Production, sterilisation and storage of biodegradable electrospun PLGA membranes for delivery of limbal stem cells to the cornea

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Abstract

Our aim was to produce, sterilize and store a synthetic, rapidly biodegrading membrane for cultured limbal stem cell transplantation. Membranes were electrospun from Poly(D,L-lactide-co-glycolide) with a 50:50 ratio of lactide and glycolide comparing 44 kg/mol and 153 kg/mol molecular weights (MW) and sterilized with \(\gamma\)-irradiation and stored for up to a year at a range of temperatures. Cells attached well on both MW membranes. The lower MW degraded faster than the higher MW membranes. \(\gamma\)-irradiation accelerated membrane breakdown when wet but sterilised membranes could be stored dry for at least a year at -20°C.

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Introduction

A number of conditions can lead to limbal stem cell loss – trauma, thermal and chemical burns, and autoimmune disorders [1]. When the avascular transparent surface of the cornea is lost, the tissue which grows over the eye comes from the conjunctiva and this essentially scar tissue results in reduced or even complete loss of vision and considerable pain [1-3].

The front line treatment is normally corneal transplantation using cadaveric corneas from tissue banks. However, these rely on there being a residual population of limbal stem cells to repopulate these donor corneas. Where patients lack any residual limbal stem cells the corneal grafts fail. In response to this a small number of specialist centres around the world over the last 15 years have developed techniques to culture and transplant limbal epithelial stem cells for the treatment of patients who have lost this population of cells which are essential for the continued regeneration of the cornea. Culture of cells from the contralateral unaffected eye is undertaken wherever possible or from donor eyes (with associated immunosuppression) when no autologous cells are available [3].

For transplantation of cells to the damaged eye this is most commonly done by culturing the cells on donor human amniotic membrane and then transplanting them to the eye (cells uppermost) where this membrane degrades over several weeks leaving these cells in place [3-4]. However, use of the amniotic membrane requires access to donor tissue banked under dedicated tissue bank conditions. This requires establishing tissue banks. Also variable clinical results have been found which might be due to variation in this donor tissue or in its processing. Critically however, all human donor tissue carries some risk of disease transmission which can be reduced with good tissue banking practices but not fully eliminated [5-7].

Accordingly, the aim of this research is to make a synthetic biodegradable alternative membrane, available as a readily stored product, to replace the use of human amniotic membrane for this purpose to reduce risks of disease transmission and improve the accessibility and reproducibility of these membranes for surgeons.

The desirable characteristics of a synthetic membrane for delivering limbal epithelial cells (LECs) are that it must be non-cytotoxic, it must provide secure attachment for LECs and support their proliferation and migration [8]. It must be as low risk for clinical use as possible, i.e. it must be sterilized using an acceptable sterilization methodology. It must be capable of being fixed to the eye post culture with cells and for it to be successful it needs to degrade within a few weeks leaving LECs securely attached to the underlying cornea. Thus we seek a membrane that will degrade predictably and within a few weeks, support LEC attachment and is capable of being produced, sterilised and stored reproducibly.

In this study poly(D,L-lactide-co-glycolide) (PLGA) was selected as it is biodegradable and biocompatible, FDA approved and has been used for many years in products such as dissolvable sutures [9]. Such sutures are currently used in the cornea. By varying the ratio of lactide to glycolide it is also possible to predict the degradation rates of these membranes in vivo [10]. For this purpose we sought a membrane that would degrade in a relatively short period of time (2-6 weeks) suitable for replacing the amniotic membrane as a carrier for cultured cells.

While there are numerous publications on the development of biodegradable electrospun membrane for clinical use there are still very few that addressed the issues of how to sterilize and store these membranes. This study focuses on the production, physical characterization, sterilization and storage of biodegradable PLGA membranes. With respect to sterilization of membranes, while it is entirely feasible to produce membranes under sterile conditions, in terms of routine manufacture, post production sterilization is more attractive and of the methodologies possible γ-irradiation is the most widely accepted sterilization methodology. All sterilization methodologies are likely to affect the physical properties of electrospun membranes; hence it is important to characterize the effect of γ-irradiation on the physical properties of the membranes. Accordingly we looked at physical characterization of the polymers (glass transition temperatures, molecular weight) pre and post spinning and pre and post sterilization. We combined the membranes with cells and looked at their rate of breakdown, how easy or difficult they were to handle with and without sterilization and we also looked at the stability of membranes when stored dry at a range of temperatures, again looking to what extent γ-irradiation affected stability on storage.

Thus in summary this study provides basic information on the characteristics of membranes which are being developed to be an off-the-shelf replacement for the amniotic membrane.
2. Materials and Methods

2.1. Polymers and electrospinning

For this study Poly (D,L-lactide-co-glycolide) with a 50:50 ratio of PLA to PGA (Purac; PLG5004 (M_w 44 kg/mol) and PLG5010 (M_w 153 kg/mol)) were electrospun. These PLGA membranes were electrospun to our specifications by The Electrospinning Company, with fibre diameters of 2-3 μm and a scaffold depth of 50 μm. We compared polymers of two MWs and incorporated sterilisation with γ-irradiation. 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) (Sigma Aldrich, Dorset, UK) was used as a solvent and polymers were dissolved at room temperature to produce solutions of suitable viscosity for electrospinning (10 wt% and 20 wt% for the low and high molecular weight, respectively).

