



This is a repository copy of *Synthetic biodegradable alternatives to the use of the amniotic membrane for corneal regeneration: assessment of local and systemic toxicity in rabbits.*

White Rose Research Online URL for this paper:
<http://eprints.whiterose.ac.uk/137983/>

Version: Accepted Version

Article:

Ramachandran, C., Sangwan, V.S., Ortega, I. et al. (4 more authors) (2018) Synthetic biodegradable alternatives to the use of the amniotic membrane for corneal regeneration: assessment of local and systemic toxicity in rabbits. *British Journal of Ophthalmology*. ISSN 0007-1161

<https://doi.org/10.1136/bjophthalmol-2018-312055>

© Author(s) (or their employer(s)) 2018. This is an author produced version of a paper subsequently published in the *British Journal of Ophthalmology*. Uploaded in accordance with the publisher's self-archiving policy.

Reuse

Items deposited in White Rose Research Online are protected by copyright, with all rights reserved unless indicated otherwise. They may be downloaded and/or printed for private study, or other acts as permitted by national copyright laws. The publisher or other rights holders may allow further reproduction and re-use of the full text version. This is indicated by the licence information on the White Rose Research Online record for the item.

Takedown

If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.



eprints@whiterose.ac.uk
<https://eprints.whiterose.ac.uk/>

Synthetic biodegradable alternatives to the use of the amniotic membrane for corneal regeneration- assessment of local and systemic toxicity in rabbits

Corresponding author: Sheila MacNeil

Department of Materials Science and Engineering,
Kroto Research Institute, North Campus,
University of Sheffield, Broad Lane,
Sheffield S3 7HQ, United Kingdom
Email: s.macneil@sheffield.ac.uk

Charanya Ramachandran¹, Virender S. Sangwan¹, Ilida Ortega², Upendra Bhatnagar³,
Sadik Mulla³, RobMcKean⁴, Sheila MacNeil⁵

¹Sudhakar and Sreekanth Ravi stem cell laboratory, LV Prasad Eye Institute,
KallamAnji Reddy campus, LV Prasad Marg, Hyderabad 500034, India

²School of Clinical Dentistry, Claremont Crescent, University of Sheffield, Sheffield
S10 2TA, United Kingdom

³ Preclinical Division, Vimta Labs Ltd., Hyderabad 500101, India

⁴ The Electrospinning Company Ltd., Rutherford Appleton Laboratory, Harwell
Oxford, OX11 0QX, United Kingdom

⁵Department of Materials Science and Engineering, Kroto Research Institute, North
Campus, University of Sheffield, Broad Lane, Sheffield S3 7HQ, United Kingdom

Synopsis- Biodegradable electrospun membranes of poly-lactico- glycolic acid 50:50 were applied to rabbit corneas for 29 days . Membranes were completely cleared from the eye by 29 days without eliciting any local or systemic toxicity

Abstract

Aim: The aim of this study was to assess the local and systemic response to poly-lactic co- glycolic acid 50:50 (PLGA) membranes, developed as synthetic biodegradable alternatives to the use of human donor amniotic membrane in the treatment of limbal stem cell deficiency.

Methods: PLGA membranes of 2cm diameter and 50 μ m thickness were placed on one eye of rabbits and secured in place using fibrin glue and a bandage contact lens, suturing the eye close with a single stitch. Control animals were treated identically, with the absence of the membranes. Plain and microfabricated electrospun membranes (containing micropockets which roughly emulate the native limbal niche) were examined over 29 days. All animals were subjected to a detailed gross and histopathological observation as well as a detailed examination of the eye.

Results: Application of the membranes both with and without microfabricated pockets did not adversely affect animal welfare. There was complete degradation of the membranes by day 29. The membranes did not induce any significant local or systemic toxicity. Conjunctival congestion and corneal vascularization were noted in a few control and PLGA treated animals. Intraocular pressure was normal and the retinal status was unaltered. The ocular surface was clear and intact in all animals by the end of 29 days.

Conclusion: Membranes of 50:50 PLGA can be safely applied to rabbit corneas without inducing any local or systemic toxicity and these break down completely within 29 days.

Introduction

Limbal stem cell deficiency (LSCD) can be caused by injuries, surgery, disease and developmental anomalies causing loss of corneal epithelial stem cells located in the limbus and leading to conjunctivalization of the ocular surface.

Treatments for LSCD range from grafting limbal tissues to transplanting cultured limbal epithelial cells (CLET) to more recently transplanting small pieces of limbal explants directly onto the ocular surface (simple limbal epithelial transplantation (SLET))[1-3]. SLET unlike CLET obviates the need for clean room facilities and cell culture technicians, therefore becoming much more accessible[4]. Both CLET and SLET procedures are successful in around 70% of patients with LSCD due to chemical burns[5-7].

