A BIOENGINEERED SEQUENTIALLY LAYERED IN VITRO CORNEAL CONSTRUCT

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Abstract - Tissue engineering intends to develop three dimensional living substitutes to restore, maintain and improve tissue functions. In addition, these biological substitutes also find application in vitro toxicity studies. Cell sheet technology enables the development of scaffold free tissue constructs from thermoresponsive culture surfaces. In this study we demonstrate the feasibility of spin coated copolymer, N-Isopropylacrylamide-co-Glycidyl methacrylate (NGMA-SC) as a thermoresponsive substrate to create an over layered construct from native corneal cell types. The corneal cells were independently cultured and characterized by immunostaining. The multilayered cornea was developed by sequential culture technique. Rabbit corneal endothelial cell monolayer on NGMA-SC was overlaid with corneal stromal fibroblasts. Corneal epithelial cell sheet was transferred on the endothelial-fibroblast bilayer to get three dimensional culture systems. The positive expression of characteristic genes such as Na\(^+\)K\(^+\)ATPase, CK12 and vimentin in the construct confirmed the presence of functionally active corneal endothelial, epithelial and fibroblast cells respectively. The layered three dimensional culture system was retrieved from NGMA-SC as an intact tissue construct by modulating thermal stimuli. We propose the sequential cell seeding and layering technique as a potential approach to develop in vitro corneal constructs.

Keywords – Cornea, Epithelial cells, Endothelial cells, Stromal fibroblasts, Tissue Engineering, Thermoresponsive polymer

1 INTRODUCTION

Cornea, the avascular transparent tissue comprises of distinguished cell types to form three major layers; the epithelium, stroma and endothelium. Epithelium is the outermost layer of the cornea and acts as a defensive barrier to infection [1]. The stromal layer is the thickest layer of the cornea primarily made of collagen. The stromal fibroblast regulates the synthesis, organization and spacing of various types of collagen and proteoglycans [2]. The inner most layer of cornea is a monolayer of endothelial cells which regulates stromal hydration keeping the cornea transparent [3]. Various reports are available on in vitro corneal equivalents developed using primary bovine corneal cells with collagen gel matrix, rabbit corneal cells with nitro cellulose membrane, immortalized human corneal cells on crosslinked collagen chondroitin sulphate matrix etc [4-9]. However, the use of scaffolds in these tissue engineered constructs can affect the transparency and also, there are issues of biocompatibility. Hence a scaffold free corneal construct without any biomaterial would be a better option in corneal tissue engineering. In vitro constructs helps in bringing three dimensional (3D) cellular and molecular microenvironment and mimics the tissue in culture systems [10]. Such models finds as an alternate for the excised tissue and small animal models in toxicity and drug screening studies [11]. Most of the reports on in vitro models of cornea proposed for drug permeation and ocular toxicity testing uses immortalized or native corneal cells in the presence or absence of artificial matrix to generate corneal equivalents [5, 12, 13]. Primary cultured corneal epithelial cells are also used as an adjunct to ocular toxicology studies as these cells retain a variety of phenotypic characteristics [14]. Cell sheet technology is an approach, where cells are expanded and retrieved as tissue construct from a thermoresponsive culture surface [15, 16]. Poly (N-Isopropylacrylamide) [PIPAAm] based thermoresponsive polymer systems are extensively studied for cell sheet technology [17]. We have previously reported that the copolymer N-Isopropylacrylamide-co-Glycidyl methacrylate (NGMA) is suitable for generating viable corneal epithelial cell sheets [18]. The polymer coating thickness ranging from nano to micrometer scale has direct effect on efficiency of cell sheet retrieval. The polymer coating thickness of 10 – 600 nm are achieved by treatment with plasma or high energy radiation [19, 20]. Spin coating is an alternate technique to obtain similar submicron coating thickness on substrates. The feasibility of ultrathin preparation of isopropylacrylamide-based stimuli-responsive films on solid surfaces by spin coating has been reported [21].

In this study an over layered cell construct from rabbit corneal endothelial cells, stromal fibroblasts and epithelial cells was developed using tissue culture grade polystyrene dishes (TCPs) spin coated with NGMA.

