Using poly(lactide-co-glycolide) electrospun scaffolds to deliver cultured epithelial cells to the cornea

Aims: To assess the potential of electrospun poly(lactide-co-glycolide) membranes to provide a biodegradable cell carrier system for limbal epithelial cells. Material & methods: 50:50 poly(lactide-co-glycolide) scaffolds were spun, sterilized and seeded with primary rabbit limbal epithelial cells. Cells were cultured on the scaffolds for 2 weeks and then examined by confocal microscopy, cryosectioning and scanning-electron microscopy. The tensile strength of scaffolds before and after annealing and sterilization was also studied. Results: The limbal cells had formed a continuous multilayer of cells on either side of the scaffold. Scaffolds with cells showed signs of the onset of degradation within 2 weeks in culture media at 37°C. Scaffolds that were annealed resulted in a more brittle and stiff mat. Conclusions: We suggest this carrier membrane could be used as a replacement for the human amniotic membrane in the treatment of limbal stem cell deficiency, lowering the risk of disease transmission to the patient.

KEYWORDS: cell carrier, cornea, electrospun, limbal, poly(lactide-co-glycolide)

The loss of limbal corneal epithelial stem cells reduces the transparency of the cornea [1,2]. To replace these lost stem cells, several methods have been developed to transplant autologous or allogeneic cultured limbal epithelial cells. To transplant cells onto the cornea, a carrier is necessary as the cultured cells are unlikely to survive if placed directly on this challenging wound bed [3]. Cells have been transferred by culturing them into intact sheets that were wrapped around contact lenses or petrolatum gauze for transplantation onto the eye [4] or by placing the cells on fibrin [1], but the most commonly used method is the use of the human amnion as a delivery dressing [5,6]. Cultured cells are placed on small pieces of the prepared amniotic membrane and this is sutured, stromal-side-down, onto the eye. The membrane degrades over a few weeks leaving the cells in place.

However, the response to the amniotic membrane is dependent on the host response to the donor tissue [7,8] and even though the amniotic membrane has been used for many years without any transmission of disease, there have been some cases of immunorejection following multiple treatments using membranes from different donors [9,10]. Furthermore, despite the tissue-banked amniotic membrane undergoing vigorous screening for approximately 6 months [11], transmission of diseases is only minimized and occult diseases may not be not detected [12]. Another problem in practice is that human amnion needs to be prepared for use, and cell performance on the amnion can vary as a function of this [13,14].

An ideal delivery carrier for corneal cells must be biocompatible with minimal risk of disease transmission (preferably none), achieve good cell adhesion and have appropriate tensile strength to secure the cornea, and undergo biodegradation leaving the transplanted cells in place [15].

Synthetic scaffolds that can achieve the former will minimize the very slight chance of viral disease transmission, which cannot be fully eliminated with the use of donated human tissue. They will also remove the inherent variability seen with donor amnion.

We recently reported on developing electrospun polymers as 3D scaffolds to replace the human dermis [16]. In this study, we now seek to develop a rapidly degradable synthetic carrier membrane for delivering cultured corneal cells to damaged corneas.

Electrospinning is simple and reproducible, and can produce fibers with diameters ranging from 3 nm to 6 µm [17,18]. Mats of electrospun scaffolds placed together can robustly mimic the extracellular matrix of tissues [19] and support cell growth. Furthermore, it is relatively simple to tune the rate of breakdown of these scaffolds by varying the ratio of polylactide acid (PLA) to polyglycolide acid (PGA) as reported by Blackwood et al [16]. In this study, scaffolds of 100% PLA survived, intact, for a year post-implantation in rats. Scaffolds of 75% PLA and
25% PGA underwent a 50% reduction after 3 months, while scaffolds of 50% PLA and 50% PGA started to lose structural integrity after only 2 weeks. These latter scaffolds degraded too fast to be used for dermal scaffolds (which will ideally be replaced by new dermal collagen, produced by the tissue-engineered skin cells, over several months) but their relatively rapid degradation seems a reasonable time scale for delivery of cultured corneal cells with progressive dissolution of the underlying membrane.

