

A novel biodegradable polymer scaffold for *in vitro* growth of corneal epithelial cells

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The shortage of donor corneal tissue worldwide has led to extensive research for alternate corneal equivalents utilizing tissue engineering methods. We conducted experiments using Poly D, L lactic acid polymer along with a copolymer (Eudragit) in varying concentrations to create a biodegradable scaffold suitable for *in vitro* growth of corneal epithelial stem cells. It was found that stable, spherical, and porous microparticles can be prepared by combining PDLLA and Eudragit RL100 polymers in the ratio of 90:10 and 70:30. The microparticles can then be fused to form scaffold membranes with porous architecture and good water retention capacity at room temperature using methanol, which can withstand handling during transplantation procedures. The scaffolds made using a 70:30 ratio were found to be suitable for the promotion of growth of laboratory corneal epithelial stem cell lines (SIRC cell lines). This innovation can pave way for further developments in corneal stem cell research and growth, thus providing for viable laboratory-derived corneal substitutes.

Key words: Corneal epithelial stem cell culture, microparticle, polymer, scaffold, tissue engineering

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The cornea is a transparent, avascular tissue of the ocular surface covered by the stratified epithelium responsible for maintaining a smooth ocular surface as well as for providing a barrier against environmental stress. Most superficial cells are regularly shed from the surface of the eyes and replaced by new cells that are ultimately provided by corneal epithelial stem cells located in the limbal area of the peripheral cornea.^[1] Pathologic conditions such as severe chemical injuries, thermal injuries, Stevens–Johnson syndrome, and ocular pemphigoid can destroy the limbal epithelium and functional blindness that cannot be treated by standard corneal transplantation.^[2] In such cases, tissue engineering techniques can be of great use by helping in *in vitro* growth of corneal epithelial cells.

Growing corneal epithelial cells *in vitro* as a sheet of cells requires a substrate. Scaffolds are engineered materials that provide a suitable environment for the growth and proliferation of cells and tissues of desired qualities. Common practices involved either the use of an amniotic membrane as a substrate or the use of other alternatives such as fibrin glue, collagen membrane, thermo-sensitive substrates, and synthetic polymer membranes. The shortage of donor corneal tissue worldwide has led to extensive research for alternate corneal equivalents utilizing tissue engineering methods. Thus, it is essential to search for promising alternatives for the growth of corneal epithelial cells that can be used for transplantation. The objective of our study was to assess the suitability of a biodegradable polymer scaffold membrane

as an ideal substrate for *in vitro* growth of viable corneal epithelial cells.

Innovation

Study design: Experimental and observational study. Study period: Two years, from October 2017 to December 2019. Location: Ophthalmic tertiary healthcare center and Immunology Research Centre in the National Capital Region.

There were three major parts to the methodology – a) particle preparation and formation of scaffolds from them, b) study of scaffold characteristics, and c) study of cell growth on the prepared scaffolds.

For the formation of microparticles, the double-emulsion solvent evaporation technique was utilized.^[3] The primary emulsion was prepared by sonicating the internal aqueous phase (IAP) and organic phase (OP) in the ratio of 1:20 and then combined with the external aqueous phase (EAP) in the ratio of 1:40 to form the secondary emulsion with the help of a homogenizer. The secondary emulsion was kept at room temperature on constant magnetic stirring for 3–4 h to facilitate the evaporation of DCM. The particles were then collected by centrifuging at 11,000 rpm for 15 min at 15°C and stored at –20°C until further use. The procedure of polymer microparticle formation is shown in Fig. 1a. To

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prepare Eudragit-blended PDLLA microparticles, the organic phase (OP), Eudragit RL 100 was dissolved in acetone and mixed with PDLLA in various volumetric ratios to obtain multiple synthetic polymer blends. The compositions of the polymer blends for OP in different ratios are given in Fig. 1c.