Human amniotic membrane (hAM) is commonly used in CLET and SLET as a sacrificial substrate. It provides support to the transplanted cells ensuring good attachment and cell survival on the ocular wound bed. SLET has radically simplified the treatment of LSCD, but further improvements could widen availability. A synthetic membrane would be more accessible and safer than using hAM.

Alternate carrier materials have been studied for the transplantation of limbal stem cells including coated contact lenses, compressed collagen and recently PLGA membranes (poly-lactic co- glycolic acid) [8,9]. PLGA has been extensively used in the clinic as dissolvable sutures[10]. Our previous work shows that electrospun PLGA membranes based on a 50:50 ratio of glycolic acid to lactic acid broke down within 4 to 6 weeks in vitro, supported limbal cell expansion as well as hAM in terms of stem cell expansion and maintenance without the need for feeder cells[11,12]. We also showed transfer of cells from these membranes to cornea organ culture models in vitro.

Limbal stem cells are believed to be located within crypts in the limbus and in contact with supporting cells which help them retain their stemness[13-15]. To mimic the native tissue, we designed a membrane incorporating microfabricated pockets to provide protection to the limbal explants that are transplanted onto the ocular surface[16].

In this study we applied PLGA membranes both with and without microfabricated pockets to the corneas of rabbits for 29 days. Experiments were done in collaboration with an accredited contract research organisation, Vimta Labs Ltd, following

Schedule 'Y', Drugs and Cosmetic (II Amendment) Rules, for assessing topical and systemic toxicity.

Both membranes broke down completely within 29 days without adverse effects.

Materials

PLGA membranes were produced by The Electrospinning Company Ltd, UK. PLGA 50:50 (Corbion, the Netherlands) of molecular weight 44 kg/mol was used in this study. Membranes were gamma irradiated (Steris AST, Swindon, UK), packaged and sent to India by World Courier (London, UK).

New Zealand rabbits were sourced from Delve Labs, Hyderabad.

Bandage contact lens and Ethicon non-absorbable surgical were from Johnson and Johnson Vision care, USA and Tisseel kit (fibrin glue) from Baxter, Austria.

Methods

The study was performed in compliance with OECD Principles of Good Laboratory Practice for the testing of chemicals as specified by International [C(97)186/Final] Legislation. The study protocol was approved by the Institutional Animal Ethics Committee of VIMTA Labs Limited and abided by the recommendations of the Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines.

Experimental Design

Rabbits (aged 10-13 weeks) were acclimatized for 5 days prior to surgery then randomized based on their weight. Fundus examination, intra ocular pressure (IOP) measurement and fluorescein dye examination were performed prior to randomization to ensure the selected rabbits had normal ocular health. Rabbits were distributed into 2 groups for both plain and microfabricated membranes; control or sham treated group (G1) of 6 rabbits (3 males + 3 females) and PLGA treated (G2) group of 14 rabbits (7 males + 7 females). Fibrin glue and a bandage contact lens were applied to the left eye of G1 rabbits. In G2 rabbits, PLGA membrane was placed on the left eye using fibrin glue, followed by the application of bandage contact lens. Before performing complete tarsorrhaphy, one drop of 2.5% povidone-iodine was instilled in the eye receiving the PLGA membrane or sham treatment. **For all animals**

tarsorrhaphy was removed a day prior to sacrifice on days 7, 14 and 28, respectively. Surgical procedures were performed under general anesthesia using a combination of Ketamine (30mg/Kg body weight) and Xylazine (5mg/Kg body weight). Ciprofloxacin eye drops were administered 4 times a day for one week and carboxymethyl cellulose drops applied daily. Four rabbits (2 male + 2 female) each, from G2 were sacrificed on day 8 and day 15 to study the degradation of the membranes in vivo. All remaining animals were sacrificed on day 29.

A schematic of the experimental protocol is given in figure 2.

Test Material

PLGA membranes, 50 μm thick with a mean fiber diameter of $2.74 \pm 0.89 \mu\text{m}$ were electrospun[12]. Polymers were dissolved in 1,1,1,3,3,3-hexafluoroisopropanol (Sigma Aldrich, Dorset, UK) at a concentration of 20 wt%. Polymer solutions were delivered at a constant feed rate of 800 $\mu\text{l/h}$, using a programmable Harvard PHD4400 syringe pump (Harvard Apparatus, Kent, UK) to a blunt tipped stainless-steel needle with an internal diameter of 0.8 mm. The tip of the needle was in turn connected to a positive high voltage unit (Glassman High Voltage Inc. High Bridge, NJ, USA) and solutions were electrospun with an applied voltage of 12.5 kV. Electrospinning was performed in an environmentally controlled, A1-Safetech, air recirculation cabinet at 25°C and a relative humidity of 25%. For the plain PLGA membranes, fibers were deposited onto a grounded, custom built rotating mandrel at a distance of 300 mm from the tip of the needle, coated in aluminum foil. For the membranes with microfabricated pockets, fibres were deposited onto templates prepared via stereolithography[16].

