

## Chapter 5

# Application of Hydrogels in Ocular Tissue Engineering

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Corneal disease is a leading cause of blindness worldwide. Transplantation of donor cornea is the preferred treatment for corneal disease. However, these are limited in supply, especially in developing countries. Therefore, there is a need for alternative treatment methods to meet the rising demand for corneal replacements. Over the past two decades, much work has been done in this realm. In this chapter, we first describe the significance of the problem associated with corneal disease, the current treatment options available and their related limitations. Following this, studies in the literature that focus on the development of alternative treatment methodologies including decellularization of xenogenic corneal tissue, development of hydrogel based polymeric bioscaffolds and microengineering are discussed in detail. Finally,

the chapter is concluded with potential future directions that need to be pursued for the realization of a fully functional bioartificial cornea as an alternative to donor cornea for corneal repair and regeneration.

## 1. Introduction

Cornea is a transparent, sparsely cellular and avascular organ that forms the outermost part of the eye. It mainly comprises of three functional layers: (i) corneal epithelium, (ii) corneal stroma, and (iii) corneal endothelium. The corneal epithelium acts as a barrier to protect the stromal layer from pathogen invasion and maintain the tear/air interface. The stromal layer forms 90% of the cornea and is responsible for its transparency and refractive index. The corneal endothelium layer comprises of approximately 400,000 endothelial cells that help conserve the transparency of the stromal layer by maintaining the fluid balance within the stroma. The three layers together regulate the function of the cornea.

Corneal disease is the second major cause for blindness and has rendered more than 10 million people blind across the world.<sup>1</sup> The most significant factors that lead to corneal disease include infection (trachoma), injury (ocular trauma) and corneal ulcerations. Keratoplasty (transplantation of donor cornea) is the current gold standard for the treatment of corneal disease. In the United States alone, approximately 72,000 corneal transplants are performed each year.<sup>2</sup> However, donor corneas are associated with a rejection rate of 18% due to immunological complications.<sup>3</sup> Further, in developing countries, donor corneas are limited in supply.<sup>4-6</sup> Application of synthetic polymer-based artificial corneas (keratoprosthesis) to restore corneal function is limited due to serious complications such as glaucoma and other inflammatory/immunological outcomes.

Over the past two decades, amniotic membrane derived from the inner wall of the fetal placenta, has been successfully applied in the clinic for ocular surface reconstruction.<sup>7-9</sup> Since the amniotic tissue is usually discarded after birth, it is easily accessible with little ethical concern. The use of the amniotic membrane as a graft for corneal repair has been reported to promote re-epithelialization, reduce inflammation and inhibit vascularization.<sup>10</sup> These key elements make the amniotic membrane a viable source for ocular surface reconstruction. However, reliable use of amniotic membrane is associated with limitations that include suboptimal transparency,<sup>11</sup> poor biomechanical strength<sup>12</sup> and donor-to-donor variation in tissue quality.<sup>13</sup> Therefore, development of bioengineered cornea by either using decellularized xenogenic tissues<sup>14</sup> or polymeric matrices as scaffolds is being widely explored for corneal repair and regeneration.

Preservation of the biochemical composition and tissue structure of the native cornea by employing optimal decellularization methods is a promising approach

to generate a biological scaffold material for corneal tissue engineering applications. Decellularized tissues present a natural microenvironment to the cells populating it and thereby modulate various facets of the host response including cell adhesion, orientation, proliferation, vascularization, and inflammation. Application of decellularized cornea for ocular applications is being widely studied both as an acellular matrix<sup>15,16</sup> relying on the host cells post implantation, and as a scaffold material seeded with cells<sup>17,18</sup> prior to implantation. Porcine corneas are most commonly decellularized for corneal applications because they are easily available and they match the size and refractive power of the human cornea.<sup>19</sup>

Efforts have also focused on the development of tissue-engineered constructs using hydrogels and biopolymeric materials for the regeneration of the native cornea. An ideal tissue engineered construct must closely mimic the native cornea to be able to perform its critical functions. Ruberti and Zeiske have defined the three design considerations that must be met for an artificial corneal construct to be functional: (i) protection, (ii) transmission, and (iii) refraction.<sup>20</sup> To be able to meet the aforementioned design criteria, the tissue engineered construct must have the following essential characteristics: (i) promote the migration and proliferation of corneal epithelial cells for the formation of a functional corneal epithelium and protect the intraocular contents from pathogenic invasion, (ii) possess tensile strength (3.8 MPa)<sup>110</sup> and viscoelastic properties comparable to the native cornea to withstand the intraocular pressure and thereby prevent rupture, (iii) mimic the nanoscale fibrillar organization of the corneal stroma to exhibit high degree of transparency (>90%) and refractive index, (iv) support the maintenance of functional keratocytes to conserve transparency and synthesize *de novo* collagenous tissue, and (v) match the swelling ratio of the native cornea to be able to retain and distribute the water content and thereby prevent the formation of an optical haze.