The scaffold fabrication was carried out by fusing the CTAB-coated microparticles with methanol.^[4] In brief, CTAB-coated particle suspension was added to a 96-well plate and incubated at 37°C to evenly dry them. Dried particles were then treated with methanol and the fused membrane obtained was then washed thrice with MQ water. We used disc-shaped membranes of about 2 mm thickness and 8 mm diameter. These scaffold membranes were then studied for their characterization and cell growth studies. The procedure for the fabrication of scaffolds is represented in Fig. 1b and the resultant membranes in Fig. 1d. Using the appropriate procedure, the particles in suspension and scaffold membranes were studied by optical microscopy and scanning electron microscopy.

Surface charge analysis of scaffolds prepared from blends of PDLLA-Eudragit 90:10 and 70:30 was compared with that of PDLLA membrane by Zeta potential analysis (based on electrophoretic mobility) using SurPASS TM 3 electrokinetic analyzer from Anton Paar instruments. Scaffold porosity index was measured by liquid displacement method and water absorption capacity was also measured. For cell culture experiments, Statens Serum Institute Rabbit Cornea (SIRC) cell

line procured from National Centre for Cell Sciences (NCCS), Pune, was used.

Media preparation: The base medium for the growth of the SIRC cell line is Eagles' minimum essential medium (MEM). To make the complete growth medium, fetal bovine serum (FBS) 10% v/v of the media concentration and antibiotic solution (penicillin-streptomycin 5,000 U/mL) 1% v/v of the final media concentration were added.

Cell culture method: Cultures were maintained in 35 mm Petri dishes at 37°C in an atmosphere containing 95% air and 5% CO₂ in an incubator. The medium was renewed twice per week. At the confluence of cell growth, cells were detached using the trypsin-EDTA solution. Complete media was then added to the petri dish, and appropriate aliquots of cell suspensions were added to new culture flasks. On obtaining enough cells, seeding experiments on the three types of scaffolds were undertaken.

Scaffolds were sterilized by overnight treatment with 70% ethanol followed by washing with Milli Q water and thereafter soaking in serum-containing media for 24 h. The SIRC cells were seeded using the static seeding method. About 3×10^3 cells suspended in 20 μ L of media were used for seeding per scaffold (surface area 0.32 cm²). Cell attachment and proliferation studies were performed on days 1, 3, 5, and 7 post seeding. Cell proliferation studies were conducted using the MTT assay. Seeding efficiency was found by seeding the same number of cells in a 96-well plate as well as the scaffold.