2 MATERIALS AND METHODS

2.1 Synthesis of the stimuli-responsive surface

Thermoresponsive NGMA having a lower critical solution temperature (LCST) of 28°C was synthesized as previously reported [18]. The polymer was spin coated on TCPs using a custom made spin coat apparatus. Briefly, a 35 mm TCPs dish (Grenier BioOne, Germany) was loaded to the spin coat
adaptor. 40 µl of the NGMA polymer solution was introduced through a sample load port and spun continuously for 10 seconds at a speed of 1000-1200 rpm. The coated plates were air dried at 60°C and rinsed with ice cold distilled water thrice to remove the unbound polymer. The plates were air dried and sterilized by ethylene oxide. The “NGMA spin coated” surface is hereafter represented as NGMA-SC.

2.2 Water contact angle
The water contact angle of NGMA-SC and TCPS dishes were analyzed using an NRL Contact Angle Goniometer (Sessile drop method) above (37°C) and below (25°C) the LCST. A minimum of four different points from each sample were analyzed using a video based contact angle measuring device (Data Physics OCA 15 plus, Germany) and imaging software (SCA 20).

2.3 Cell adhesion on NGMA-SC
The cell adhesion on spin coated surface was studied using L929 fibroblast cell line (NCCS, Pune). Cells were seeded at a density of 1 x 10^4 cells/ml on NGMA-SC and normal TCPS dishes and cultured in Modified Eagles Medium (MEM, Sigma) supplemented with 10% Foetal Bovine Serum (FBS, Gibco) at 37°C, inside a CO₂ incubator (Sanyo) set with 5% CO₂ and > 90% relative humidity. The cell adhesion, morphology and growth of the L-929 cells were monitored using an inverted phase contrast microscope (Lieca; DMIL or DMI6000B) and compared with cells on TCPS.

2.4 Thermoresponsive efficacy of NGMA-SC
The efficacy of NGMA-SC surface to respond to temperature and enable cell retrieval was determined using L-929 cells by a previously reported method with slight modification [17]. Around 1 x 10^4 cells were seeded on the modified surface and allowed to form monolayer. A hydrophilic polyvinylidene fluoride (PVDF, Millipore, India) membrane conditioned in serum free culture medium was placed on the monolayer. The plates were kept below 10°C for 1 min and monitored under a phase contrast microscope for change in morphology from spindle to round. The PVDF membrane was removed using forceps and transferred to a normal TCPS with cell side facing down. The membrane was kept in wet condition by adding 100 µl culture medium containing serum and incubated for one hour at 37°C. Excess medium was added till the membrane floated and was removed using forceps. The transferred cells were cultured for 48h.

2.5 Cell culture
Epithelial cells, stromal fibroblasts and endothelial cells were isolated from cornea collected from cadaveric New Zealand white rabbits (Sctb: NZW) with prior approval of the Institute Animal Ethics Committee. Cornea with a portion of the sclera were excised and collected in Phosphate Buffered Saline (PBS) containing penicillin-streptomycin antibiotics (PAA, Austria) and were used for cell isolation within two hours.

2.5.1 Corneal epithelial cells
For epithelial cell culture the limbus was cut into 2 mm size explants and placed on the NGMA-SC dish. The explants were allowed to attach for two hours in a CO₂ incubator with a small amount of culture medium (DMEM, 10% FBS). Sufficient medium was added and the culture was maintained for five days. The explants were removed and the cells were cultured until cell sheet retrieval.

2.5.2 Corneal endothelial cells
The inner side of the cornea was treated with 200 µl of 0.25% Trypsin-EDTA solution (Gibco, Germany) for 10 min at 37°C in an incubator. The endothelial cell layer with descemet’s membrane was peeled off using a blunt end forceps. The cell layer was transferred to a petri dish containing 0.1% collagenase (Sigma, India) solution and incubated at 37°C for 4 h. The cells were dislodged by gentle pipetting and the cell suspension was centrifuged at 900 g for 10 min at 4°C (Hermel, Germany). The cell pellet was resuspended in 1 ml Iscove’s Modified Dulbeccos Medium (IMDM : Sigma, India) supplemented with 10% FBS, growth factors such as 5 ng/ml Epidermal Growth Factor, 5 µg/ml Insulin, 0.5 µg/ml Hydrocortisone, 0.1 mM Ethanolamine, 0.1 mM Phosphoethanolamine, 25 µg/ml Bovine Pituitary Extract (PAN Biotech, Germany) and Penicillin/Streptomycin antibiotic. Cells were seeded on TCPS precoated with 1% gelatin (Sigma, India) and maintained at 37°C in a CO₂ incubator.