This chapter is a comprehensive summary of the various studies in the literature that focus on the development of decellularized corneas or polymeric hydrogels as substrates for corneal tissue engineering applications. Further, some of the key studies that employ stem cells for corneal regeneration have been highlighted. Finally, studies that employ microengineering to pattern polymeric substrates and mimic the highly organized structure of native cornea have been described.

## 2. Decellularization of Biological Tissues for Corneal Repair

Decellularization is a process by which cellular and nuclear material is removed from the tissue via chemical, physical, and biological means whilst retaining the extracellular matrix (ECM) of tissue.<sup>14,22,111</sup> The ECM is a biological scaffold with an intact tissue microstructure comprising of proteins arranged in a unique architecture specific to the tissue. By presenting a substrate that is akin to the native tissue, decellularized

scaffolds modulate the host tissue response by influencing cell adhesion, cell–cell signaling, and cell proliferation. Further, the tissue specific architecture has been shown to guide cell differentiation and maintain the phenotype of the differentiated cells to enable the regeneration of a functional tissue.<sup>21</sup>

Unlike other tissues in the body, cornea has the specific requirement of being optically clear and transparent. Therefore, optimal decellularization of the tissue must not only result in the removal of the cellular material and antigens but also protect the transparency of the tissue. Further, the decellularized tissue must support the maintenance and growth of corneal cells.<sup>24</sup> While the most effective decellularization method that can be reliably used for corneal applications is yet to be determined, there are a plethora of methods in use today that have shown promise in the development of decellularized corneal scaffolds.<sup>14,22,111</sup> Most researchers use a combination of methods to facilitate complete decellularization while maintaining the key attributes of the tissue to enable their applicability for a functional cornea.<sup>22</sup> Currently, a great deal of interest has been placed on decellularization of porcine corneas as they are easily available and closely mimic the ECM of the native human cornea.<sup>19</sup>

## 2.1. Chemical methods

Acids and bases are commonly employed for decellularization of tissues.<sup>22,30</sup> Treatment with acids, such as peracetic acid, and bases, such as ammonium hydroxide, results in the digestion of the cell cytoplasmic contents and disruption of nucleic acids within the cell. However, these are associated with limitations that include altering the tissue microstructure and weakening the mechanical properties of the tissue.

Detergents are another class of chemicals also used for decellularization of tissues.<sup>22,30</sup> They are classified into three basic categories (non-ionic, ionic and zwitterionic) based on the polar head group (charge). Non-ionic detergents (uncharged head groups) such as Triton X-100 are mild and non-denaturing. They work by disrupting lipid–lipid and lipid–protein interactions, while leaving protein–protein interactions intact<sup>22</sup> and thus removing the biological information (antigens, etc.) that could illicit an immune response while preserving the unique protein–protein architecture of the tissue. However, non-ionic detergents have limited ability to fully decellularize the tissue.<sup>16</sup> Ionic detergents (charged head groups) such as sodium dodecyl sulfate (SDS) are much stronger than Triton X-100 resulting in more efficient solubilization of cytoplasmic and nuclear membranes.<sup>22</sup> However, they have a tendency to denature proteins and reduce the transparency of the tissue.<sup>25</sup> Zwitterionic detergents have a net charge of zero and exhibit properties of both ionic and non-ionic detergents. While zwitterionic reagents such as CHAPS (3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate)

protect the native state of proteins, they are not commonly used because they are not as effective in removing nuclear information and cell fragments which can lead to immunological rejection after implantation.<sup>16</sup>