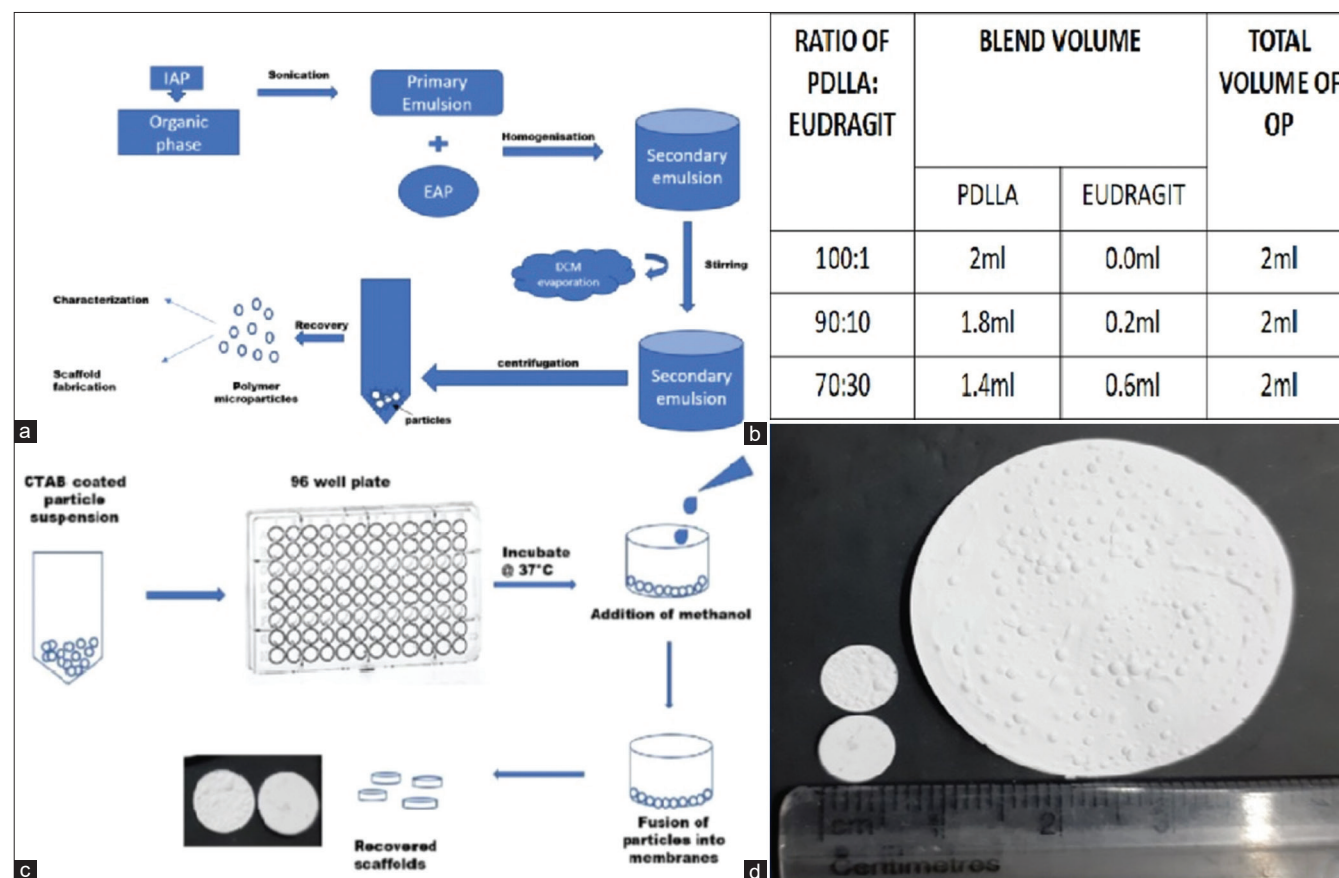


Figure 1: (a) Formulation of polymer microparticles, (b) Fabrication of scaffolds from microparticles. (c) Synthetic polymer blends in different volumetric ratios for microparticle formation. (d) Scaffold membrane of desired shape and size

MTT assay was performed for the cell-seeded scaffold as well as cell-seeded tissue culture well. Morphology of seeded cells on the scaffold was studied using SEM.

Results

Optical microscopy of particles: Under the Nikon Eclipse Ti-S microscope at $20\times$ magnification, PDLLA-Eudragit particles prepared in the ratio of 90:10 and 70:30 showed a spherical, stable, and porous structure [Fig. 2-c]. Heterogeneity in particle size was seen to increase with increasing concentrations of Eudragit. P: E 70:30 particles showed the highest heterogeneity in their particle size as compared to PDLLA and P: E 90:10.

SEM imaging of particles supported the observation made on optical microscopy, that with the increasing concentrations of Eudragit polymer, the heterogeneity of particle size also increased. The particles prepared showed porous, spherical, and stable structures [Fig. 2d-f].

The addition of Eudragit in 90:10 and 70:30 ratios to PDLLA did not significantly alter its gross physical appearance as the membranes resembled the PDLLA scaffolds in

their appearance [Fig. 1d]. The SEM analysis of scaffold surfaces [Fig. 2g-i] revealed porous architecture on their surface, which is a very important property of scaffold to provide for the infiltration of cells, permeation of nutrients and oxygen inside the scaffold, and also for removal of cellular debris.

The zeta potential analysis revealed that scaffold surface net charge increased to positive with the addition of increasing concentration of Eudragit [Fig. 3a].