2.5.3 Corneal stromal fibroblasts
The remanant corneal stroma was minced into 1 mm bits and treated with 0.1% Collagenase solution for 3 h at 37°C. The cell suspension were centrifuged at 1800 rpm for 10 min. The pellet was resuspended in DMEM supplemented with 10% FBS, 4 mM L-glutamine and Penicillin -streptomycin antibiotics. and transferred to T-25 flasks (Nunc, USA) . The cells were cultured to confluence inside a CO₂ incubator.

For all the cultures the medium change was performed every alternate day. The stromal fibroblasts and endothelial cells were subcultured using trypsin solution [Gibco, (0.5 g/L)-EDTA (0.2g/L)] at 37°C.

2.6 Characterization of corneal cells
Cultured corneal cells were screened for their characteristic markers Cytokeratin 3/12 (epithelial cells), vimentin (fibroblast and endothelial cells), and Na⁺K⁺ATPase (endothelial cells). The cells were fixed in paraformaldehyde for 1h and permeabilized with 0.1% Triton X-100 (Sigma, India). Non-specific binding was blocked by incubating with freshly prepared 1% bovine serum albumin (Sigma, India) for 10 min. Cells were incubated with primary mouse antibodies against CK3/12 [1:100, (Chemicon International, Germany)], vimentin [1:100, (Dako, Germany)] and Na⁺K⁺ATPase [(1:25) (Abcam, Germany)] for 1 h in a humidified chamber at room temperature. The cells were then incubated with fluorescein isothiocyanate (FITC) tagged secondary antibody (antimouse IgG 1:100, Sigma India) for 1 h at room temperature inside a humidified chamber. The nucleus was counter stained with 0.5 µg/ml Hoechst 33258...
(Sigma, India) or 0.1 µg/ml propidium iodide (Sigma, India) and observed under a fluorescence microscope (Leica DMI 6000, Germany). All steps besides protein blocking were followed by rinsing with PBS.

2.7 Layered corneal construct

The multi-cell layered construct was developed by a combination of overlay culture method and cell sheet transfer. Firstly, endothelial cells were cultured as monolayer on NGMA-SC. The stromal fibroblast cells were used as the second layer of cells by direct seeding over the endothelial monolayer. The stromal cells were labeled using PKH26 Red Fluorescent Cell Linker Kit (Sigma, India) prior to seeding for easy detection. The corneal epithelial cell sheet retrieved from another NGMA-SC dish served as the final layer over the endothelial-stromal bi-layered construct. The corneal construct was maintained in vitro for 48 h in DMEM containing 10% FBS inside CO₂ incubator.

2.7.1 Reverse Transcription Polymerase Chain Reaction

The construct was characterized by Reverse Transcription Polymerase Chain Reaction (RT-PCR). The RNA was extracted in TRIzol (Merk - Genei) and isolated using chloroform-ethanol method. The cDNA was synthesised using Moloney murine leukemia virus Reverse Transcriptase Polymerase Chain Reaction (M-MuLV RT-PCR, Merck-Genei) kit. and amplified (Genei Red dye PCR kit, Merck-Genei) using their respective primers (Table 1) by PCR. Amplification for the target genes Cytokeratin-12 [22], Vimentin [23] and beta actin [24] was done at 30 cycles at their respective annealing temperatures. Na⁺ K⁺ ATPase target genes, ATP1A1 [24], were amplified using the following parameters: 5 min at 94 °C, 10 cycles of step-down PCR consisting of 1 min at 94 °C, 50 s at 55 °C then decrease 0.5 °C each cycle until 50 °C; 1 min at 72 °C, followed by 27 cycles of 1 min at 94 °C, 50 s at 50 °C, 1 min at 72 °C, with a final extension of 5 min at 72 °C. Beta actin was used as a loading control. The PCR products were electrophoresed in 1.5 % agarose gel.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Forward</th>
<th>Size</th>
</tr>
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<tbody>
<tr>
<td>Vimentin</td>
<td>ATGCTTCTTTGGCACGTCTTGACCT</td>
<td>395</td>
</tr>
<tr>
<td>ATP1A1</td>
<td>CTCTGTAACAGGGCGGTATT</td>
<td>219</td>
</tr>
<tr>
<td>CK-12</td>
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<td>150</td>
</tr>
<tr>
<td>β Actin</td>
<td>ATGCTCGATGAGCGAGCG</td>
<td>350</td>
</tr>
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2.7.2 Retrieval of layered corneal construct

The layered corneal construct from the NGMA-SC dish was retrieved by incubating below 10°C for 1 min followed by incubation at 22-24°C for 10 min.