A programmable Harvard PHD4400 syringe pump (Harvard Apparatus, Kent, UK) was used to deliver polymer solutions from a 5 ml plastic syringes (Becton Dickinson, Oxford, UK). Polymers were delivered at a constant rate of 800 μl/h, via PTFE tubing (1/16" O.D.), to a blunt tipped stainless steel needle with an internal diameter of 0.8 mm (Intertronics, Kidlington, Oxfordshire, UK). The tip of the needle was 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. Fibres were deposited onto a grounded, custom built rotating mandrel (120 mm in diameter, 250 mm in length) at a distance of 300 mm from the tip of the needle, coated in 50 μm thick aluminium foil.

Electrospinning was performed in an environmentally controlled, A1-Safetech, air recirculation cabinet (a1-envirosciences GmbH, Düsseldorf, Germany) at an air temperature of 25°C and a relative humidity of 25%. In order to make sure no residual solvent remained in the membrane a vacuum oven was used to dry membranes at room temperature for 48 hours. Membranes cut into 22 mm diameter discs and placed in 12-well, non-tissue culture treated plates (BD Falcon, Beckton Dickinson, Oxford, UK). The plates were then individually sealed in polypropylene bags (Andrew James UK LTD, Bowburn, UK) before storage.

2.2. γ-Irradiation of membranes

γ-irradiation was used to sterilise PLGA membranes which previously fabricated and mounted in 12-well plates by The Electrospinning Company. This process was carried out at Synergy Health Plc. (Moray Road, Swindon, UK), with an external dose range of 25-40 kGy. The low and high molecular weight membranes (sterilised and non-sterilised) were then compared throughout to assess the impact of sterilisation on their physical properties, their ability to support cells and also their breakdown when stored without cells at a range of temperatures.

2.3. Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry (DSC) was used to obtain the glass transition temperature (T_g). Thermal transitions were recorded on a Perkin-Elmer Pyris-1 connected to a controller model CCA7 (Perkin-Elmer, Wellesley, MA, USA). The samples were heated in an inert atmosphere of Nitrogen (30 ml/min flow rate) from 0–100°C at a rate of 10°C/min; each sample was re-heated. Samples of 5–10 mg of each PLGA membrane were placed in a crimped aluminium pan and subjected to the heat gradient. Thermograms were obtained and subsequently analysed.

2.4. Gel Permeation chromatography (GPC)

The polymer molecular weight distribution were determined by gel permeation chromatography (GPC) (Perkin-Elmer, Wellesley, MA, USA) equipped with a refractive index detector (Perkin-Elmer, Wellesley, MA, USA). In this work the PLGA (50:50) samples were tested either as crystals (before spinning) or polymer discs (after spinning). Tetrahydrofuran (THF, Fisher Scientific, Loughborough, UK) was used as a solvent and dried samples were dissolved in this solvent with a flow rate marker of toluene, at a concentration of 8 mg/ml and eluted through
the column at a flow rate of 1 ml/min at 37°C. Polystyrene standards (Polysciences, Warrington, PA, USA) were used to obtain a primary calibration curve. All samples of the same polymer type were analyzed in a single run.

2.5. Wettability of Membranes

A contact angle goniometer (ramé-hart, USA) was used to measure the wettability of PLGA membranes. In this study 3 μl drop of deionised water (dH₂O), phosphate buffer saline (PBS) or media (DMEM) were used. Five samples were used for each test and the average value was reported with a standard deviation (±SD).

2.6. Biomechanical Properties

A Bose Electroforce 3100 tensile test machine (Bose, MN, USA) was used to carry out mechanical tests on PLGA membranes. PLGA membranes were cut into rectangles with dimensions of 30x10 mm² and membranes were clamped in place via stainless steel clamps and pulled apart at a rate of 1 mm/10 sec with grip distance of 7 mm. In this study the force exerted was measured via a 22 N small-load cell and the sample thicknesses measured using a micrometer. A total number of five samples were used for each experimental condition.