Other chemicals commonly used for decellularization include alcohols, hypotonic and hypertonic solutions, and chelating agents. Alcohols work via two mechanisms: dehydration of the tissue and removal of the lipids (solvation). Since alcohols are typically used as fixatives, their use for decellularization may result in altering the ultrastructure of the corneal tissue.<sup>22</sup> Hypotonic and hypertonic solutions rely on osmotic shock resulting from high differences in ion concentration to disrupt the cell membrane and lyse cells. Most commonly, deionized water or sodium chloride solutions have been used for cell lysis.<sup>22</sup> Chelating agents such as EDTA bind to metallic ions (divalent cations) and disrupt cell adhesion causing dislocation of cells.<sup>22,26</sup>

Most of the aforementioned chemicals have been used either by themselves or in combination for effective decellularization of corneal tissue. Findings from some of the key studies have been elucidated here. Choi *et al.*, employed a combination of Triton X-100 and ammonium hydroxide to produce decellularized human corneal stroma scaffolds with intact ECM proteins and biomechanical properties comparable to normal cornea.<sup>27</sup> Further, they reported that culture of human corneal endothelial cells on decellularized human corneal stromas resulted in the regeneration of the corneal endothelium with potential for transplantation. Du *et al.*, developed a full thickness acellular porcine matrix for corneal tissue engineering applications by decellularizing porcine cornea via immersion in 0.5 wt% sodium dodecyl sulfate.<sup>16</sup> They reported that the process resulted in efficient decellularization while preserving the major structural components and strength of the cornea suggesting that acellular porcine matrix can be used for corneal transplantation and tissue engineering applications. Gonzalez-Andrades *et al.* compared two different chemical methods (NaCl and SDS) to identify the most optimal method of decellularization.<sup>24</sup> The results indicated that while treatment with 0.1% SDS treatment resulted in high level of fibril disorganization and poor optical behavior of the corneas, 1.5 M NaCl preserves the histologic structure, composition, and optical behavior of porcine corneas. Other research groups have also reported a similar negative outcome including loosening of collagen fibrils and removal of glycosaminoglycans (GAGs) with the use of SDS as a decellularization agent.<sup>28,29</sup> GAGs are highly polar and attract water which is essential for the health of the eye. Removal of GAGs during decellularization is not optimal as it may cause loss of hydration in the corneal stroma resulting in corneal haze.

Ponce Marquez *et al.*, studied the effect of three different chemical methods to decellularize bovine corneas.<sup>30</sup> The three methods included: (1) treatment with 1%

SDS followed by 75% ethanol, (2) treatment with 75% ethanol followed by trypsin-EDTA, and (3) treatment with varying ratios of peracetic acid and ethanol mixtures. Histological analyses revealed that while treatment with peracetic acid and ethanol mixtures (method 3) resulted in only partial decellularization, no nuclei or cell debris were found when the corneas were treated with methods 1 and 2. Further, corneas treated with method 2 were reported to maintain their transparency. Keratocytes cultured on corneas treated with method 2 were observed to be more metabolically active and produce more collagen compared to method 1. Mechanical characterization results indicated that the Young's modulus of corneas treated with method 1 was significantly higher compared to method 2; however, the mechanical properties of corneas treated with method 2 were comparable to native cornea. Glycerol has also been used in the decellularization of cornea.<sup>14,31</sup> It has been shown to maintain and even restore corneal transparency after edema (over swelling due to high water content).

## 2.2. Physical methods

Physical methods most commonly employed for decellularization of tissues include ultrahigh hydrostatic pressure (UHP), freeze-thaw cycling, lyophilization, and mechanical agitation. UHP works via physical disruption of the cell membrane such that the cellular contents are exposed and washed out as debris. A simple and benign cultured medium wash may be used to remove the compromised cellular material out of the ECM of the tissue, thus making this method non-cytotoxic unlike the detergents.<sup>32</sup> Sasaki *et al.*, have shown that while treatment of porcine corneas with detergents Triton X-100 and SDS resulted in decrease in GAG content and denaturation of the superstructure of the cornea, UHP treatment had no such effect and maintained the corneal structure.<sup>29</sup> Further, more efficient removal of cell components was observed with the UHP method compared to detergents. However, the corneas treated with both detergents and UHP were extremely cloudy. Upon treatment with glycerol, the transparency was recovered for corneas treated with UHP but the corneas treated with detergents were found to be irreversibly cloudy.<sup>29</sup> The mechanical properties of corneas decellularized using UHP were not altered and comparable to native cornea.