The construct was peeled off using a sterile polyethylene terephthalate film while observing under a microscope.

3 RESULTS

3.1 Water contact angle

Water contact angle of NGMA-SC was compared with TCPS at 25°C (below LCST) and 37°C (above LCST) (Fig. 1). The contact angle of NGMA-SC showed a difference of 6° at the set temperature above and below the LCST. Whereas above LCST, the difference in contact angle of NGMA-SC and TCPS was 1.78° (Table 2). Statistical analysis of the water contact angle showed that there was significant difference in the contact angle of NGMA-SC above and below LCST (p = 0.0004). Whereas, TCPS did not show any significant shift in the water contact angle (p = 0.28).

<table>
<thead>
<tr>
<th>Surface</th>
<th>Contact angle Below LCST (± SD)</th>
<th>Contact angle Above LCST (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGMA-SC</td>
<td>61.65 ± 1.33°</td>
<td>67.73 ± 2.39°</td>
</tr>
<tr>
<td>TCPS</td>
<td>68.20 ± 0.89°</td>
<td>69.50 ± 2.03°</td>
</tr>
<tr>
<td>SD</td>
<td>68.20 ± 0.89°</td>
<td>69.50 ± 2.03°</td>
</tr>
</tbody>
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3.2 Cell adhesion on spin coated plates

The L-929 cells cultured on NGMA-SC retained their characteristic spindle morphology and formed a monolayer similar to cells cultured on TCPS. This indicated the non-cytotoxic and cell adhesion promoting nature of the NGMA-SC plates (Fig. 2).
3.3 Thermoresponsive efficacy

The cell sheet retrieval from NGMA-SC plate was successfully carried out using a PVDF membrane as illustrated in Fig. 3.

The L-929 cells adhered to the PVDF membrane could be removed from NGMA-SC at temperature below LCST and leaving no trace of the cells in the culture dish. The cells on the PVDF membrane that were transferred to another TCPS formed a monolayer within 48 h (Fig. 3D).

3.4 Isolation and Culture of Rabbit Corneal cells

Three major types of corneal cells – epithelial cells, stromal fibroblasts and endothelial cells were isolated and cultured separately.

Corneal epithelial cells were obtained from limbal explant cultures on NGMA-SC dish. Cells started migrating from the explants within 3 days and reached confluency in 7-10 days with typical epithelial morphology (Fig. 4).

The fibroblasts isolated from the stroma exhibited the typical spindle morphology and could be maintained by subculturing. The cells proliferated and reached confluency within 4-5 days in a split ratio of 1:3. There was no difference in cell morphology during in vitro maintenance (Fig. 5).

Corneal endothelial cells successfully isolated and cultured on gelatin coated dishes expressed their characteristic morphology (Fig. 6).

The isolated cells formed patches initially (24h) and subsequently formed a monolayer within 5 days of culture. Endothelial cells could be maintained in culture upto six passages and cells at passage 3 or 4 were used for the experiment.

Fig 2. Cell morphology on NGMA-SC coated plates, L929 cells seeded on TCPS A- 1 day, B – 2 days, C – 3 days. L929 cells seeded on NGMA coated plates D – 1 day, E – 2 days, F – 3 days

Fig 3. Schematic representation of L-929 cell retrieval, transfer and corresponding phase contrast images. (A) PVDF membrane (white arrows) placed on the L-929 monolayer cultured on NGMA-SC dish. (B) Retrieval of the cells at a temperature below the LCST. Black arrows indicates cells retrieved with the PVDF membrane (C) Cells transferred to a fresh TCPS dish was immediately observed after floating off the PVDF the membrane. (D) Transferred cells on the new TCPS dish observed after 48 h incubation.

Fig 4. Limbal explant culture on NGMA-SC plates (A) Cells migrating from the explant in 24 h, (B) Cell migration from explants observed on the 4th day and (C) Epithelial cells covered the culture surface in 10 days.