It was observed that the addition of Eudragit in the 90:10 concentration did not bring major changes in the porosity of the scaffold formed. The scaffolds fabricated from the P: E 70:30 polymer blend seemed to have a better porosity index as compared to PDLLA and P: E 90:10. Thus the P: E 70:30 scaffold is better suited for cell growth as compared to the others [Fig. 3b].

It was seen that out of the three scaffolds the water absorption capacity of the P: E 70:30 scaffold was lowest while that of the P: E 90:10 scaffold was highest [Fig. 3c].

SIRC cell culture studies: Cell attachment efficiency was assessed using the MTT assay on the scaffolds seeded with

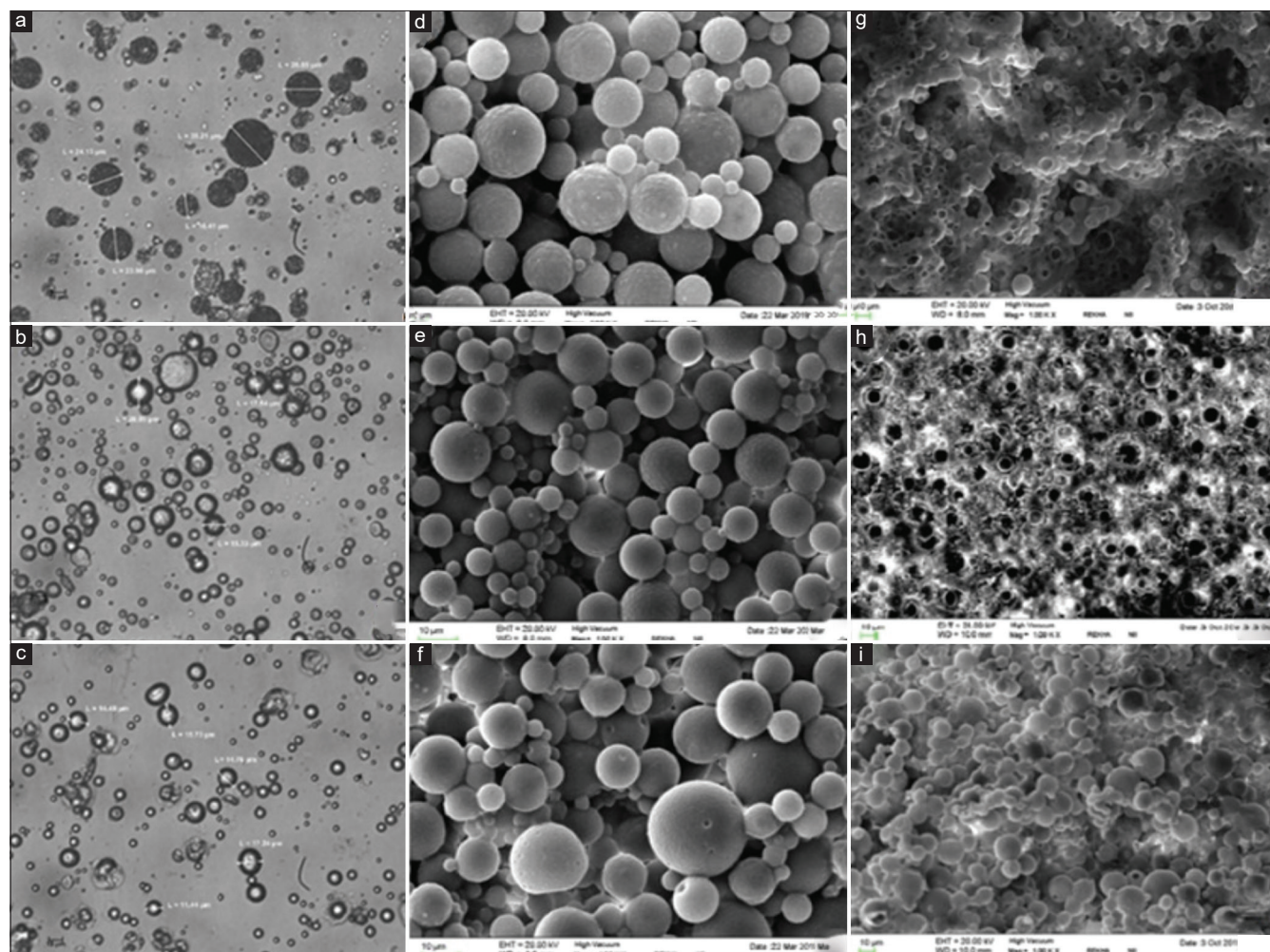


Figure 2: Surface morphology of particles and scaffolds. Optical microscopy study at $20\times$ magnification of (a) PDLLA microparticles, (b) PDLLA:Eudragit 90:10 microparticles, (c) PDLLA:Eudragit 70:30 microparticles. Scanning electron microscopy imaging of polymer microparticles. (d) PDLLA, (e) PDLLA-Eudragit 90:10, (f) PDLLA-Eudragit 70:30. Scanning electron microscopy imaging of scaffold surfaces at $1000\times$ magnification. (g) PDLLA, (h) PDLLA:Eudragit 90:10, (i) PDLLA:Eudragit 70:30

SIRC cells. MTT assay was read on days 1, 3, and 5 of seeding. The absorbance measured at 570 nm was then read and plotted [Fig. 4a].

The results from the MTT assay suggested that with increasing concentration of Eudragit RL 100 polymer in membranes, cells sustained were better. Of the two scaffolds fabricated from the PDLLA-Eudragit blended polymer, the P: E 70:30 showed better cell proliferation [Fig. 4b and c].

For primary corneal epithelial cell culture, corneal buttons were extracted from Balb/C mice in accordance with the ethical guidelines, using the explant culture method. The lack of pigment in the iris tissue of Balb/C mice seemed advantageous as it did not interfere in dissecting the corneal tissue. Due to the extremely small size of mice eyes, multiple corneal buttons were explanted to obtain a sufficient cell population to perform the seeding experiments [Fig. 5].