Accordingly, the aim of the current study was to begin to evaluate the use of a 50:50 poly(lactide-co-glycolide) (PLGA) electrospun fiber mat as a potential carrier for limbal epithelial cells as a lower-risk clinical option to the use of human donor amnion. We investigated the effect of heat annealing and sterilization on the mechanical properties of the scaffold and assessed how well the corneal epithelial cells attached to the scaffold. We also assessed how well scaffolds with cells could be handled and sutured, and looked at the initial signs of scaffold breakdown within the first 2 weeks of placing cells on these membranes.

Methods

Rabbit limbal epithelial cells

Rabbit limbal epithelial cells were isolated from commercial white rabbits derived from the New Zealand white rabbit. The rabbits typically weighed 2.4–2.6 kg. As described earlier [20], the limbal rim of the cornea was excised into four segments and placed into 2.5 mg/ml of dispase II (in 10% Dulbecco’s modified Eagle medium) for 45 min. The limbal cells were then scraped off the tissues into phosphate-buffered saline using a pair of blunt forceps. The solution was collected and centrifuged at 1000 rpm; the supernatant was discarded and the cells were resuspended in fresh media.

The cells were seeded into flasks along with growth-arrested 3T3 murine fibroblasts as a feeder layer. The culture media for the rabbit limbal epithelial cells was Green’s media [21], consisting of Dulbecco’s modified Eagle medium and Ham’s F12 medium (Gibco®, Invitrogen Life Technologies, CA, USA) in a 3:1 ratio supplemented with 10% foetal calf serum, 10 ng/ml EGF (R&D Systems, MN, USA), 0.4 mg/ml hydrocortisone, 1.8 × 10⁻⁴ mol/l adenine, 5 mg/ml insulin, 5 mg/ml transferrin, 2 × 10⁻⁷ mol/l glutamine, 2 × 10⁻⁷ mol/l triiodothyronine (Sigma-Aldrich, UK), 0.625 mg/ml amphotericin B, 100 IU/ml penicillin and 100 mg/ml streptomycin (Gibco).

Fabrication of PLGA scaffolds

Poly(lactide-co-glycolide) (50:50; Sigma-Aldrich) of molecular weight 40,000–75,000 was electrospun as previously described [16]. Briefly, the polymers were dissolved in dichloromethane at a concentration of 25% (wt/wt). The polymer was passed through a 2 ml syringe with a stainless steel needle with an internal diameter of 0.8 mm. The solution was delivered at a constant flow rate of 1.5 ml/h using a syringe pump. The polymer was electrospun at a voltage of 10 kV, supplied by a high-voltage source (Alpha series III, Brandenburg, Germany). Sheets of the scaffold were collected onto aluminum foil sheets wrapped around an earthed aluminum rotating collector (300 rpm). Jet formation was assisted by placing an aluminum ring at 10 kV approximately 5 mm behind the tip of the syringe needle.

Sterilization of scaffolds

Scaffolds were sterilized by either immersion in 70% cold ethanol for 10 min or 0.01% peracetic acid for 3 h using a protocol we previously assessed for human donor skin [22].

Culture of cells on the PLGA scaffolds

Primary rabbit limbal epithelial cells were seeded onto a double-layered sheet of the nontreated scaffold sterilized by immersion into 70% ethanol.

The cells were seeded onto the scaffolds (two single sheets of scaffold placed together) using a stainless steel ring, and after 24 h in culture, the ring was removed. After 2 days under submerged conditions, the scaffolds were placed on a steel mesh grid, bringing the scaffolds to an air–liquid interface with a media change every 2 days. On day 14, the cells on the scaffolds were fixed using 10% buffered formaldehde. The rabbit limbal epithelial cells were stained with CellTracker™ Red (Invitrogen, Paisley, UK) according to the manufacturer’s protocol. The cells were visualized using the Zeiss LSM 510 META (Zeiss, Welwyn, UK) confocal microscope histology and scanning-electron microscopy (SEM).