Fig 5. Corneal fibroblast cells at different passages (A) Passage 1, (B) Passage 2 and (C) Passage 3.

Fig 6. Corneal endothelial cells at different passages (A) Passage 1, (B) Passage 2 and (C) Passage 3.
Fig 6. Primary corneal endothelial cells at different time periods - (A) 1 day and (B) 5 days. Images C and D shows confluent homogeneous monolayer of the first passage and fourth passage respectively expressing the characteristic cobble stone morphology.

3.5 Characterization of the cultured corneal cells

Corneal cells were characterized by immunostaining of specific markers such as CK 3/12 for epithelial cells, vimentin for fibroblasts and endothelial cells and Na⁺K⁺ATPase for endothelial cells (Fig. 7).

Fig 7. Immunostaining of corneal cells (A) Limbal Epithelium positive for Cytokeratin 3/12 (green) counterstained with propidium iodide (red) (B) Stromal fibroblasts positive for vimentin (green) with nucleus counter stained with Hoechst, (C) Endothelial cells expressing vimentin (green) and (D) Endothelial cells expressing Na⁺K⁺ATPase (green) with nucleus counter stained with Hoechst.

The positive staining for CK-3/12 staining confirmed the epithelial cells in the explant culture. Stromal fibroblasts cells exhibited spindle morphology and positive staining for vimentin. Positive expression of vimentin and Na⁺K⁺ATPase confirmed the endothelial cells.

3.6 Layered corneal construct

The layered corneal construct required the use of two NGMA-SC dishes. On one dish corneal endothelial were established and allowed to form a monolayer. The cells expressed the characteristic cobble stone morphology on the modified surface (Fig. 8).

Fig 8. Primary culture of endothelial cells on gelatin coated NGMA-SC surface formed a monolayer in 5 days.

On the second NGMA-SC dish epithelial explants culture was established in parallel (Fig. 4). The cells from the stromal component were PKH26 labeled stromal fibroblast cells. These fibroblasts were seeded onto the endothelial monolayer to obtain a bilayer. The fibroblast growth and migration was visualized by fluorescence microscopy. PKH26 stained fibroblasts expressed spindle morphology and an increase in cell number on unstained endothelial cells (Fig. 9).

Fig 9. PKH26 labeled corneal fibroblast cells on the endothelial monolayer observed immediately after seeding by phase contrast (A) and fluorescence microscope (C). Cells after 48h, phase contrast (B) and fluorescence microscope (D). Fibroblasts attained subconfluence within 48 h covering the endothelial cells.

Epithelial cells cultured on NGMA-SC was retrieved as a cell sheet by peel off technique after temperature variation. The epithelial cell sheet transferred on the bilayer attached and spread within 48 h making it a complete multi-cell layered construct. Additionally, the fibroblast cells in the layered construct could be visualized by the positive fluorescence of PKH26 labeling (Fig. 10).
Fig 10. Phase contrast images of Epithelial cell sheet on the endothelial-fibroblast bilayer observed (A) immediately after transfer (B) after 48 h. (C) PKH26 stained fibroblasts in the multi-cell layered construct observed at the end of 48 h after epithelial cell sheet transfer.

The gene expression of characteristic markers of the individual cells in the layered construct showed the expression of CK12, Vimentin and Na’K’ATPase (Fig. 11). This proved both the presence and active metabolic state of all three cell types in the layered construct.

Fig 11. Gene Expression Profile of multicell layered corneal construct.

3.7 Retrieval of layered corneal construct

The corneal construct developed on NGMA-SC was retrieved as an intact tissue construct, by peel off technique. Incubation at a temperature below LCST of NGMA allowed the detachment of the construct with high efficiency. This confirms that the different cell types in layers maintained cell-cell contact and could organize into a single multilayered construct enabling physical peeling from the culture surface (Fig. 12).

Under phase contrast microscope the construct appeared transparent and at high magnification, the multi layered nature was evident (Fig. 13).

Fig 12. Schematic representation and corresponding phase contrast images of the Multicell layered corneal construct retrieval from the NGMA-SC surface. (A) Multi-cell layered construct on NGMA. (B) Incubation of construct below LCST (10 – 27 °C) for 10 min followed by mechanical peeling from the culture dish using a PET film. (C) Corneal construct in the process of retrieval.