Following production and quality control assessment by SEM (Phenom G2 Pro, The Netherlands), membranes were dried under vacuum at room temperature for 48hrs to remove residual solvent, before being die cut into 22 mm diameter discs. The membranes were then vacuum sealed in bags prior to terminal sterilization via γ -irradiation (25-40 kGy) at Steris AST Plc. (Moray Road, Swindon, UK). A schematic representation of the production process is given in figure 1.

Observations

1. Clinical signs

Clinical examinations were performed prior to treatment and weekly thereafter. These included changes to skin, fur, eyes, mucous membrane, secretions, excretions, autonomic activity, gait, posture and response to handling and presence of clonic or tonic movements.

1.1. Body Weight

Body weights were recorded on days 1, 8, 15, 22 and 29. Fasting body weights for interim sacrificed animals were taken one day prior to necropsy.

2. Ophthalmic investigations

2.1 Gross ocular observations were performed by visual assessment for signs of infection or inflammation. Treated eyes were examined for degradation of scaffold on the day of sacrifice after removal of bandage contact lens.

2.2 Fundus examination and IOP of all rabbits was carried out using a Kowa Digital Ophthalmoscope (Welch Allyn, USA) and a Reichert Tono-Pen XL Tonometer (Reichert, USA) prior to randomisation and sacrifice.

2.3 Fluorescein was applied to the ocular surface to assess health prior to randomisation and on sacrifice days after removal of contact lens and PLGA membrane where applicable.

3. Blood investigations

Clinical laboratory investigations were performed on days 8, 15 or 29. Blood was collected from the overnight fasted rabbits from the marginal ear vein. Haematological parameters were determined using ADVIA, 2120 (Siemens, Germany).

Clinical chemistry parameters were analysed with the help of an automatic biochemical analyser (Vitros 250, Johnson & Johnson) using standard kits.

4. Tissue investigations

The animals were fasted overnight, weighed and euthanised under over anesthesia of thiopentone sodium.

4.1 Organ weights

Adrenals, brain, heart, lungs, kidneys, liver, spleen, testes/uterus and thymus were dissected free of fat and weighed wet as soon as possible to avoid drying.

4.2 Histopathology

Tissues from sacrificed rabbits were processed in an automatic tissue processor (Microm spin tissue processor STP 120-3, Thermo Scientific, USA) and embedded in paraffin wax using Microm Embedding center EC350 (Thermo Scientific, USA). Tissue sections of 3-6 μ were stained with haematoxylin and eosin in an automatic tissue stainer (Microm HMS 740 robotic stainer, Thermo Scientific, USA). A 4-step grading system of minimum, mild, moderate and marked was used to rank microscopic findings for comparison among groups.

Statistical Analysis

The data generated from the rabbits subjected to interim sacrifice on day 8 and 15 was not considered for statistical analysis along with the animals sacrificed on day 29 (due to variance in the day of sacrifice compared to control animals). The data is expressed as mean \pm SD. Normality of the data was confirmed using D'Agostino and Pearson omnibus test. ANOVA and Kruskal-Wallis tests were used for group comparison of non-homogenous data. Parameters showing significance in the Kruskal-Wallis test were further analysed with Wilcoxon test to compare each group individually over the respective control arm. The data analysis was performed using SAS[®] 9.2, Enterprise Guide version 4.2 (SAS Institute Inc., Cary, NC). All statistical tests were performed at 5% level of significance.

Results

Rabbits from control and PLGA treated groups did not show any treatment related abnormal clinical signs during the experimental period. There were no deaths in any of the groups of rabbits.

Body Weight

No statistical differences were observed in the mean body weights (Supplementary table 1) of the different groups of rabbits throughout this study.