The optimization for sub-culturing and passage could not be done despite multiple attempts at various time periods within the given time frame of our experimental study. The cells obtained were insufficient to be taken up for seeding and characterization experiments.

Discussion

For a long time, the immune-privileged corneal tissue, owing to its avascularity has given surgeons the option of replacing the diseased cornea with cadaveric tissue, but this has its own limitations. To find avenues in tissue engineering that can overcome this limitation, multiple experiments are being conducted to search for better alternatives and substitutes to promote corneal epithelial wound healing in cases of limbal stem cell deficiency. In recent times, emphasis has been given to tissue engineering using external support systems to aid in

cellular growth. In this context, various natural and synthetic polymer scaffolds have made their way into the field of tissue engineering.^[5]

The materials derived from natural polymers suffer from fast degradation rates, difficulty in sterilization and purification, high variability, high contamination potential, and with strong propensity to induce immune response upon implantation. The synthetic polymer scaffolds, in contrast, can be suitably modified as per the requirement for desired properties and offer the potential for improved control, repeatability, and safety.^[6]

Our study thus aimed to search for a viable, laboratory-grown, non-immunogenic, biodegradable synthetic polymer scaffold for the growth of corneal epithelial stem cells *in vitro*, which can then be successfully transplanted onto the diseased cornea after optimization. Such a novel technique will not only reduce the burden of the eye banks in the country but also will give us the advantage of offering a better vision to patients who have lost hope due to the severity of their disease.