Proulx *et al.*, have reported the reconstruction of a healthy corneal endothelium using devitalized human corneas.<sup>33</sup> Native human corneas were decellularized by using three consecutive freeze-thaw cycles that resulted in cell lysis via the formation of ice crystals within the cellular membrane. Feline corneal endothelial cells cultured on denuded Descemet's membrane of devitalized human corneas maintain endothelial morphology and form a high cell density monolayer. Further, the healthy corneal endothelium expressed functional proteins such as ZO-1, Na<sup>+</sup>/

$K^+$ -APTase and  $NA^+/HCO_3^-$  cotransporter, thus demonstrating potential to be used as a replacement for diseased endothelium. Corneal stroma from pig, rabbit and human source was decellularized by freezing using liquid nitrogen followed by thawing rapidly at  $37^\circ C$ .<sup>34</sup> While the rabbit corneas became opaque, pig and human corneas were found to be transparent after three freeze thaw cycles. Further, the collagen fibrils were severely damaged in rabbit corneas versus well preserved collagen fibrils in pig and human corneas. These findings suggest that techniques for successful decellularization are not necessarily transferable across species. Further, it is important to note that the effect of freeze-thaw method on the mechanical properties of cornea were not reported in these studies. Fu *et al.*, used a combination of Triton X-100 and freeze drying to reconstruct tissue engineered cornea from porcine acellular matrix.<sup>35</sup> The decellularized scaffold was seeded with keratocytes followed by epithelial and endothelial cells on either side of the scaffold to form a viable biological corneal equivalent for corneal tissue engineering applications. Recently, porcine corneas were decellularized using a combination of freeze-thaw cycles and pepsin treatment to generate optically transparent and mechanically robust hydrogels for corneal stroma regeneration.<sup>112</sup>

Lyophilization is another common method to fabricate acellular scaffolds. It differs from freeze-thaw cycling in that the process itself produces pores in the tissue which are useful for cell seeding and distribution within the decellularized tissue. The diameter of the pores resulting from the freezing process can be controlled by varying the temperature: smaller ice crystals form at lower freezing temperatures while larger ice crystals form when there is a larger temperature gradient during freezing. Xiao *et al.*, employed the lyophilization method to fabricate porous acellular porcine corneal stroma scaffolds and reported that keratocytes remain viable and proliferate well on these scaffolds.<sup>15</sup> Light transmittance of these scaffolds after transplantation in rabbit eyes was found to be comparable to native cornea. Lee *et al.*, have shown that lyophilized acellular porcine cornea have better survival compared to acellular porcine cornea in rat model.<sup>36</sup> In a follow-up study, Lee *et al.*, showed that lyophilized acellular porcine cornea repopulated with cells showed better optical transparency and higher expression of corneal markers suggesting that recellularization of scaffolds prior to transplantation may be beneficial for ocular surface reconstruction.<sup>36</sup>

Mechanical agitation is generally used in combination with other methods for more effective decellularization. The use of mechanical agitation alone is largely ineffective as it results in removal of cells only from the surface of the tissue. Additionally, the mechanical agitation greatly affects the integrity of the unique architecture of the tissue itself.<sup>26</sup> Therefore, it is not a method that should be used in whole-layer tissues' decellularization, where maintaining structure and fibril orientation is of utmost importance, such as in corneal tissue scaffolding.

### 2.3. Biological methods

Biological methods for decellularization entail the use of enzymatic agents. Trypsin is commonly used as a pretreatment during the decellularization process and it works via hydrolytic degradation and breakdown of proteins into smaller amino acids resulting in disruption of the ECM.<sup>26,31</sup> However, prolonged exposure to trypsin can destroy the cornea's unique ECM including the removal of collagen and GAG which affects the transparency and strength of the cornea. Therefore, the decellularization of corneal tissue via trypsin needs to be carried out judiciously to preserve the unique architecture of the cornea.<sup>14</sup>

Nuclease is a type of enzyme that can cleave phosphodiester bonds which make up the backbone of DNA and RNA. There are two primary categories of nucleases: endonuclease and exonuclease. Endonucleases are enzymes that catalyze the hydrolysis of bonds between nucleic acids in the interior of DNA or RNA chains. Exonucleases are enzymes that catalyze the hydrolysis of nucleotides at the ends of DNA or RNA chains. One of the limitations of using nucleases for decellularization is some amounts of the enzyme remains in the tissue even after the decellularization process which could elicit an immune response that could result in the rejection of a corneal scaffold.<sup>26</sup> Additionally, nucleases also tend to require additional treatment by a different decellularization method for complete removal of cellular and nuclear content.