Preparation of scaffolds for histology

After fixation in 10% buffered formaldehyde for 24 h, samples were cut in half with a scalpel and mounted in optimal cutting temperature compound (VWR International, West Susse, UK) to expose the center of the scaffold. Samples were sectioned using a Leica CM3050 S cryostat...
(Leica Microsystems, Germany) at a thickness of 8 µm. Sections were mounted on 3-aminopropytriethoxysilane (Sigma-Aldrich)-coated slides that were prepared by a brief dip in 3% 3-aminopropytriethoxysilane (in acetone), washed three times in water and allowed to dry. For histological staining, slides were washed gently in water for 1 min to remove optimal cutting temperature compound, followed by staining in hematoxylin for 1 min 30 s and washed in water again for 2 min. Sections were then immersed briefly in water and a coverslip was placed on the section using aqueous mountant. Sections were examined under light microscopy.

Preparation of scaffolds for SEM
Scaffolds were prepared for SEM, as described previously [16]. Approximately 25 nm of gold was sputter-coated onto the scaffolds and then the samples were examined using a SEM (FEI XL-20 SEM, Philips, Guildford, UK) at an accelerating voltage of 10–15 kV.

Mechanical testing of the scaffolds
Scaffolds (heat treated at 60°C for 3 h or non-heat treated) were cut into dogbone shapes as shown in Figure 1. Using the Bose Electroforce® 3100 tensile test machine (Bose, MN, USA), the scaffolds were clamped in place via stainless steel clamps and pulled apart at a rate of 1 mm/10 s over a distance of 6 mm. The force exerted was measured via a 22 N small-load cell. A total of six samples were used for each experimental condition. Scaffolds were also tested poststerilization in 0.01% peracetic acid for 3 h compared with dry scaffolds presterilization.

Statistics
The difference between the mechanical properties of scaffolds was assessed using analysis of variance (ANOVA) and p < 0.05 were considered significant.

Results
Primary rabbit limbal epithelial cells were used in this study as in the UK there is a scarcity of human donor corneas for experimental research. Investigating corneal cell growth on the scaffolds by confocal microscopy showed a continuous layer of rhodamine-stained epithelial cells on the surface of the scaffold (Figure 2A–C). Cryosectioning, and hematoxylin staining of the scaffolds after 2 weeks of cell culture demonstrated that the control scaffolds without cells measured approximately 100 µm in thickness and there was, as expected, no hematoxylin staining (Figure 3A). Scaffolds with epithelial cells cultured on them for 14 days showed clear hematoxylin nuclei staining (Figure 3B) and the thickness of the scaffold was approximately 150 µm. A multilayer of cells was observed on both the top and bottom surfaces of the scaffold with a lower density of cells throughout the scaffold.

Scanning-electron microscopy of the scaffolds without cells showed random fibers of approximately 3–8 µm (Figure 4A) and that a cross-section of the scaffolds measured approximately 100 µm in thickness after 2 weeks in media (Figure 4B). The cultured corneal cells formed a layer on the surface of the scaffold (Figure 4C & D), confirming both the histology and confocal microscopy results, and the thickness of the scaffold was now approximately 70 µm. The reduction in the thickness of the scaffold assessed postcell culture by SEM (but not seen with hematoxylin staining where it apparently increased) requires further investigation. A comparison of SEM sections of scaffolds, with and without cells, suggested that the fibers without cells appeared more loosely
they appeared to be slightly opaque (Figure 5C). During the second week of culture, the scaffolds with cells on them were slightly brittle to handle (and difficult to suture) compared with the first week when they were easy to handle and suture.

It has been reported that heat treating the scaffold reduces the shrinkage of PLGA scaffolds [23]. In addition to this, peracetic acid was used to sterilize the scaffolds in the tensile study as it has been previously used in sterilization of dermis [22].