Fig 13. Multi-cell layered corneal construct retrieved from NGMA surface observed under phase contrast microscope. (A) Transparent multi-cell layered construct floating in the culture medium. (B) Construct under high magnification showing multi-layered nature and cell-cell contact.

4 DISCUSSION

Tissue engineering aims to develop living substitutes to restore, maintain and improve tissue functions. In addition, the 3D biological substitutes thus resulted also finds application in in vitro toxicity studies. Stimuli responsive polymers have been studied extensively for biomedical applications [25]. Over the past two decades there are many reports on the use of thermoresponsive PIPAAm based polymers for tissue engineering [26]. Cell sheet technology is a distinct non-destructive method where thermoresponsive culture surface are used to develop in vitro constructs. We have previously reported that the thermoresponsive NGMA is a suitable substrate to generate multilayered corneal epithelial cell sheets. In this study a multi layered corneal construct was developed from three different cell types of rabbit cornea using NGMA.
Two well established approaches for isolation of corneal epithelial cells are explant culture and enzymatic dissociation of epithelial cells, each having their own advantages [27, 28]. Corneal epithelial cells in vitro require growth factors and hormone enriched medium together with either a non-mitotic feeder layer or denuded amniotic membrane [29, 30]. Very early studies on understanding the growth potential of corneal cells from different areas of human cornea showed that cells from the periphery grow better in culture than central cornea [31]. Later on it was found that the epithelium in the limbus grows better than the peripheral corneal epithelium [32]. Here limbal explant culture method was preferred over enzymatic method due to its simplicity in requirements to establish in vitro culture within a short duration. The limbal epithelial culture could be established directly on NGMA-SC by explant culture method. The result is similar to our earlier report on goat corneal epithelial culture on surfaces modified with NGMA by hand coating technique [33]. The cytokeratin - 3/12 is a characteristic cytoskeletal protein known to be expressed with distinct pattern during epithelial differentiation[34]. Immunostaining confirmed CK-3/12 positive cells in the explant culture.

Corneal stroma forms the thickest layer of the cornea comprising of densely packed collagen matrix containing fibroblast cells. The lattice arrangement and spacing between the collagen fibrils accounts for the transparency of cornea [34, 35]. The fibroblasts also synthesize corneal crystalin, similar to those present in the lens which maintains transparency and optical refraction [36]. There are different protocols described forstromal fibroblast isolation with or without digestive enzymes [37]. Since the corneal fibroblasts are present within collagen matrix, we adopted the use of trypsin and collagenase to isolate these cells. The primary fibroblast cells showed spindle morphology and proliferated on normal TCPS expressing the characteristic marker vimentin.

The final and innermost layer of cells in the cornea is the endothelial monolayer present along the posterior side and is essential for maintaining corneal transparency [3]. Literature review on corneal endothelial cells suggests that both explant and isolated cells methods can be successfully established in in-vitro culture [23, 38, 39]. A combination of enzyme digestion and growth factors supplemented medium was used in this study to isolate and culture primary corneal endothelial cells. The endothelial cells were maintained upto six subcultures without any morphological changes. The cells were positive for Na’K’ATPase which is an important transmembrane protein that controls hydration of stroma and maintains the corneal transparency.

Here we explain the development of multilayered corneal construct prepared by overlaying epithelial cells on to endothelial-fibroblast bilayer using cell sheet technology. Our earlier reports on thermoresponsive polymers have shown that NGMA is a suitable substrate for obtaining scaffold free bioengineered constructs. In the present study, spin coating was opted over hand coating to reduce batch variation and coating thickness. A custom made spin coater was used to apply a thin film of NGMA polymer on a 35 mm culture dish. At temperatures above the LCST of NGMA, water contact angles exhibited minor variation of 1.78° whereas below LCST; the difference was 3 fold (Table 2). Cell adhesion analysis showed that the spin coated surface supports excellent adhesion and spreading. This result is similar to the cell adhesion on NGMA coating applied manually [33]. The NGMA-SC surface also proved to exhibit thermoresponsiveness in retrieval of fibroblast cells using PVDF membrane similar to our previous report [40].