2.7. Culture of rabbit limbal epithelial cells (rLEC)

Rabbit limbal epithelial cells (rLEC) were isolated from New Zealand white rabbits (Hook Farm, Hook, Berkshire UK). Initially, excess fat and tissue were removed and 3% videne antiseptic solution (Ecolab, Swindon, UK) was used to disinfect the eyes. As previously described [10,12] the limbal rim of the cornea was excised into four segments and placed into 2.5 mg/ml of Dispase II (Roche Diagnostics Ltd., Burgess Hill, UK) (in Dulbecco’s modified Eagle medium) (Biosera, Ringmer, UK) for 45 minutes. The limbal cells were then scraped off the tissues into phosphate buffered saline using a pair of blunt forceps. The solution was centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and the cells were resuspended in fresh media epithelial culture medium. The cells were seeded into flasks containing growth-arrested 3T3 murine fibroblasts as a feeder layer. The culture media for the rabbit limbal epithelial cells consisted of Dulbecco’s modified Eagle medium (DMEM GlutaMAX™, Gibco, Life Technologies Ltd., Paisley UK) and Ham’s F12 medium (Biosera, Ringmer, UK) in a 1:1 ratio supplemented with 10% foetal calf serum, 10 ng/ml EGF (R&D systems, MN, USA), 5 μg/ml insulin, 2.5 μg/ml Amphoterin (Sigma-Aldrich, Poole, UK) and 100 IU/ml penicillin and 100 mg/ml streptomycin (Gibco, Life Technologies Ltd., Paisley UK). For explant culture, the limbal tissue was chopped into small pieces (approximately 0.5mm) and placed on electrospun membranes and cultured for 2 weeks.

2.8. In vitro degradation of membranes

PLGA membranes were cut into 18 mm diameter disks using a stainless steel cork borer and placed in 12 well plate. rLEC were then seeded (10⁴ cells/well) into stainless steel rings of 10 mm internal diameter on membranes and cultured for a period of up to 6 weeks at 37⁰C. Stainless steel rings was used to hold the membranes flat (without any contraction) and provided a well within which cells could be successfully seeded. Post periods of culture, specimens were fixed using 3.7% formaldehyde solution and prepared for SEM imaging. Membranes were also examined using light microscopy and physical changes in the membrane mats were recorded daily by taking optical micrographs of the membranes as well as analysis of scaffold weight loss.

2.9. Preparation of membranes for SEM

Scanning electron microscope (Philips/FEI XL-20 SEM) was used in this study to examine the morphology, microstructure, fibre diameter, fibre integrity and degradation rate of both low and high molecular weight PLGA membranes (non-sterile and γ-irradiated) at an accelerating voltage of 10–15 kV. 3.7% formaldehyde solution was used to fix the specimens. Specimens were washed with 2 ml of 0.1 M sodium cacodylate buffer for 5 min at room
temperature. Additionally, specimens were fixed once more in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 30 minutes at 4°C. The remaining glutaraldehyde was removed with an additional wash with 2 ml of sodium cacodylate. 1% osmium tetraoxide was used as a secondary fixation for 2 hours. A final wash was performed with sodium cacodylate and samples were dehydrated in ascending grades of alcohol, freeze dried (overnight), bisected and mounted on 12.5 mm stubs. Finally the samples were sputter coated with approximately 25 nm of gold and then examined using SEM.

2.10. Weight loss

The extent of degradation was expressed as percentage of weight loss of the dried membranes after incubation with cells for period of 6 weeks. Membranes were weighed at the beginning of the experiment and then each membrane was weighed on a weekly basis after removing it from culture. Membranes were dried using vacuum oven before measurement. The loss of weight was calculated and expressed as a percentage of the initial weight. The weight loss percentages of the specimens were calculated from the weights obtained before and after degradation using the following formula (1) in which \( W_1 \) and \( W_2 \) are the sample weights before and after degradation, respectively.

\[
\text{Weight loss (\%)} = \frac{W_1-W_2}{W_1} \times 100
\]  

(1)

2.11. Membranes handling

rLEC were cultured on membranes for 6 weeks at 37°C. Physical changes in the membranes were recorded daily by taking optical micrographs. Membrane handling was carried out simply by using a pair of forceps after they were removed from culture (in wet conditions) and scored as easy to handle (+++), fragile (++) or very brittle (+).

2.12. Storage of membranes

PLGA membranes were stored at a wide range of temperatures from -80°C to +50°C in order to assess the storage shelf life of the membranes over 12 months using SEM to assess fibre integrity. Additional membranes were stored at 37°C (both in a dry oven and a humidified incubator). In the case of the latter, membranes were placed in an empty petri dish within an incubator containing 5% CO\(_2\) with high humidity. A similar storage experiment was carried out at the same range of temperatures for 12 months and membranes were vacuumed within polypropylene (PE/PA composite) vacuum sealed bags (Andrew James UK LTD, Bowburn, UK) with thickness of 0.12mm. Water absorption by membranes was detected using silica orange, cobalt (II) chloride and cooper (II) sulphate desiccants. Fibre integrity was assessed by using SEM.

2.13. Explant culture on stored membranes

Initially, for the fibrin coating, a 1:1 mixture of fibrinogen (18.75 mg/ml) and thrombin (2.5U/ml) was pipetted onto the membranes. A cell scrapper was used to spread the drops on the membranes. Eight to ten explants of approximately 0.5mm were placed in the centre of the membrane at a distance of about 2mm from each other into the centre of the stainless steel rings and cultured for 14 days. Cells were fixed with 3.7% formaldehyde and processed for immunocytochemistry.