Ophthalmic examination

The PLGA membranes (plain and microfabricated) appeared intact on the ocular surface on day 8. Partial breakdown of the membrane was noted in all animals on day 15. By day 29, there was complete breakdown of the respective membranes in all animals (Fig.3)

Mild to moderate conjunctival congestion was noted in all animals sacrificed on day 8 but resolved completely by day 29. In all animals sacrificed on day 8 (both plain and microfabricated) PLGA membrane was found adhered to the ocular surface. Vascularization (<2 clock hours) was noted in 1/4 and 3/6 animals that received the plain PLGA membrane at the end of 15 and 29 days, respectively. Control animals showed epithelial defects and stromal thinning in 2/6 (day 15) and 1/6 (day 29) animals, respectively. Corneal vascularization (2-4 clock hours) was noted in 3/6 animals of the pocket PLGA treated animals and in 3/6 of control animals at the end of the 29 days.

There was no significant change to the IOP when compared to the control animals (Supplementary figure 1). This was true for both membranes with and without microfabricated pockets. Similarly, no abnormalities were detected in the retina of any of the animals before or after treatment with the PLGA membranes. A summary of the ophthalmic observations is given as a table in figure 3.

Hematology

In animals that received the plain membrane, a significant decrease in MCHC ($p \leq 0.01$), RBC distribution width ($p \leq 0.05$), relative and absolute monocyte counts ($p \leq 0.05$) and absolute lymphocyte counts ($p \leq 0.05$) was observed compared to control group. A significant increase in relative basophil count ($p \leq 0.05$) was also observed in rabbits treated with the test membrane compared to the control group (Table 3).

In animals that received the pocket membranes, a significant increase in absolute lymphocyte counts ($p \leq 0.05$) was observed in rabbits treated with the test items compared to the control animals. No other parameter varied significantly between the two groups (Table 1).

Clinical Chemistry

ALKP ($p \leq 0.05$) and GGT ($p \leq 0.01$) were significantly increased in rabbits treated with plain membranes compared to control rabbits and there was a significant decrease in creatinine ($p \leq 0.05$) and total bilirubin ($p \leq 0.01$) values when compared to control rabbits (Table 2).

In animals that received pocket membranes, there was a significant decrease in ALT values ($p \leq 0.05$) and a significant increase in BUN ($p \leq 0.01$) values when compared to control rabbits. All other parameters were comparable in both groups (Table 2)

Organ Weights

There were no significant changes in the absolute or relative organ weights in male and female rabbits that received the plain membrane compared to the control animals. In the animals with the pocket membranes, absolute ($p \leq 0.01$) and relative ($p \leq 0.05$) weights of the liver were significantly higher in the treated group compared to the control group (Supplementary table 2).

Gross Pathology

There were no gross pathological findings indicative of inflammation or immune response in any of the rabbits treated with plain or pocket membranes or with sham treatment.

Histopathology

Hyperplasia of corneal epithelium and remodeling of anterior stromal collagen fibers were noted in 1/6 control animals (sacrificed on day 29) of the plain membrane group (Supplementary table 3). The same was noted in 2/6 control animals and 4/6 animals that received the pocket membrane (day 29; supplementary table 4). Three rabbits that had the pocket membrane showed infiltration of heterophils at the filtration angle (Supplementary table 4).

There were no lesions observed in the retina in any of the animals examined irrespective of whether animals received PLGA membranes or not.

In the liver, focal chronic inflammation was noted in 1/6 (plain PLGA) and mineralization of hepatocytes in 1/6 (pocket PLGA) animals. Basophilic tubules and mineralization was noted in the kidneys of 1/6 animals that received the plain and pocket PLGA membranes, respectively. Increased alveolar macrophages were noted in 1/6 animals that received both plain and pocket PLGA membranes. All other histological changes were comparable between the control and PLGA treated animals.

Discussion

PLGA is biodegradable, biocompatible, FDA approved and has been used in several applications including drug delivery. The success of PLGA as a product lies in the fact that its degradation is largely driven by hydrolytic mechanisms rather than enzymatic breakdown and that it breaks down to lactic acid glycolic acid both readily handled by the body. The rate of breakdown of PLGA will depend on the ratio of polylactic acid to polyglycolic acid, its location in the body and its bulk composition. Thus there is minimal systemic toxicity reported with the use of PLGA in the clinic[10,17,18]. In addition, the ability to alter its degradation properties by simply altering the ratio of the individual monomers has made this the material of choice for a range of applications.

PLGA based materials have also been tested for a number of applications in the eye especially in developing ocular drug delivery systems such as implants and micro-or nano-particles. The efficacy of PLGA in delivering anti-inflammatory agents to reduce inflammation following cataract surgery has been reported[19]. A study in rabbits showed that the PLGA films implanted in the sub-conjunctival space were well tolerated during the study period (6months) with no significant inflammatory reaction indicative of a rejection[20]. Further PLGA scaffolds were shown to support the porcine and rabbit derived corneal endothelium and retinal pigment epithelial cells[21] suggesting that the material might be suitable for use in cell therapy since it offered sufficient support for the cells to grow and mature exhibiting normal cellular characteristics with minimal cell toxicity.