Over the years various corneal equivalents have been developed including substrates like fibrin, fibrin – agarose, collagen, etc as the stromal matrix [8, 39, 41]. Yet, a fully functional corneal equivalent requires specific cell types in addition to stromal matrix. There are reports on development of bioengineered cornea from all three types of corneal cells as in vitro model for ocular drug absorption analysis [12]. This study aimed to use the technique of cell sheet engineering to create a multi layered corneal construct by sequentially layering the three major corneal cells. The corneal endothelial cells were seeded on gelatin precoated NGMA -SC and allowed to form a monolayer. The endothelial cells were overlaid with PKH26 labeled stromal fibroblast cells while limbal epithelial cell culture was established concurrently on another NGMA-SC plate. Since the fibroblasts are seeded directly on the endothelial cells, which is a different atmosphere from in vivo cornea, the fibroblasts covered the endothelial monolayer. The experiment was designed such that, when the fibroblast cells cover the underlying endothelial layer, the limbal explant would be ready for cell sheet retrieval. The multilayered epithelial cell sheet retrieved by temperature variation was transferred to the bilayered construct. The epithelial cells could attach and spread on the endothelial-fibroblast bilayer. PKH26 labeling of stromal fibroblasts facilitated the visualization of its migration in the multi layered construct. Since PKH26 irreversibly binds to the cell membrane, the fluorescent dye is equally distributed between daughter cells upon division [42]. The spindle morphology and increase in number of fluorescent stained cells in the multilayered construct indicated that stromal fibroblasts were proliferating as depicted in Fig 11 &12. Gene expression analysis of the multi-layered construct was positive for CK-12, Vimentin and Na’K’ATPase. This showed that all three types of corneal cells were present in the construct and they were biologically active.

In vitro tissue reconstruction is not only demanding for tissue regeneration therapy but also in developing model systems for drug screening. Cell sheet tissue engineering is fairly new scaffold-free approach to repair damaged tissues in cell based therapy using thermoresponsive polymers [43]. Multiple layering of cell sheets is also possible to develop tissue constructs having homo or hetero-cell types. Three dimensional tissue reconstruction by cell sheet layering has been achieved in myocardial tissue engineering [44]. However this approach has not been extended for the construction of multi layer corneal equivalent. In this study we demonstrated the feasibility of NGMA-SC in multilayering of corneal cells to develop in vitro corneal models. Epithelial cell sheet transferred to endothelial – fibroblast bilayer was retrieved as an intact tissue construct via thermal stimuli. The epithelium of the cornea in vivo forms a tissue of about 6-8 cell layers. We have in previous reports on corneal epithelial cell culture by explant method shown that cell outgrowth from explants is multilayered [33]. Since an in vitro corneal construct would comprise of the different cornea cell types, a triple co-culture system was
constructed and maintained in the basic culture medium DMEM with 10% FBS. It is known that like other coculture systems, co-culture of different cell types in the cornea can influence each others growth and function in vitro [8]. The present model was engineered by overlaying the three corneal cell types without a stromal matrix to prove that the cell types can be individually cultured and over-layered to create a bioengineered cornea. The exact replication of the ultrastructural arrangement of stromal extracellular matrix is a major challenge in corneal tissue engineering [45]. However the extent of ECM production by the stromal fibroblasts in our in vitro construct remains to be examined. The final construct developed in this study differs from ex vivo corneas and existing artificial systems. Proulx et al have shown that corneal fibroblasts in presence of serum and ascorbic acid produces extracellular matrix and forms thick sheets of collagenous tissues which can be superimposed to reconstruct a corneal stroma [46]. Development of such scaffold free 3D corneal fibroblast construct support the possibility of self generated storma [47, 48]. We speculate that if the culture conditions are suitable, the stromal fibroblasts are expected to synthesize its own ECM in the multilayered construct. This will eliminate the need for artificial stromal equivalents and resemble the original corneal tissue. Thus overlaying cells and cell sheet constructs is presented as a potential approach to develop in vitro corneal constructs. More studies by including the stromal component in the multilayered construct and confirmation of 3-dimensional cell distribution by histological analysis will elevate its performance as an alternate for the excised tissue and small animal models in toxicity and drug screening studies.

CONCLUSIONS

The results confirmed the feasibility of NGMA spin coated on to TCPS dishes as an efficient substrate for developing a scaffold free multi-layered corneal construct. All three corneal cell types, epithelium, fibroblast and endothelium were sequentially layered to form a 3D construct that exhibited characteristic gene expression. We propose this multi-cell layering technique as a promising method for developing in vitro corneal constructs.

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