Gonzalez-Andrades *et al.*, employed Dispase to remove the corneal epithelium and endothelium layers prior to employing chemical means to fully decellularize porcine cornea.<sup>24</sup> The phospholipase class of enzymes is characterized by the hydrolysis of phospholipids into fatty acids. Wu *et al.*, employed phospholipase A2 to decellularize porcine corneal scaffolds. Unlike other enzymatic decellularizing agents, phospholipase A2 preserved the collagen and fibril architecture of the tissue without breaking down the proteoglycans. Further, phospholipase A2 did not significantly affect the transparency of the decellularized cornea and the mechanical strength was also maintained.

Aprotinin has been used together with EDTA and SDS for efficient elimination of corneal cells from porcine corneas.<sup>17</sup> The resultant decellularized porcine cornea supported the growth of corneal epithelial, stromal and endothelial cells and thus demonstrating promise to be used for corneal tissue engineering applications.

### 3. Hydrogels and Polymeric Matrices for Corneal Repair

While application of xenografts (porcine and bovine corneas) post decellularization for corneal repair is meritorious, they are associated with several limitations that include immune response and risk of disease transmission. Additionally, a fully efficient decellularization process that completely removes the cellular material and

also maintains the microstructure and transparency of the corneal tissue is challenging and yet to be realized. Tissue engineering using natural or synthetic biomaterials with or without cells has been widely studied as a promising approach for the regeneration of cornea. Efforts in the corneal tissue engineering realm have centered on two different strategies: 1) development of thin sheet like matrices for the regeneration of specific layers (i.e., epithelium or endothelium) of the cornea, 2) fabrication of multi-layered scaffolds for the development of hemi-corneas (stroma + epithelium) or full-thickness tissue engineered cornea. Various materials, both natural and synthetic, have been used for the development of a corneal scaffold. This section presents a summary of some of the key studies on the development of hydrogels and biopolymeric materials for corneal tissue engineering applications.

### 3.1. Collagen hydrogels

Type-I collagen is the primary component of native cornea and hence is of significant interest as a biomaterial for corneal tissue engineering applications. In 1999, Griffith *et al.*, developed functional human corneal equivalents using a collagen–chondroitin sulfate substrate.<sup>37</sup> In this study, human cells isolated from the individual layers of the cornea were immortalized, well-characterized and then seeded on the substrate. Stromal cells were seeded within the substrate and the epithelial cells and endothelial cells were seeded on the top and bottom of the substrate, respectively. The resultant corneal equivalent resembled the human cornea in morphology, histology, and transparency. Further, the corneal equivalents were also comparable to the human cornea in terms of the stromal swelling, physiological endothelium activity, and response to chemicals. However, despite the positive outcome *in vitro*, these scaffolds lacked the mechanical robustness required to withstand surgery and implantation procedure. In a follow-up study, Griffith *et al.*, fabricated collagen-based composites by blending collagen and a synthetic acrylamide-based polymer [poly(N-isopropylacrylamide)] (pNIPAAm). To assess the feasibility of these composites in ocular surface reconstruction, a 3 mm circular wound was made in the central cornea area of Japanese white rabbits and the collagen-pNIPAAm scaffolds were implanted.<sup>38,39</sup> The results showed regrowth of epithelium within three days for 87.5% of the rabbits (21 out of 24). While rapid epithelialization was observed on the composite scaffold, histological findings revealed that epithelial differentiation overlaying the polymer was abnormal as indicated by disarrayed cell stratification on the scaffold. To improve this outcome, Li *et al.*, synthesized a hydrogel by blending collagen and a copolymer poly(N-isopropylacrylamide-coacrylic acid-coacryloxysuccinimide) (TERP).<sup>40</sup> A laminin adhesion pentapeptide motif (YIGSR) was then grafted onto the hydrogel (TERP 5). Upon implantation of TERP 5 in pig corneas, rapid recruitment of the host corneal epithelial and stromal cells was observed together with functional innervation within

the implant. This mechanically robust and suturable scaffold was the first physiologically functional tissue substitute reported for corneal applications.