It is important in designing material for the clinic to use a recognised sterilisation technique. In developing this PLGA membrane for clinical use we used gamma radiation at 25 to 40 kGy with an average dose per run of 29.4 kGy. In our previous publication which formed part of the data which was submitted to the Indian health regulatory authority to obtain approval for a first in man study we studied the impact of gamma radiation on membrane stability and on the ability of the membranes to support outgrowth of limbal cells from limbal tissue explants[12].

Gamma radiation accelerated the degradation of the PLGA membranes however, their ability to support limbal epithelial cell expansion, especially of the stem cell population, was unaffected. We show in the study that 50% of the cells cultured on gamma irradiated membranes were proliferating (as determined by positive BrdU staining) and there was good outgrowth from explants placed on the irradiated membranes as assessed using DAPI staining and Rose Bengal staining[12]. Thus

gamma radiation while accelerating the breakdown of the membranes (which was actually beneficial for this application) had no adverse effects on limbal explanted growth.

We also showed that these membranes in addition to being sterilised could be packaged and stored at -20C for more than 2 years and shipped long distance without altering their material properties[12].

The motivation behind the current study is that prior to any first in man safety study it is necessary to demonstrate that these membranes do not induce local or systemic toxicity when applied to the cornea of a relevant animal model. The results we obtained showed that membranes degraded within 29 days without causing significant topical or systemic toxicity.

While temporary conjunctival congestion was noted in all animals on day 8, persistent vascularization of the corneal surface (2-4 clock hours) was noted in both sham and treated animals indicating the response was primarily to the surgical procedure. Fluorescein staining showed clear and intact ocular surface in all animals except one control animal that had stromal thinning. Hematological parameters showed significant but minor changes in lymphocyte and monocyte counts in the test animals- both decreases and increases but all within the normal expected range of clinical values for these animals. As the gross and individual organ histology findings did not indicate sustained inflammation or infection, these changes were considered to be adaptive, consistent with the expected immune response to the breakdown of the PLGA material.

Similarly, while there were significant decreases in the creatinine and total bilirubin levels in plain PLGA treated animals, significant increases in ALKP and GGT values, an increase in BUN values and a decrease in the ALT values in the pocket treated animals compared to the control animals these were again within the normal range for these animals and there was no histological evidence of any adverse reaction to the PLGA membranes.

There was a significant increase in the liver weight for animals that received the pocket membrane but again no evidence of any correlation with clinical histopathological findings as would occur with hepatocellular hypertrophy which can occur in response to toxicity. We suggest the increase in the liver weight in all the animals of pocket PLGA treated animals might be indicative of a mild inflammatory response. In an earlier study[11] in which we implanted electrospun sheets of various

ratios of PLA to PGA into the flanks of rats we found a vigorous macrophage response associated with scaffold breakdown but little evidence of any lymphocytic response.

In conclusion, these toxicity studies showed no evidence of topical or systemic toxicity in response to the placing of these membranes on the cornea of rabbits.

Acknowledgement

We acknowledge the Wellcome Trust (Affordable Healthcare in India) for the funding support. We are thankful to Dr. Jomy Jose from Vimta Labs Ltd for her help with the interpretation of the animal data.

Contributors: CR: conception of study, data analysis, interpretation of data and manuscript preparation; VSS: conception of study and interpretation of data; IO: design of PLGA materials and manuscript preparation; UB: design of study, data collection and analysis; SM: design of study, data collection and analysis RMK: manuscript preparation; SMN: conception and design of study, data interpretation and manuscript preparation and approval

Competing Interests: None

References

1. Kenyon KR, Tseng SC. Limbal autograft transplantation for ocular surface disorders. *Ophthalmology* 1989;**96**(5):709-22; discussion 22-3
2. Rama P, Bonini S, Lambiase A, et al. Autologous fibrin-cultured limbal stem cells permanently restore the corneal surface of patients with total limbal stem cell deficiency. *Transplantation* 2001;**72**(9):1478-85
3. Sangwan VS, Basu S, MacNeil S, et al. Simple limbal epithelial transplantation (SLET): a novel surgical technique for the treatment of unilateral limbal stem cell deficiency. *The British journal of ophthalmology* 2012;**96**(7):931-4 doi: 10.1136/bjophthalmol-2011-301164[published Online First: Epub Date].
4. Vazirani J, Ali MH, Sharma N, et al. Autologous simple limbal epithelial transplantation for unilateral limbal stem cell deficiency: multicentre results. *The British journal of ophthalmology* 2016;**100**(10):1416-20 doi: 10.1136/bjophthalmol-2015-307348[published Online First: Epub Date].
5. Basu S, Sureka SP, Shanbhag SS, et al. Simple Limbal Epithelial Transplantation: Long-Term Clinical Outcomes in 125 Cases of Unilateral

