well^{-1}$). Some wells were left blank to serve as cell-blank controls. The plates were returned to the incubator for 4 h prior to the addition of the PHF conditioned media and PHF degradation product solutions. The prepared stock solutions (50 mg mL^{-1}) were twice diluted by a factor of 10 using fresh complete medium. 20 µL of the two dilutions thereof was added to the 96 well plates in triplicate (to obtain concentrations of 100 and 1000 ppm), gently mixed by orbital movement of the plates and then the plates were returned to the incubator for a further 72 h incubation. Subsequently, CelltiterAqueousOne solution was added to the plates (20 µL $well^{-1}$), the plates were gently rocked to facilitate mixing and then returned to the incubator for 30 min to 4 h. In periodic intervals, the UV–vis absorbance of the plates was read at 490 and 700 nm using a Cary 50 Bio UV–vis Spectrophotometer equipped with a micro plate reader. Usually sufficient colour had developed after ca. 2 h incubation. Absorbance values at 490 nm were corrected for background absorbance (700 nm) and absorbance of the medium alone (cell-blank controls), and then normalized to the growth control.

Preparation of Film for Cell Culture Studies: Dried PHFs (5 wt% PCL) were rehydrated in PBS (20 mL) and cut into circular disks (diameter 16 mm) using a circular hole puncher. Subsequently, the films were placed in 80 % v/v ethanol (20 mL) for 1 h. The films were then washed with sterile PBS solution (3×20 mL) at intervals of 15 min. The PHFs were then placed into a 24-well plate with sterile glass rings (external diameter 15 mm) placed on top of the films. PBS (2 mL) was added into wells containing the films and the well plate was stored at 8 °C in a refrigerator prior to cell culture studies. Neither surface modification nor protein conjugation was carried out on the PHFs prior to cell culture studies.

CEC Harvesting and In Vitro Cell Seeding of PHFs: Eye orbs from fresh scavenged experimental cadavers of merino sheep were washed with proviodine (1:50, 8 min), methanol (20% v/v, 60 s), peracetic acid (0.1% v/v, 5 min), and in PBS with antibiotic–antimycotic. Corneas were then dissected and transferred to thinning media (1:1 DMEM:F12, insulin (0.5 µg mL^{-1}), transferrin (0.275 µg mL^{-1}), selenium (0.25 mg mL^{-1}), EGF, 2% FCS, antibiotic–antimycotic and dextran) for 16 h. Descemet's membrane was dissected from corneal tissues, treated with collagenase (2 mg mL^{-1}) for 60 min, and trypsin (0.05 %)–EDTA (0.02 %) for 5 min. Descemet's membrane in the samples was then cut into small pieces and triturated to produce a single cell suspension, subsequently the decellularized Descemet's fragments were removed. 5×10^4 cells were seeded onto PHFs or Thermanox TCP coverslips in corneal media (1:1 DMEM:F12, insulin (0.5 µg mL^{-1}), transferrin (0.275 µg mL^{-1}), selenium (0.25 mg mL^{-1}), EGF, 10% FCS, and antibiotic antimycotic). Cell culture was performed under standard conditions (37 °C, 5% CO_2).

Immunofluorescence: Descemet's membranes dissected from sheep corneas were used as positive controls. Test samples consisted of sheep CECs on PHFs. All samples were fixed with 4% paraformaldehyde (PFA) for 10 min before washing in PBS and stored at 4 °C until use. Immunofluorescence was performed as follows. Samples were permeabilized with 0.3% Triton X-100 for 15 min before washing with PBS. Blocking was with 3% normal goat serum for 30 min. Samples were incubated with the primary antibody (Anti-Na^+–K^+ ATPase IgG antibody) in PBS for 2 h in a humidified chamber. The negative controls were incubated without a primary antibody. After PBS washing, the samples were incubated with the secondary antibody for 1 h (Alexa Fluor 488). Following another PBS washing, samples were incubated with DAPI for 5 min. After a final PBS wash, samples were mounted with aqueous medium.

Determination of Cell Density: Computer-assisted stereographic tomography (CAST, Olympus) was used to determine cell density from haematoxylin-labelled nuclei. For comparison, the cell density of freshly dissected ovine corneas was determined by phase contrast microscopy (Lions Eye Donation Service, CERA).

In Vivo Evaluation of PHFs: Dehydrated PHFs (5 wt% PCL) were cut into disks (10 mm diameter) using a hole puncher, dried at 40 °C for 24 h and then sealed in double sample bags. The films were sterilized at a minimum dose of 25 kGy at Steritech (Dandenong, Victoria, Australia). The sterilized films were used for the evaluation of in vivo toxicity and inflammatory responses towards the PHFs. All procedures were conducted according to the guidelines of the National Health and Medical Research Council (NHMRC) of Australia and were approved by Faculty of Veterinary Science Animal Ethics Committee (ID: 101197), The University of Melbourne. Merino sheep ($n = 4$) were fasted for 24 h prior to surgical procedures. The pupil of the eye to be operated on was dilated with one drop of topical 1% atropine sulphate and one drop of 10% viscous phenylephrine hydrochloride for 1 h prior to the procedure. Anaesthesia was induced with 25 mg kg^{-1} sodium thiopentone intravenously into the external carotid vein, and maintained after intubation of the airway with 1.5% halothane in 2:1 air/oxygen. A 3-mm slit was made into the cornea using an ophthalmic slit knife and the PHFs were inserted using fine forceps. The incision made into the anterior chamber was sutured and the sheep were allowed to recover. Following surgery, the corneal morphology was examined using a hand-held slit-lamp every 2–3 d and scored for clarity, oedema, and indices of inflammation, using a validated proforma. Specifically, clarity and oedema were each scored on a scale of 0–4, with 0 being completely transparent (for clarity) or thin (for oedema), and 4 being completely opaque (for clarity) or maximally thick (for oedema). 28 d after implantation, the sheep were euthanized via intrajugular lethabarb injection and the eye orbs were harvested. For end-point histology, tissues were fixed in buffered formalin, embedded in paraffin wax, cut at 5 µm and stained with haematoxylin and eosin (H&E). Sections were examined to determine inflammatory responses, degradation of the PHF, maintenance of the CEC monolayer, and any blockage of the trabecular meshwork.

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