A simple crosslinked collagen tissue substitute for corneal implantation<sup>41</sup> was synthesized in contact lens molds by mixing porcine collagen (pH adjusted to 5.5) and aqueous solutions of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) followed by curing at 21°C for 24 h and 37°C for 24 h. The optical transparency of the crosslinked collagen matrices was reported to be superior to the human cornea. Further, *in vivo* studies in rabbit and porcine models showed that 23 out of 24 crosslinked collagen matrices remained transparent six months post implantation and showed stable host-graft integration. Further, the crosslinked collagen matrices promote corneal cell regeneration and innervation. Together, these findings suggest that stable implantable collagen matrix substitutes can be synthesized via simple crosslinking using water soluble carbodiimides.

Since the use of xenogenic sources of collagen (bovine, porcine etc.) is associated with several limitations including disease transmission and many immunological and allergic reactions, recombinant human collagen is a viable source for the synthesis of collagen hydrogels for corneal repair. Comparative studies to assess the efficacy of recombinant human type I collagen (RHC I) and recombinant human type III collagen (RHC III) for use as corneal substitutes revealed that both RHC I and RHC III had similar optical clarity to the human cornea.<sup>42,43</sup> However, RHC III hydrogels were found to be optically and mechanically superior to RHC I hydrogels. Twelve months post implantation in pig corneas, both RHC I and RHC III hydrogels showed good integration with the host tissue. Confocal microscopic images showed regeneration of corneal cells and nerve and also the retention of optical clarity by the hydrogels.<sup>42</sup> Re-innervation of these RHC substitutes one year post implantation in a pig model was found to be comparable to porcine allograft and biosynthetic porcine collagen based grafts.<sup>44</sup>

The promising outcome of the RHC III hydrogels, as described above, led to the first Phase I clinical study. EDC-NHS crosslinked RHC III corneal substitutes were implanted into 10 patients with vision loss.<sup>45</sup> Six to seven months post implantation, the RHC III corneal substitutes were found to be well-integrated accompanied by regeneration of corneal epithelium, stroma and nerves. No evidence of neovascularization, inflammation or rejection was observed. Remodeling and seamless integration of the RHC III collagen substitutes with the host tissue was witnessed as seen in the prior animal studies. A follow-up study 24 months post-implantation confirmed that the RHC III corneal substitutes remain integrated to the host tissue with no evidence of infection, inflammation or vascularization.<sup>46</sup> Visual acuity test revealed that the vision of 6 out of 10 patients improved, two remained unchanged and two deteriorated when compared with values prior to implantation of the RHC III collagen substitutes.<sup>46</sup> A recent assessment four

years post-implantation revealed that the RHC III collagen substitutes outperformed donor human corneas in many realms.<sup>47</sup> While one out of nine patients implanted with donor human cornea had a rejection episode, the regenerated neo-corneas for patients implanted with RHC III corneal substitutes were found to be stably integrated with no episodes of rejection. Further, unlike donor human corneas, corneal cell and nerve regeneration in RHC III corneal substitute was comparable to healthy human corneas. However, visual acuity of RHC III corneal substitutes was lower (20/54) compared to donor human corneas (20/36) suggesting that further optimization of the corneal substitutes is needed to improve vision. This Phase I clinical study demonstrates that RHC III corneal substitutes have significant potential to be used for the treatment of corneal blindness in the future.

Collagen matrices have been formed as gels, foams and sponges for corneal applications. A tissue-engineered collagen sponge matrix has been shown to maintain the phenotype of human corneal cells and support the co-culture of different corneal cell types.<sup>48</sup> The transparency of the collagen sponge matrix was five times greater than conventional collagen gels but only 50–60% of the native cornea. Highly porous collagen-based foams were developed as biodegradable scaffolds using lyophilization followed by EDC-NHS crosslinking.<sup>49</sup> Human corneal keratocytes penetrated and populated the scaffold over one month and synthesized corneal ECM composed of collagen types I, V, and VI. Reconstructed full-thickness corneal substitute was developed using porous collagen-chondroitin sulfate scaffold seeded with stromal keratocytes followed by epithelial and endothelial cells on the top and bottom of the scaffold, respectively.<sup>50</sup> Results showed that keratocytes populate and synthesize *de novo* ECM on the scaffold. Further, the epithelial cells form a stratified epithelium and the endothelial cells remain viable suggesting that collagen-chondroitin sulfate scaffolds are good substrates for artificial cornea. Duan and Sheardown developed a dendrimer (polypropyleneimine octaamine) based crosslinking approach of concentrated collagen solutions and reported that the transparency of dendrimer crosslinked gels was significantly greater compared to glutaraldehyde and EDC crosslinked gels.<sup>51</sup> Further, dendrimer crosslinked collagen gels were significantly stiffer (5 MPa) than EDC crosslinked gels (0.1 MPa) and comparable to human cornea (up to<sup>3–13</sup> MPa) thereby demonstrating promise to be used for corneal tissue engineering applications. However, the suture strength of dendrimer crosslinked collagen gels was significantly lower than the native cornea.