2.14. Characterisation of corneal cells by immunochemistry

Stem cell markers p63 was used to characterise the cells cultured on membranes. Cells were treated with 0.5% triton X for 10 minutes after which they were blocked with 5% BSA in PBS (Sigma-Aldrich, USA) for 1 hour. The cells were incubated in the primary antibody diluted in 1% blocking medium overnight at 4°C and then incubated
in a biotin conjugated secondary antibody for one hour followed by a streptavidin conjugated tertiary antibody for 30-45 minutes.

2.15. Statistical analysis

Results were tested for normality using a Kolmogorov Smirnov test. Results that showed normal distribution (p > 0.05) were analysed using SPSS via a One-way Analysis of Variance (ANOVA) followed by a post Hoc Bonferroni test. Kruskal-Wallis test and serial Mann Whitney tests were used for non-normally distributed results (p < 0.05). Statistical tests were performed such that a p value of < 0.05 was considered as indicating a significant difference.

3. Results

3.1. Transition temperature

As shown in Figure 1a, the initial Tg for PLGA with low and high molecular weight polymer before spinning was 44.5°C and 47.7°C respectively. After spinning these temperatures dropped to 31.1°C and 40.4°C respectively. The reduction in Tg post spinning was significant for both low and high molecular weight polymers (p < 0.01), but γ-irradiation of these electrospun membranes did not cause any further effect on transition temperature.

3.2. Molecular weight and polydispersity

The molecular weights of the 153 and 44 kg/mol were determined by GPC and assessed at 102 and 39 kg/mol respectively (see Fig 1b). Electrospinning did not significantly affect the molecular weight of either of these polymers but γ-irradiation post spinning significantly reduced the molecular weight estimate for the 153 kg/mol to 27 kg/mol. For the 44 kg/mol the reduction was from 38 to 26 kg/mol.

The polydispersity index for the 153 kg/mol PLGA was 1.38 and this significantly increased post spinning to 1.86, but was not significantly increased by subsequent γ-irradiation (2.05). For the 44 kg/mol PLGA, the polydispersity index was 1.64. Post-spinning there was only a slight increase to 1.72, but it significantly increased to 1.97 by subsequent γ-irradiation (p < 0.05) (see Fig 1c).

3.3. Biomechanical properties

Figures 1 (d) and (e) show the ultimate tensile strength and Young’s modulus for the membranes before and after γ-irradiation. For the high molecular weight membrane γ-irradiation significantly increased tensile strength (from 3.68 ± 0.16 to 4.59 ± 0.17 N/mm²) (p < 0.05). For the low molecular weight membrane ultimate tensile strength was slightly increased from 4.13 ± 0.79 to 4.96 ± 1.76 N/mm² by γ-irradiation but this was not significant. With respect to Young’s modulus, γ-irradiation did not affect Young’s modulus for the high molecular weight membrane, but did significantly increase the Young’s modulus from 62.98 ± 4.4 to 95.5 ± 0.98 N/mm² (p<0.001) for the low molecular weight membrane.
3.4. Wettability of membranes

The use of contact angles to indicate the wettability of the membranes was assessed using deionised water (dH₂O), PBS and media (DMEM) and results are shown in Figure 2. For the 153 kg/mol PLGA it was clear that γ-irradiated membranes showed a more rapid decrease in contact angle with time when PBS or DMEM were used. These were completely wet after 10 minutes for PBS and 20 minutes for DMEM. For the 44 kg/mol PLGA all membranes showed a similar reduction in contact angle over 20-25 minutes. Again, results were slightly faster if DMEM was used rather than PBS or dH₂O. For these sterilised membranes they were largely wet within 15 minutes. Accordingly, all membranes were soaked in media for 30 minutes before use with cells or explants.
Figure 2. Contact angles of non-sterile and $\gamma$-irradiated electrospun PLGA (50/50) membranes with low (44 kg/mol) and high (153 kg/mol) molecular weight polymer using a 3μl drop of deionised water (dH2O), media (DMEM) and PBS. The average value was reported with a standard deviation ($\pm$SD) but these were too small to be seen within the size of the symbols used.

3.5. In Vitro breakdown of membranes and weight loss

Figure 3 shows that PLGA membranes with 50:50 of lactic and glycolic acid rapidly broke down as expected when placed in media at 37°C in a CO2 gassed incubator. Similar results were seen with membranes both with and without cells and the data shown throughout Figure 3 are for those with cells present. Membrane fibre diameters were initially 2-3μm and the membranes had a depth of 50μm and these measurements were very consistent from batch to batch. Results for membrane breakdown (Figure 3) and loss of mass (Table 1) show that $\gamma$-irradiation significantly accelerated breakdown. There was no apparent change in the fibre diameter by day 7 but as early as day 14 it was evident that polymer fibres had absorbed moisture and some swelling was evident. Sterilised fibre diameters increased to 13.10 μm ± 0.86 SD and 7.84 μm ± 0.52 SD for lower MW and higher MW polymers respectively, compared to 10.10 μm ± 1.67 SD and 5.28 μm ± 0.84 SD for these non-sterilised fibres (Table 1). Over 6 weeks fibres clearly lost integrity and coalesced. Breakdown of the 44 kg/mol membranes was faster than that of the 153 kg/mol and the impact of sterilisation was highly significant ($p<0.001$) as summarised in Table 1.