- Chronic Ocular Surface Burns. *Ophthalmology* 2016;**123**(5):1000-10 doi: 10.1016/j.ophtha.2015.12.042[published Online First: Epub Date]].
6. Rama P, Matuska S, Paganoni G, et al. Limbal stem-cell therapy and long-term corneal regeneration. *The New England journal of medicine* 2010;**363**(2):147-55 doi: 10.1056/NEJMoa0905955[published Online First: Epub Date]].
 7. Sangwan VS, Basu S, Vemuganti GK, et al. Clinical outcomes of xeno-free autologous cultivated limbal epithelial transplantation: a 10-year study. *The British journal of ophthalmology* 2011;**95**(11):1525-9 doi: 10.1136/bjophthalmol-2011-300352[published Online First: Epub Date]].
 8. Bobba S, Chow S, Watson S, et al. Clinical outcomes of xeno-free expansion and transplantation of autologous ocular surface epithelial stem cells via contact lens delivery: a prospective case series. *Stem cell research & therapy* 2015;**6**:23 doi: 10.1186/s13287-015-0009-1[published Online First: Epub Date]].
 9. Mi S, Chen B, Wright B, et al. Ex vivo construction of an artificial ocular surface by combination of corneal limbal epithelial cells and a compressed collagen scaffold containing keratocytes. *Tissue engineering Part A* 2010;**16**(6):2091-100 doi: 10.1089/ten.TEA.2009.0748[published Online First: Epub Date]].
 10. Orr JW, Jr., Montz FJ, Barter J, et al. Continuous abdominal fascial closure: a randomized controlled trial of poly(L-lactide/glycolide). *Gynecologic oncology* 2003;**90**(2):342-7
 11. Blackwood KA, McKean R, Canton I, et al. Development of biodegradable electrospun scaffolds for dermal replacement. *Biomaterials* 2008;**29**(21):3091-104 doi: 10.1016/j.biomaterials.2008.03.037[published Online First: Epub Date]].
 12. Deshpande P, Ramachandran C, Sefat F, et al. Simplifying corneal surface regeneration using a biodegradable synthetic membrane and limbal tissue explants. *Biomaterials* 2013;**34**(21):5088-106 doi: 10.1016/j.biomaterials.2013.03.064[published Online First: Epub Date]].
 13. Dziasko MA, Daniels JT. Anatomical Features and Cell-Cell Interactions in the Human Limbal Epithelial Stem Cell Niche. *The ocular surface* 2016;**14**(3):322-30 doi: 10.1016/j.jtos.2016.04.002[published Online First: Epub Date]].
 14. Dziasko MA, Tuft SJ, Daniels JT. Limbal melanocytes support limbal epithelial stem cells in 2D and 3D microenvironments. *Experimental eye research* 2015;**138**:70-9 doi: 10.1016/j.exer.2015.06.026[published Online First: Epub Date]].
 15. Nowell CS, Radtke F. Corneal epithelial stem cells and their niche at a glance. *Journal of cell science* 2017;**130**(6):1021-25 doi: 10.1242/jcs.198119[published Online First: Epub Date]].
 16. Ortega I, Ryan AJ, Deshpande P, et al. Combined microfabrication and electrospinning to produce 3-D architectures for corneal repair. *Acta biomaterialia* 2013;**9**(3):5511-20 doi: 10.1016/j.actbio.2012.10.039[published Online First: Epub Date]].
 17. Eppley BL. Use of resorbable plates and screws in pediatric facial fractures. *Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons* 2005;**63**(3):385-91 doi: 10.1016/j.joms.2004.11.011[published Online First: Epub Date]].

18. Weisberger EC, Eppley BL. Resorbable fixation plates in head and neck surgery. *The Laryngoscope* 1997;**107**(6):716-9
19. Eperon S, Bossy-Nobs L, Petropoulos IK, et al. A biodegradable drug delivery system for the treatment of postoperative inflammation. *International journal of pharmaceuticals* 2008;**352**(1-2):240-7 doi: 10.1016/j.ijpharm.2007.10.054[published Online First: Epub Date]].
20. Peng Y, Ang M, Foo S, et al. Biocompatibility and biodegradation studies of subconjunctival implants in rabbit eyes. *PloS one* 2011;**6**(7):e22507 doi: 10.1371/journal.pone.0022507[published Online First: Epub Date]].
21. Hadlock T, Singh S, Vacanti JP, et al. Ocular cell monolayers cultured on biodegradable substrates. *Tissue engineering* 1999;**5**(3):187-96 doi: 10.1089/ten.1999.5.187[published Online First: Epub Date]].