Final experiments focused on the physical properties of the scaffolds after heat annealing and sterilization with peracetic acid. Heat annealing is a simple way to put together several layers of thin scaffold to make a thicker membrane and sterilization is necessary in preparing to take biomaterials such as these to the clinic.

Wet-annealed scaffolds led to a more brittle sheet of fibers that broke on the application of a smaller force compared with the dry-annealed scaffolds (p < 0.05) as summarized in Table 1. Annealing also increased the stiffness (Young’s modulus) of the dry scaffolds (p < 0.05); however, there was no significant difference in Young’s modulus on wetting the scaffolds. Sterilizing the scaffolds with peracetic acid also led to a significant reduction in the tensile strength of the heat-annealed scaffolds (p < 0.01), as shown in Table 1, but reduced the stiffness. It was also observed that the nonannealed scaffolds were easier to handle compared with the annealed scaffolds, which became brittle by day 14.

Discussion

The aim of this study was to begin to assess the potential of rapidly degrading PLGA scaffolds as an alternative to the human amniotic membrane to treat corneal diseases. Increasingly, patients who have lost limbal epithelial stem cells are being treated with laboratory-expanded limbal stem cells (from the contralateral eye where this is unaffected or allogeneic cells where both eyes are affected). When it comes to delivering these cells back to the cornea, the most popular methodology is that of growing them on small pieces of human donor amniotic membrane and then suturing the membrane back onto the eye. Many hundreds of patients have now been treated using this approach with very encouraging results overall [24–26].

Once sutured onto the eye, the amniotic membrane usually disintegrates within 1 month after surgery. However, it has been reported that, owing to donor variations and handling before surgery, treatment outcomes using the amniotic membrane are not consistent [13,14]. Another
issue is of risk management. Blood samples are taken from donors at the point of delivery of the baby and then again some 6 months later to screen for the presence of the HIV. During this time the amniotic membrane must be banked but not released, hence, it requires careful banking procedures to reduce the risk of viral disease transmission. This can be achieved with good tissue banking practices but the real costs of these are substantial. In addition, reports suggest that the amniotic membrane not only functions as a carrier but also provides growth factors and cytokines, which may be important in epithelial–stromal interactions on the ocular surface, as well as maintenance of the microenvironment of the limbal cell niche [27].

In view of these issues, many groups are working towards an alternative to the amniotic membrane, and materials such as recombinant collagen gels [28], synthetic polymer gels [29], contact lenses [20,30] and temperature-sensitive synthetic polymers [31] have all been developed as cell carriers.

In this study, we propose a very simple approach of using a PLGA scaffold that degrades rapidly. PLGA scaffolds undergo a hydrolytic chain scission of the ester bonds in their backbone, eventually leading to the production of carbon dioxide and water [32]. The rate of breakdown of the scaffolds can be controlled to a useful degree by varying the ratio of PLA (which has a hydrophobic methyl backbone and tends to degrade very slowly) to PGA (more hydrophilic and degrades more rapidly). In general, cells attach well to these scaffolds. It is thought that, in the presence of serum, the fibronectin and vitronectin adhere to the polymer surface and the cells, in turn, adhere to the proteins [33,34].

**Figure 4.** Scanning-electron microscopy images of scaffolds. (A) Front view and (B) cross-sectional view of cell-free scaffolds. (C) Front view and (D) cross-sectional view of scaffolds cultured with limbal epithelial cells for 14 days.