Additionally, each week a sample was sacrificed to assess membrane mass by drying membranes and weighing them to calculate the percentage weight loss. Table 1 which shows a steady increase in weight loss for non-sterile low MW membranes (44 kg/mol) throughout the 6 weeks studied with a mean % loss of 48.32% ± 1.2%SD by week 6, whereas, $\gamma$-irradiated samples had a mean % weight loss of 78.43% ± 2.73% SD by week 6 ($p<0.01$).

Similarly there was steady increase for the non-sterile higher MW membranes (153 kg/mol) throughout the 6 weeks studied with a mean % loss of 37.29% ± 1.81%SD by week 6, whereas, $\gamma$-irradiated samples had a mean % weight loss of 75% ± 2.81% SD by week 6 ($p<0.01$).
Table 1. Membrane mass, mean % of weight loss (±SD) and fibre diameter (μm ± SD) of non-sterile and γ-irradiated electrospun PLGA (50/50) membranes with low (44 kg/mol) and high (153 kg/mol) molecular weight polymer over periods of 6 weeks. The approximate mass of membranes was assessed as follows: membranes of 75-100% of the original mass are indicated as ‘++++’; 50-75% of the original mass as ‘+++’; 25-50% of the original mass as ‘++’; and 0-25% of the original mass as ‘+’; no membrane detected is indicated as ‘0’.

| PLGA (50:50) Membranes | Time point | Day 0 | Week 1 | Week 2 | Week 3 | Week 4 | Week 5 | Week 6 |
|-------------------------|------------|-------|--------|--------|--------|--------|--------|--------|
| Non-Sterile (MW: 153 Kg/mol) | Membrane mass | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| Mean % of weight loss (±SD) | 0.0 | 0.0 | 0.01 | 0.13 | 0.14 | 0.20 | 0.37 | 0.50 |
| Fiber diameter (μm ± SD) | 2.39 | 4.16 | 5.28 | 6.57 | 6.94 | 7.34 | 8.67 | 1.11 |
| Gamma-irradiated (MW: 153 Kg/mol) | Membrane mass | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| Mean % of weight loss (±SD) | 0.0 | 0.29 | 0.91 | 1.35 | 1.46 | 1.71 | 2.94 | 73.41 |
| Fiber diameter (μm ± SD) | 2.55 | 6.11 | 7.84 | 12.1 | 13.72 | 11.53 | 9.21 | 1.02 |
| Non-Sterile (MW: 44 Kg/mol) | Membrane mass | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| Mean % of weight loss (±SD) | 0.0 | 2.43 | 11.53 | 15.26 | 22.41 | 38.49 | 48.32 | 1.20 |
| Fiber diameter (μm ± SD) | 0.0 | 0.36 | 0.31 | 0.54 | 0.79 | 1.20 | 1.20 | 1.20 |
| Gamma-irradiated (MW: 44 Kg/mol) | Membrane mass | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| Mean % of weight loss (±SD) | 0.0 | 9.1 | 13.81 | 21.93 | 31.45 | 70.18 | 78.43 | 2.73 |
| Fiber diameter (μm ± SD) | 0.0 | 0.43 | 0.47 | 1.51 | 0.93 | 2.39 | 2.73 | 2.73 |

Figure 3. In vitro degradation of four sets of electrospun PLGA (50/50) membranes in the presence of rabbit limbal epithelial cells over a period of 6 weeks at 37°C in culture, Scale bar = 20 μm.
3.6. Membrane handling

Membrane handling was assessed by simply picking them up with forceps. Membranes incubated with cells could be handled for 2-5 weeks depending on their molecular weight and whether they were irradiated or not (see Table 2).

The low molecular weight membrane could be handled for up to 2 weeks with care but only 1 week if γ-irradiated. The higher molecular weight membrane could be handled for up to 5 weeks with care, but only 3 weeks if γ-irradiated. From this it appears that γ-irradiation increased the brittleness and fragility of the membranes and reduced the period for which they could be handled by approximately a week.

Table 2. Membrane handling of non-sterile and γ-irradiated PLGA (50:50) membranes (wet) with low (44 kg/mol) and high (153 kg/mol) molecular weight polymer in the presence of rabbit limbal epithelial cells over 6 weeks at 37°C. Membrane handling was simply carried out using a pair of forceps and scored as easy to handle (+++), fragile (++) or very brittle (+). Above images are examples of membranes dried prior to handling while data in the above table were collected from wet membranes which were removed from culture over 6 weeks.

| PLGA Membrane       | Handling         | Week 1 | Week 2 | Week 3 | Week 4 | Week 5 | Week 6 |
|---------------------|------------------|--------|--------|--------|--------|--------|--------|
| Non-Sterile (MW: 153 kg/mol) | +++              | +++    | +++    | +++    | +++    | +      |
| Gamma Irradiated (MW: 153 kg/mol) | +++              | +++    | +++    | ++     | ++     | +      |
| Non-Sterile (MW: 44 kg/mol)    | +++              | +++    | ++     | ++     | +      | +      |
| Gamma Irradiated (MW: 44 kg/mol) | +++              | ++     | +      | +      | +      | +      |

3.7. Effect of temperature on membrane storage

The storage of membranes under a range of temperatures (-80°C, -20°C, +4°C, room temperature, +37°C moist incubator, +37°C dry oven and +50°C) was investigated looking at membranes stored in the presence of desiccant for periods of up to 12 months. On the basis of SEM images, fibre integrity was assessed as fully intact (+++), some indication of fibre swelling (++), fibres merging (+) or no evidence of intact fibres remaining (-). Table 3 summarises all storage experiments for these biodegradable membranes.