Figure legends

Figure 1: Schematic of the production and sterilisation route for PLGA electrospun membranes (plain and microfabricated).

The membrane design (top panel) involved the fabrication of the membranes in collaboration with The Electrospinning Company which ensured both reproducibility and scaling-up possibilities. For the production of the microfabricated membranes, first polymeric ring templates of 1.6 cm of diameter were created using a layer-by-layer photocuring approach in which a blue laser (473 nm) was directed into a bath of Polyethylene glycol diacrylate (PEGDA) containing camphorquinone (CQ) during intervals of 15-60 seconds. The process involved the use of a computer aided design comprising two layers (Layer 1 acted as a base and it did not contain microfeatures; Layer 2 contained a total of 6 horse-shaped microfeatures of sizes ranging 350-500µm). The combination of these two layers resulted in the creation of the polymeric rings which acted as templates. These were mounted onto a 2 mm thick grounded electroplated aluminium sheet and then membranes were electrospun over them. This resulted in microfabricated PLGA electrospun rings (pocket membranes). The manufactured membranes were characterised and tested in vitro using both rabbit and human corneal cells. After product design optimisation and in vitro testing, prototype membranes were produced and sterilised using gamma-irradiation. PLGA membranes were then vacuum-packed with a range of desiccants to ensure moisture stability. The optimised and sterilised membranes (plain and microfabricated) were then used for the evaluation of local and systemic toxicity in rabbits.

Figure 2: Schematic of the experimental protocol in rabbits

This protocol was used for both the study of the plain PLGA membrane and of the PLGA membrane containing microfabricated pockets which were conducted as two separate experiments with individual control groups.

Figure 3: Breakdown of plain and pocket PLGA membranes on corneas of rabbits

Representative images of the residual plain PLGA membrane on the ocular surface of the rabbit on days 8,15 and 29 is shown in figure 3A. Representative image of the sham treated eye on day 29 is shown for comparison. In panel B are shown representative images of the ocular surface of control eyes and eyes treated with PLGA membranes after fluorescein staining on days 8, 15, and 29. The staining seen in these images are of the PLGA membranes that remained attached to the ocular surface. Panel C is a summary table of the ocular findings in the animals treated with plain or pocket PLGA and their respective control animals.

Supplementary figure 1: Examination of the effect of plain and pocket PLGA membranes on the fundus of the eye and on intra-ocular pressure.

A shows photographs of the retina of the rabbit eyes before and after application of plain membranes and membranes containing microfabricated pockets.

B shows the intraocular pressures recorded on days 7, 15 and 29 for rabbits which did not receive membranes and for rabbits which received plain membranes or membranes with microfabricated pockets.

Table 1: Hematological values

Parameter	Sham Treated	Plain membrane	Sham Treated	Pocket membrane
WBC (10 ³ Cells/ μ L)	7.73 \pm 1.10	6.46 \pm 2.09	6.62 \pm 2.01	8.63 \pm 1.30
RBC (10 ⁶ Cells/ μ L)	5.43 \pm 0.61	5.66 \pm 0.25	5.43 \pm 0.47	5.44 \pm 0.67
Hb (g/dL)	11.6 \pm 1.1	11.9 \pm 0.7	11.3 \pm 0.7	11.2 \pm 0.7
Hct (%)	36.3 \pm 3.4	39.1 \pm 1.8	37.9 \pm 2.2	38.1 \pm 2.7
MCV (fL)	67.1 \pm 4.0	69.1 \pm 2.1	69.9 \pm 3.9	70.4 \pm 5.2
MCH (pg)	21.5 \pm 1.5	21.1 \pm 0.9	20.8 \pm 0.8	20.8 \pm 1.2
MCHC (g/dL)	32.0 \pm 0.6	30.5 \pm 0.5 (** \pm)	29.8 \pm 0.5	29.6 \pm 0.7
RDW (%)	15.8 \pm 0.9	14.3 \pm 1.3 (* \pm)	15.4 \pm 0.8	14.5 \pm 1.2
Platelets (10 ³ Cells/ μ L)	634 \pm 197	605 \pm 93	589 \pm 184	505 \pm 122
Relative DLC (10³ cells/μl)				
Neut (%)	17.9 \pm 9.4	18.6 \pm 11.0	16.1 \pm 7.3	17.8 \pm 8.1
Lymp (%)	74.4 \pm 9.9	73.5 \pm 10.5	75.8 \pm 8.2	74.0 \pm 8.3
Mono (%)	3.2 \pm 0.5	2.5 \pm 0.6 (* \pm)	2.3 \pm 1.1	3.2 \pm 1.7
Eosi (%)	1.0 \pm 0.5	0.9 \pm 0.7	1.1 \pm 0.4	1.1 \pm 0.3
Baso (%)	3.1 \pm 1.0	4.4 \pm 0.9 (* \pm)	4.3 \pm 1.8	3.4 \pm 1.5
Luc (%)	0.4 \pm 0.5	0.2 \pm 0.1	0.5 \pm 0.3	0.4 \pm 0.2
Absolute DLC (x10³ cells/μl)				
Neut (10 ³ cells/ μ l)	1.46 \pm 1.06	1.37 \pm 1.26	1.15 \pm 0.82	1.61 \pm 0.88
Lymp (10 ³ cells/ μ l)	5.55 \pm 0.35	4.58 \pm 0.88 (* \pm)	4.93 \pm 1.16	6.32 \pm 0.48
Mono (10 ³ cells/ μ l)	0.25 \pm 0.06	0.16 \pm 0.05 (* \pm)	0.15 \pm 0.07	0.29 \pm 0.15
Eosi (10 ³ cells/ μ l)	0.08 \pm 0.05	0.06 \pm 0.05	0.08 \pm 0.05	0.10 \pm 0.04
Baso (10 ³ cells/ μ l)	0.24 \pm 0.07	0.28 \pm 0.09	0.27 \pm 0.12	0.28 \pm 0.11
Luc (10 ³ cells/ μ l)	0.04 \pm 0.05	0.01 \pm 0.00	0.03 \pm 0.02	0.04 \pm 0.02