To initiate our experimental study, we used the SIRC cell line that was originally derived from the cornea of a normal hare, *Oryctolagus cuniculus*, in 1957 by M. Volkert of the Statens Serum Institut, Copenhagen, Denmark. SIRC cell line does not display the typical characteristics of corneal epithelial cells and possesses features that are more consistent with a fibroblastic cell phenotype such as the corneal keratocyte.^[7] The immunocytochemical analysis reveals that SIRC cell lines stain strongly positive for vimentin but lack cytokeratin expression, which is considered specific for corneal epithelial cells.^[8] Thus, although this cell line has been used in laboratories as a corneal correlate due to its keratocyte origin, we proceeded with primary corneal cell culture from a suitable mammalian species such as the Balb/C mice to find answers to the suitability of our

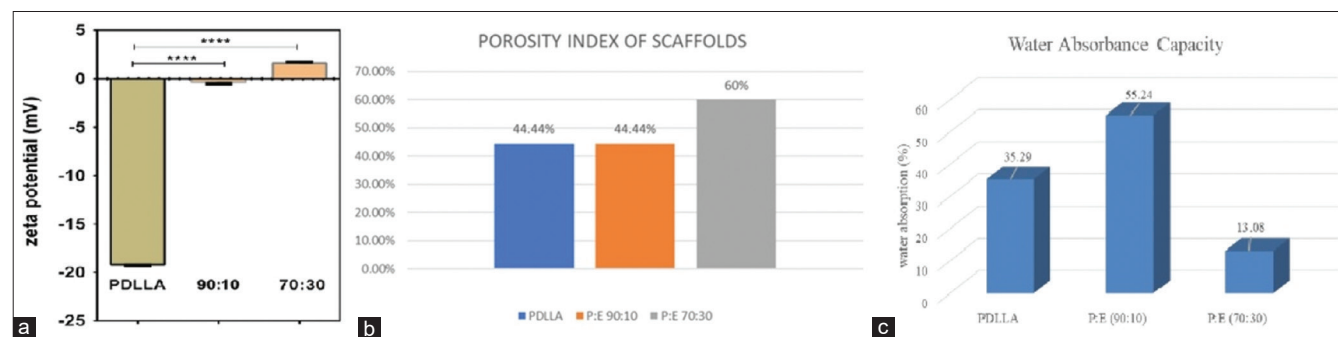


Figure 3: Scaffold characterization. (a) Zeta potential analysis and comparison of different PDLLA-Eudragit-blended scaffolds. Scaffold surface charge increases with increasing concentration of Eudragit. (b) Porosity Index. (c) Water absorption capacity of scaffolds

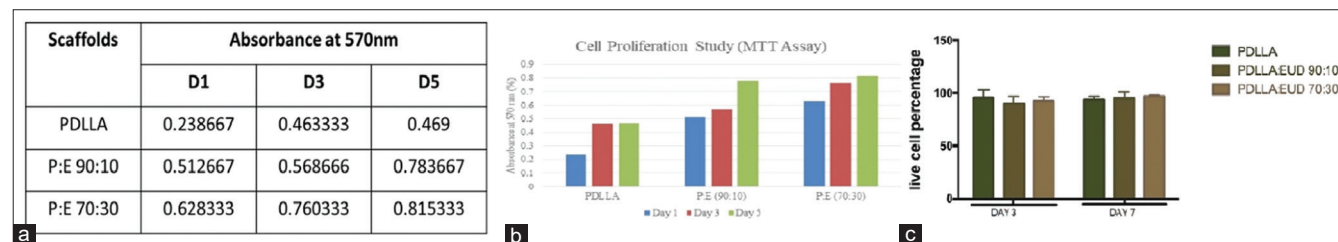


Figure 4: (a) Absorbance values for MTT assay for growth of SIRC cells on scaffold membranes. (b) Cell proliferation study using the MTT assay on different scaffold membranes on various days by reading the absorbance at 570 nm. (c) Live-dead cell percentage on various cell-seeded scaffold surfaces on day 3 and day 7