There was no visible or measurable change in fibre diameter irrespective of MW or whether membranes were γ-irradiated or not. Similar results were seen at -20°C and -80°C (See Table 3). The inclusion of silica orange desiccant proved useful in seeing changes in water content in the environment. At 50°C the 153 kg/mol membranes were clearly breaking down by 2 weeks whether sterilised or not and the 44 kg/mol had completely collapsed. At 37°C (under dry conditions) membranes showed some evidence of breakdown by 2 weeks and under moist conditions fibres had completely merged by 2 weeks. Placing membranes within a moist environment at 37°C resulted in a rapid collapse of the fibres, whereas the same membranes maintained in culture media (with a bicarbonate pH buffering system) survived for several weeks both with and without cells. In the absence of any buffering system it is likely that these membranes became highly acidic due to the presence of CO2 and H2O. At room temperature (21°C in our laboratory) membranes started to take up water and become brittle after 2 months. At 4°C γ-irradiated membranes were stable for 3 months (both 44 kg/mol and 153 kg/mol). The non-sterile membranes were also stable for 3 months (for the 153 kg/mol) and less stable for the 44 kg/mol (only 1 month).
For membranes stored for a year at -20°C in the presence of desiccant there was no measurable change in fibre diameter irrespective of MW or whether membranes were γ-irradiated or not. Similar results were seen at -80°C (see Table 3). An additional storage experiment was carried out at similar range of temperatures for 12 months and membranes were placed within a polypropylene vacuum sealed bag (Andrew James UK LTD, Bowburn, UK) comparing membranes within bag with no vacuum (control). Fibre integrity for various range of temperatures was shown in Figure 4.

![Figure 4](image-url)  
Figure 4. Effect of temperature and time on storage of vacuumed and non-vacuum PLGA (50/50) membranes (44 kg/mol) over periods of 12 months. Membrane integrity was scored as fully intact fibres (+++), some fibre swelling (++), fibre merging (+) or no intact fibres (-).

Results showed that vacuum packing significantly improved the storage of PLGA membranes. There was no visible or measurable change in fibre diameter for membranes stored at -20°C and -80°C (see Figure 6). There was no visible colour change was also seen for -20°C and -80°C using silica orange, cobalt (II) chloride and cooper (II) sulphate desiccants. Finally, to confirm the suitability of membranes stored at -20°C to support cell culture, limbal explants were grown on membranes of 44kg/mol (non-sterilised) which had been stored at -20°C for 12 months. The results clearly show cell outgrowth from explants onto these membranes after 2 weeks (Figure 5 a, b) and that cells expressed the proliferation marker p63 (Figure 5 c, d) suggesting that the membrane is able to support the growth of limbal epithelial cells even after 12 months in storage.

![Figure 5](image-url)  
Figure 5. Explants outgrowth in cells from explants (magnification 10X) (A,B) and expression of P63 (C,D) (shown in green) on low molecular weight membranes (44kg/mol) at -20°C during storage over periods of 12 months, Scale bar = 100μm.
Table 3. Comparison of fibre integrity of non-sterile and γ-irradiated electrospun PLGA (50/50) membranes with low (44 kg/mol) and high (153 kg/mol) molecular weight polymer at various temperatures during storage over periods of 12 months. Membrane integrity scored as fully intact fibre (+++), some fibre swelling (++), fibre merging (+) or no intact fibre (-).

| PLGA Membrane | Temperature (°C) | Fiber Integrity Months |
|---------------|-----------------|------------------------|
| Non-Sterile (44 kg/mol) | 50°C | +++ - - - - |
| | 37°C (Dry) | +++ - - - - |
| | 37°C (Mist) | +++ - - - - |
| | 21°C (Room) | +++ ++ ++ ++ ++ |
| | 4°C | +++ ++ ++ ++ ++ |
| | -20°C | +++ ++ ++ ++ ++ |
| | -40°C | +++ ++ ++ ++ ++ |
| γ-irradiated (55 kg/mol) | 50°C | +++ - - - - |
| | 37°C (Dry) | +++ - - - - |
| | 37°C (Mist) | +++ - - - - |
| | 21°C (Room) | +++ ++ ++ ++ ++ |
| | 4°C | +++ ++ ++ ++ ++ |
| | -20°C | +++ ++ ++ ++ ++ |
| | -40°C | +++ ++ ++ ++ ++ |

Figure 6. Comparison of fibre integrity of vacuumed and non-vacuum electrospun PLGA (50/50) membranes with low (44 kg/mol) molecular weight polymer at -20°C during storage over periods of 12 months. Scale bar = 20μm. Changes in water absorption by membrane were detected using silica orange, cobalt (II) chloride and cooper (II) sulphate desiccants.
4. Discussion

In producing membranes for tissue engineering purposes it is clearly important that the membranes are fit for purpose in supporting cell attachment, the growth of cells and production of new extracellular matrix proteins.