Pure collagen-based hydrogels are mechanically weak and degrade rapidly. While crosslinking improves the strength and stiffness of collagen hydrogels to some extent, additional processing methods have been developed and employed along with crosslinking to further enhance the mechanical properties and biodegradability of the hydrogels for corneal applications.<sup>52–55</sup> One of the primary reasons for the weak mechanical strength of traditional collagen hydrogels is the high water

content. Plastic compression of traditional collagen gels has been reported to remove excess water and form dense and mechanically strong collagen matrices. Mi *et al.*, have reported that plastically compressed collagen scaffolds are transparent with fiber diameter and orientation comparable to native cornea.<sup>52</sup> Further, photochemical crosslinking of plastically compressed collagen gels using riboflavin significantly improved the mechanical properties and suturability of the scaffold without altering the structural and optical properties.<sup>53</sup> In a follow-up study, Xiao *et al.* implanted the plastically compressed collagen gels in rabbit corneas and reported that the biocompatibility of the semi-transparent gels was comparable to the amniotic membrane.<sup>54</sup> Levis *et al.*, developed plastically compressed collagen constructs incorporated with human limbal fibroblasts as a substrate for the expansion of human limbal epithelial cells.<sup>55</sup> The fibroblasts remained viable within the collagen constructs post plastic compression and the epithelial cells expanded well on the surface of the constructs. Further, the epithelial cells formed a multilayered epithelium with polygonal morphology similar to that of the native central cornea suggesting that plastically compressed collagen constructs may be used for limbal epithelial cell transplantation. Levis *et al.*, have also shown that plastically compressed collagen maintain the cobblestone morphology and support the expansion of corneal endothelial cells.<sup>56</sup> Additionally, the ease of handling during transplantation make plastically compressed collagen a promising carrier for corneal endothelial cell transplantation.

Incorporation of natural or synthetic polymers within the collagen network is another method that is widely employed to improve the mechanical properties and biodegradability of collagen hydrogels. A collagen-phospholipid corneal substitute was developed using a combination of EDC-NHS crosslinked collagen and poly(ethylene glycol) diacrylate crosslinked 2-methacryloyloxyethyl phosphorylcholine (MPC).<sup>57,58</sup> The resultant hybrid hydrogel was mechanically superior and more stable compared to hydrogels made from individual components. Further, the optical transparency and cellular compatibility of these hybrid hydrogels was comparable to traditional collagen hydrogels. Implantation of these hybrid hydrogels into the corneas of minipigs resulted in regeneration of the corneal epithelium and stroma together with nerve ingrowth suggesting that these hydrogels can be used as corneal substitutes in clinical applications. Rafat *et al.*, synthesized hybrid polymer network scaffolds using collagen and chitosan and crosslinked the scaffold with EDC-NHS along with poly (ethylene glycol) dibutylaldehyde (PEG DBA).<sup>59</sup> The resultant scaffolds were found to be transparent, optimally strong and biocompatible. Silica-collagen biohybrid materials have shown to be transparent and exhibit better mechanical properties than collagen crosslinked scaffolds.<sup>60</sup>

The high level of transparency and robust mechanical properties of native cornea are attributed to the orthogonal arrangement of aligned collagen fibrils

within the corneal stroma. Development of bioengineered corneas that mimics such structural organization would greatly enhance their potential to be used in corneal regeneration applications. Torbet *et al.*, gelled neutralized type I collagen solution in the presence of a horizontal magnetic field of 7 Tesla to assemble collagen molecules and form oriented collagen fibrils within the gel.<sup>61</sup> A series of gelation–rotation–gelation cycles was employed to produce a scaffold of orthogonal lamellae composed of aligned collagen fibrils. Keratocytes seeded onto the scaffold composed of three orthogonal lamellae were found to rapidly proliferate, penetrate the scaffold and align along the direction of the collagen fibrils. Incorporation of proteoglycans improved the transparency of the scaffold and enhanced the proliferation of keratocytes. In a follow-up study, human keratocytes were seeded onto magnetically aligned collagen scaffolds and cultured for 30 days to form the reconstructed stroma.<sup>62</sup> Human limbal stem cell-derived epithelial cells seeded on the surface of the reconstructed stroma exhibited a well-differentiated epithelium consistent with the epithelial structure of the native cornea. Additionally, in a preliminary study, the scaffolds were implanted into five rabbits to assess the *in vivo* response.<sup>62</sup> Four of the five rabbits lost their scaffolds despite extensive suturing. However, the retained scaffold showed good outcomes in case of re-epithelialization, regaining transparency and minimal neo-vascularization. Further work is required in terms of crosslinking of these scaffolds and suturing for better scaffold retention.