**Figure 5.** Images of the scaffolds with limbal epithelial cells on (A) day 0 sutured to a metal grid, (B) day 1 and (C) on day 14.
Table 1. Mechanical testing of poly(lactide-co-glycolide) scaffolds.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Maximum yield (×10⁶) (N/m²)</th>
<th>Maximum strain (%)</th>
<th>Young’s Modulus (×10¹⁰)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nonheat treated</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry</td>
<td>0.53 ± 0.19</td>
<td>26 ± 14%</td>
<td>5.1 ± 2.3</td>
</tr>
<tr>
<td>Wet</td>
<td>0.29 ± 0.08</td>
<td>17.7 ± 6.5%</td>
<td>3.8 ± 1.32</td>
</tr>
<tr>
<td><strong>Heat treated (60°C for 3 h)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry</td>
<td>0.88 ± 0.28</td>
<td>22.4 ± 3.2%</td>
<td>10.5 ± 2.2</td>
</tr>
<tr>
<td>Wet</td>
<td>0.38 ± 0.29</td>
<td>9 ± 4.7%</td>
<td>7.6 ± 2.9</td>
</tr>
</tbody>
</table>

Dry: No treatment; Wet: Sterilized with 0.01% peracetic acid for 3 h prior to testing.

Recent studies have also demonstrated the use of PGA scaffolds in corneal tissue engineering [35,36], where PGA has been used to replace the stroma of the cornea. Our results from culturing skin cells on PLGA scaffolds demonstrated that the cells became well organized, and both keratinocytes and fibroblasts rapidly (within 7 days) produced new collagen on these fibers [16]. Accordingly, our aim in this study was to evaluate whether scaffolds of 50% PLA and 50% PGA would be suitable candidates to replace amniotic membrane.

We used rabbit corneal epithelial cells, which attached well to the scaffolds, and we looked at the handling of the scaffolds with cells at an early time point (within the first few days of seeding of the cells) and after 2 weeks. The scaffolds supported good growth of corneal epithelial cells, achieving a confluent layer within 2 weeks and the scaffolds could be readily sutured (in this experimental protocol to metal mesh grids) within the first week of culture. This would be ideal for clinical use—cells could be seeded on the scaffolds and then a day or two later, the scaffolds could be sutured to the eye.

The next issue was the breakdown of the scaffolds and it was obvious that there were major changes happening by week 2, the scaffolds became opaque and more brittle in their handling and appeared to be visibly shrinking. We interpreted these changes as evidence that the scaffolds are beginning to break down.

Initially, we used ethanol sterilization for our experimental work but, while convenient for laboratory experiments, this is not a recognized sterilization methodology that can be taken to the clinic. We then introduced peracetic acid sterilization, which is clinically approved and is also a wet-sterilization methodology. This also reduced scaffold strength but improved pliability and did not affect the interaction of cells with the scaffold fibers. A recent study from our laboratory has demonstrated that there is no significant difference in the viability of epithelial cells and fibroblasts on scaffolds sterilized with either peracetic acid, γ-irradiation or ethanol [37].

Tensile studies looked at the pros and cons of annealing thin layers of electrospun scaffolds to make a thicker material for handling. While this worked well in terms of producing a thicker scaffold, the mechanical properties and flexibility of the scaffold were compromised making these harder to handle.

In conclusion, these initial in vitro results are very encouraging for using simple electrospun scaffolds that could be stored sterilized and dry as an off-the-shelf product and then seeded with cells and sutured (or fibrin glued) onto the patient’s cornea within a few days. The next challenge will be to assess the performance of these scaffolds with cells in a cornea model (ex vivo, as we have recently used [20], or in vivo) to assess the fate of the corneal cells as the membrane breaks down.

Financial & competing interests disclosure
The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research
The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary
- Simple, rapidly degrading electrospun scaffolds of 50% polylactide acid and 50% polyglycolide acid were used to support the culture of rabbit corneal epithelial cells.
- A continuous layer of corneal cells formed within 2 weeks, as assessed by cryosectioning, confocal microscopy and scanning-electron microscopy.
- Scaffolds could be readily handled and sutured within the first week of culture with cells.
- These synthetic off-the-shelf scaffolds could be a lower risk replacement for the amniotic membrane in delivering limbal epithelial cells to treat corneal diseases.
Bibliography