However, beyond confirming this there are other key issues that need to be addressed before membranes can move to the clinic. In this manuscript we looked at the reproducibility of membrane production, the use of γ-irradiation to sterilise membranes, the handling abilities of the membranes with cells on them and the storage of the membranes cell-free at a wide range of temperatures. All of this information needs to be known before one can take these membranes to the clinic.

The main findings of this study are as follows. The higher molecular weight (153 kg/mol) polymer proved more stable than the lower molecular weight (44 kg/mol). Membranes were initially developed in house and then their production was outsourced to The Electrospinning Company where reproducible membranes of 2-3 μm were produced. We selected γ-irradiation as a recognised sterilisation technique for these membranes, aware that it is quite likely that γ-irradiation will cause a certain amount of damage to the membranes. However, in this particular application we are seeking to develop membranes which will support cell culture for a couple of weeks in the laboratory but will then degrade rapidly when the cells plus membranes are transferred to the human cornea. Hence any acceleration of membrane breakdown by γ-irradiation could be accommodated within this protocol.

The physical characteristics of the membranes showed that spinning did slightly reduce the transition temperature but this was not further affected by γ-irradiation. The molecular weight of the membranes was not affected by spinning (104 for 153 kg/mol and around 38 for 44 kg/mol). Sterilisation with γ-irradiation reduced this slightly but significantly for the 44 kg/mol membrane and markedly reduced this for the 153 kg/mol. Again, γ-irradiation slightly (non-significantly) affected the polydispersity. Furthermore, γ-irradiation increased the ultimate tensile strength of both the 44 and 153 kg/mol membranes. It had no significant effect on the Young’s modulus for the 153 but increased the Young’s modulus for the 44 kg/mol.

Membranes were initially slightly hydrophobic (contact angles of around 100°) but rapidly took up liquid. It was interesting to note that membranes in media (DMEM) or to a lesser extent PBS showed significantly faster reduction in contact angle compared to membranes in deionised water. Membranes need to be wet (become hydrophilic) to promote cell attachment and results showed that both membranes became very hydrophilic within 10-30 minutes. Membranes showed much faster wetting when phosphate buffered saline was used or media, suggesting that it is the ionic salts within these that contributes to this more rapid wetting. This was not studied further in this study but the implication is that membranes should be soaked in media for at least 30 minutes before cells are added.

Cells were cultured on membranes and maintained at 37°C for periods of up to 6 weeks in a CO2 incubator and from this it was clear that there was a window of a few weeks, possibly 2-3 weeks, for the 44 kg/mol in which the membrane maintained some physical integrity, whereas this was much greater for the 153 kg/mol PLGA membrane. Table 2 illustrates that as membranes take up water they become very brittle and hard to handle. There was some indication that γ-irradiation increased the fragility of the membrane and reduced the period when it could be handled by approximately a week. Thus, for the low molecular weight membrane 44 kg/mol, these membranes when irradiated were already becoming hard to handle at 2 weeks, whereas without irradiation they could be handled readily at 2 weeks but started to become fragile at week 3. In contrast the 153 kg/mol membranes could be handled reliably for at least 3 weeks whether non-sterile or γ-irradiated but by 4 weeks the γ-irradiated membranes showed evidence of increased fragility.

In looking at the breakdown of the membranes we looked at fibre diameter, which increased as membranes took up water, and we also measured a crude indication of membrane mass (qualitatively) and a quantitative assessment of weight lost. As shown in Figure 3, there was a clear impact of γ-irradiation on breakdown in that membranes showed accelerated weight loss post γ-irradiation compared to non-irradiated membranes. This was evident after 3 weeks culture of cells on membranes and very evident by week 6.

The next study we undertook was that of storing the dry membranes at a range of temperatures deliberately chosen to span very cold (-80°C and -20°C) through to very warm (50°C). To the best of our knowledge there are no publications on storage of membranes for clinical use. The results were very clear. Membranes were stable at -
20°C and -80°C for at least 12 months (the longest period examined in this investigation). At 4°C membranes were stable for 2-3 months. At higher temperatures, however, it was clear that membranes began to swell and lose integrity after few weeks and membranes deliberately stored at 50°C showed rapid loss of integrity (very evident by 2 weeks) and membranes stored at 37°C under dry conditions showed some loss of structure by week 2. The same membranes stored under wet conditions had completely lost their structure by 2 weeks. The difference between these results and those where membranes were cultured at 37°C in media (buffered to pH 7.4) with cells for up to 6 weeks must we suggest be explained by the pH bicarbonate buffered environment of the cell cultures (pH 6.8-7.2), whereas the membranes stored at 37°C under moist conditions had no pH buffering and broke down very quickly. We suggest that this is due to a build up of carbonic acid on the membrane, thus leading to acid catalysed hydrolysis of the polymer membrane. Additionally, storage of PLGA membranes at a similar range of temperatures for 12 months within vacuum packed bags showed that vacuum packing significantly improved the storage of this membrane.