N=6 (each of sham treated and PLGA treated animals), * \pm Significant increase when compared to control (p<0.05); * \pm Significant decrease when compared to control (p<0.05); ** \pm Significant increase when compared to control (p<0.01); ** \pm Significant decrease when compared to control (p<0.01)

Table 2: Clinical chemistry values

Parameter	Sham Treated	Plain membrane	Sham Treated	Pocket membrane
Glucose (mg/dl)	140 ± 10	136 ± 19	143 ± 19	172 ± 27
BUN (mg/dl)	19.8 ± 3.6	16.9 ± 3.4	19.8 ± 2.2	25.1 ± 2.9 (**+)
Creatinine (mg/dL)	0.91 ± 0.16	0.69 ± 0.11 (*-)	0.78 ± 0.10	0.77 ± 0.12
Na+(mmol/L)	142 ± 4.0	146 ± 3.0	154 ± 16	156 ± 4.0
K+ (mmol/L)	4.1 ± 0.4	3.6 ± 0.2	3.8 ± 0.4	4.6 ± 0.3
Ca+ (mg/dL)	14.02± 0.42	14.64± 0.69	13.81± 0.25	15.52± 0.27
Phosphate (mg/dL)	5.32 ± 0.57	5.60 ± 0.76	6.08 ± 0.27	5.54 ± 0.79
T.Cholesterol(mg/dl)	55 ± 10	47 ± 3.0	58 ± 21	54 ± 18
Triglyceride (mg/dL)	53 ± 6.0	73 ± 33	91 ± 57	139 ± 80
T.Protein(g/dL)	6.30 ± 0.40	6.53 ± 0.64	6.41 ± 0.33	6.35 ± 0.36
Albumin (g/dL)	4.16 ± 0.31	4.39 ± 0.53	4.48 ± 0.27	4.46 ± 0.53
A/G Ratio	2.04 ± 0.57	2.11 ± 0.47	2.35 ± 0.25	2.41 ± 0.54
AST(U/L)	35 ± 10	45 ± 32	38 ± 16	27 ± 5
ALT(U/L)	62 ± 17	72 ± 23	60 ± 13	42 ± 13 (*-)
ALP(U/L)	98 ± 26	131 ± 16 (*+)	156 ± 28	126 ± 31
GGT (U/L)	8.0 ± 1.0	11 ± 1.0 (**+)	9.0 ± 2.0	9.0 ± 1.0
T.Bilirubin(mg/dL)	0.55 ± 0.09	0.40 ± 0.04 (**-)	0.41 ± 0.07	0.42 ± 0.06
Glo(g/dL)	2.14 ± 0.46	2.14 ± 0.41	1.92 ± 0.18	1.88 ± 0.19

N=6 (each of sham treated and PLGA treated animals), *-significant decrease compared to sham treatment (p<0.05); **- significant decrease compared to sham treatment (p<0.01); *+ significant increase compared to sham treatment (p<0.05); **+ significant increase compared to sham treatment (p<0.01)