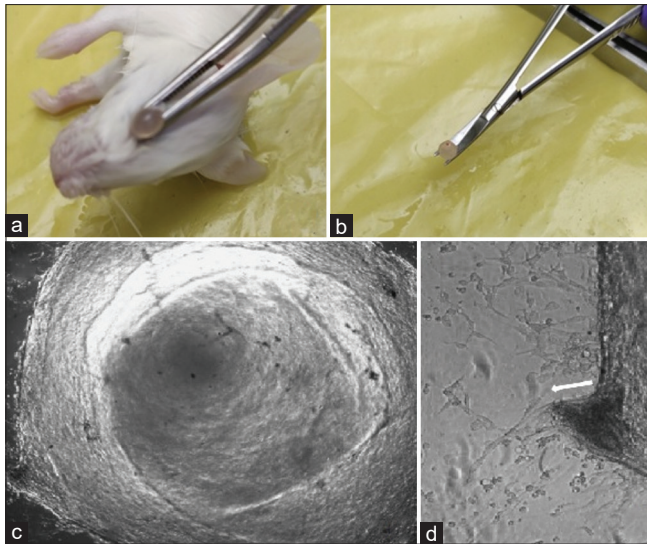


Figure 5: (a) Corneal tissue extraction from mice. (b) Enucleation of the eyeball from Balb/C mice. (c) Optical microscopic images of the excised complete corneal button on attachment to tissue culture plate surface, (d) Cells seen migrating from the edge of explant on day 5 (white arrow)

modified scaffold in promoting the growth and proliferation of the corneal epithelial cell lineage.

We modified the PDLLA polymeric scaffold by introducing another polymer, Eudragit RL100 in various concentrations using the double-emulsion-solvent evaporation method to form microparticles, which were then used to fabricate scaffolds of varying compositions by the addition of methanol, causing particle fusion and membrane formation. The scaffold membranes formed were then studied for their properties such as particle physiochemical characterization, scaffold surface morphology, porosity, surface charge, water retention capacity, and promotion of cell growth. The addition of Eudragit polymer seemed to increase the positive charge of the scaffold membrane surface as shown by the cell proliferation assay results and the surface charge study results. Our study shows that although PDLLA polymer has a negative charge on its surface, the addition of the cationic polymer Eudragit RL 100 in increasing concentrations increases the surface charge toward the positive side. Thus, it shows that the addition of Eudragit RL100 to PDLLA acts to enhance its surface properties by increasing its surface charge and thus promoting cell attachment. Of all the three polymer particles studied, P: E 70:30 had the highest positive charge. On studying the porosity of the scaffolds derived from PDLLA and P: E 90:10, we noticed that there was no significant difference in the porosity index of PDLLA and P: E 90:10 scaffolds, both having 44% porosity. However, on increasing the Eudragit RL 100 polymer concentration in the ratio of 70:30, the porosity improved significantly to 60%. This contributed to the enhanced cell growth and proliferation over the P: E 70:30 scaffold as the porosity allows for the seepage of nutrients and better cell growth. Thus, the P: E 70:30 polymer-derived scaffolds support better cell survival, which was also confirmed by the augmented growth of cells seen on its surface in SEM imaging. The next step was to culture primary corneal epithelial cells from a suitable mammalian species *in vitro* and then study their growth characteristics on the modified scaffold membrane.

The primary corneal epithelial cell culture from mice corneas showed cells migrating and proliferating from the explant edge onto the petri dish surface. However, the cell culture showed a very slow cell growth. Multiple attempts were made to subculture the primary corneal cells for seeding and characterizing experiments, but it was observed that the cells were unable to grow further. The optimization for sub-culturing and passage could not be done despite repeated attempts at various time periods.

Conclusion

Tissue engineering and stem cell culture can pave the way to innovations in ophthalmology that can help us combat blindness. Our study concluded that scaffolds suitable for corneal epithelial stem cell growth can be fabricated at room temperature in a laboratory using PDLLA and Eudragit RL 100 polymers in the ratio of 70:30. These scaffolds also supported the growth of primary corneal epithelial stem cells from mice cornea. The limitation of our study was that we were unable to passage and subculture the primary stem cells, given the time constraints and because they are very delicate. Future studies in this direction are needed.

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Conflicts of interest

There are no conflicts of interest.

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