A number of studies have been carried out over the years to replace the use of the amniotic membrane to treat LSCD. The use of contact lenses [12,15-16] to deliver cells to damaged corneas has been explored. Also the use of natural polymers such as recombinant cross-linked collagen [17], fish scale collagen [18], fibrin [19], myogel [20] and silk [21] and synthetic polymers such as poly (N-isopropylacrylamide) [22] and a copolymer of poly(N-isopropylacrylamide-co-n-butyl methacrylate) and polyethylene glycol (PEG) [23-24]. Some of these various delivery systems have been used in patients and have been successful [15,19,22] however, the long term outcome of such techniques are only now being examined as sufficient numbers of patients become available for assessment. For example Sangwan et al [25] reported that after about 4 years, the success rate of delivering cultured LEC using the amnion as a delivery system drops down to about 40%. As acknowledged in this study, there may be many factors which explain how patients vary in their long term response to the treatment –the aetiology of the condition that led to stem cell loss for example may be a major factor and the state of the eye pre-transplantation and the inflammatory response to the cultured LEC cells. Also the success rate is thought to be dependent on the type of cells used to treat LSCD. Patient’s own oral mucosal [26] or hair follicle stem cells [27] in the case of bilateral injuries have been used but the use of the oral cells has not been very successful leading to neovascularisation, stromal melting and perforation [28-29]. In addition, variability in the amniotic membrane used for cell delivery will also be a factor in this picture thus a synthetic off the shelf cell delivery system that is predictable and effective could be a useful addition to this transplantation technology.

Safety and risk management in preparing material for clinical use dictate that any cell carrier needs to be as low risk as possible for patients. Biodegradable polymers can be sterilised differently depending on their applications. The common sterilization methods are ethylene oxide (EO) gas [30], dry heat [31], steam [32], organic solvents (ethanol) [33], plasma treatment [34], and γ-irradiation [35]. Typical sterilization methods are often not suitable for polymer membranes [36]. For instance, standard sterilisation techniques such as autoclaving and ethylene oxide treatment have been shown to adversely affect polymer structures with deformation at elevated temperatures, and deterioration due to decreased molecular weight [36-37]. Similarly dry heat and steam sterilization can cause extensive degradation. Plasma treatment sterilization used recently may change the structure of the polymer due to the heat generated during the process. A previous study showed that ethanol sterilisation is convenient method to sterilise membranes, however, this is not a recognised sterilisation methodology that can be taken to the clinic [36]. Peracetic acid was also tested, which is clinically approved and is also a wet-sterilisation methodology, but this also reduced membrane strength. [36]. Another study from our laboratory found there was no significant difference in the viability of epithelial cells and fibroblast on membranes sterilised with either peracetic acid, ethanol or γ-irradiation [13].

Although γ-irradiation remains a widely accepted methodology for biodegradable polymers [38-40], it has been reported that γ-irradiation induces free radicals in polymer matrix by chain scission and decreases the molecular weight of the polymers. The extent of molecular weight reduction was dose dependent and it affected polymer degradation and the release profile of drug from polymer device [39-40]. In this study we have shown that γ-irradiation does affect PLGA membranes in terms of a reduction in their molecular weight, and transition temperature resulting in an accelerated rate of breakdown. Fortunately in producing carrier membranes for cornea tissue engineering these effects of γ-irradiation are an advantage for this application as they increase the rate of
breakdown of the PLGA membranes (which is clinically desirable) without affecting the culture of cells on these membranes.

5. Conclusion

Our study shows that the PLGA (50:50) has the potential to support rLEC for periods of at least of 2-3 weeks in culture. Thereafter it begins to breakdown rapidly by taking up water. A higher MW polymer took a little longer (4-5 weeks) to achieve an equivalent breakdown compared to a lower MW PLGA. PLGA with higher molecular weight (153 kg/mol) is more stable than that with lower molecular weight (44 kg/mol). Sterilisation with γ-irradiation increased the rate of breakdown of both membranes by approximately a week but did not adversely affect the attachment of cells to membranes. Membranes could be stored dry at -20°C for at least a year without any loss of function. Vacuum packing significantly improved the storage of PLGA membranes. The results also show cell outgrowth from explants onto these membranes after 2 weeks and that cells expressed the proliferation marker p63 suggesting that these membranes is able to support the growth of limbal epithelial cells even after 12 months in storage. In conclusion, the physical characteristics of this sterilised cell carrier are suitable for further evaluation as a clinical alternative to the amniotic membrane in the treatment of limbal stem cell deficiency. This synthetic sterilised carrier offers a lower disease risk alternative for clinical use to the use of donor amniotic membrane for cell delivery.

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