### 3.2. Non-collagenous hydrogels

Biomaterials for the development of hydrogels and polymeric matrices for corneal applications are not limited to collagen alone. Other materials that include gelatin, keratin, chitosan and silk have also been used. Some of the key studies that focus on the development of non-collagenous matrices for corneal regeneration are elucidated here.

#### 3.2.1. Keratin

Human hair keratin in nanoparticulate form was mixed with a softening agent (glycerol) and subjected to a curing process to develop a transferable, stable, and transparent keratin film for corneal applications.<sup>63</sup> The transparency and stiffness of keratin films were reported to be significantly higher than amniotic membrane (currently in use for ocular surface reconstruction). Further, growth behavior of human corneal epithelial cells was comparable between keratin films and amniotic membrane. Based on these findings, the authors suggest that keratin films are promising candidates for ocular surface reconstruction. In a follow-up study,

Borrelli *et al.*, employed different sterilization protocols to assess the effect of sterilization on the optical transparency and biomechanical properties of keratin films.<sup>64</sup> The sterilization process showed no significant differences in both optical transparency and biomechanical properties of keratin films. However, suturing of keratin films onto enucleated porcine eyes was found to be more challenging than amniotic membrane due to poor elasticity leading to concerns of irritation and neovascularization post implantation *in vivo*. Therefore, the authors suggest that additional work to improve the elasticity of the material is needed for the use of keratin films in ocular surface reconstruction.

### 3.2.2. Chitosan

A blend of chitosan, gelatin and chondroitin sulfate was used to synthesize optically transparent membranes with a tensile strength of 1.48 MPa and elongation at break of 45.64%.<sup>65</sup> These membranes support the growth of corneal endothelial cells and may be used as carriers for corneal endothelial cell transplantation. Immobilization of hyaluronic acid onto chitosan membranes was reported to maintain the transparency (99%) and improve the hydrophilicity and growth of corneal epithelial cells suggesting that these membranes are promising candidates for corneal regeneration.<sup>66</sup> The transparency of ultrathin chitosan films (~50  $\mu\text{m}$  thick) crosslinked with diepoxy-polyethylene glycol (PEG) and cystamine was reported to be on par with human cornea.<sup>67</sup> Further, the films were biodegradable and supported the growth of corneal endothelial cells. Optimization of PEG concentration for crosslinking resulted in mechanically robust films that possessed excellent characteristics for physical manipulation and implantation demonstrated by an *ex vivo* ovine surgical model. The smooth topology together with excellent optical and biomechanical properties suggest that ultrathin chitosan-PEG films are promising candidates to be used for corneal transplantation procedures.<sup>67</sup> Grolik *et al.*, reported that the genipin crosslinked chitosan-collagen hydrogels were significantly stronger (46.93 MPa) than amniotic membrane (2.3 MPa).<sup>68</sup> Further, growth of corneal epithelial cells on genipin crosslinked chitosan-collagen hydrogels was comparable to amniotic membrane. However, the hydrogels exhibited a bluish–brown color due to genipin crosslinking. The authors suggest that the bluish–brown color of the hydrogels should not pose a problem as the hydrogels would be resorbed post implantation and instead can be used as an indicator of resorption of the hydrogels.

### 3.2.3. Silk Fibroin

Several studies have reported the merit of using silk fibroin as a biomaterial for ocular tissue reconstruction.<sup>69</sup> Silk fibroin membranes have been reported to be

transparent, easy to handle and support the growth of human limbal epithelial cells.<sup>70</sup> Further, the morphology and phenotype of corneal epithelial cells on the silk fibroin membrane has shown to be comparable to the amniotic membrane.<sup>71,72</sup> Madden *et al.*, were the first to report that primary human corneal endothelial cells exhibit a polygonal morphology and grow to confluency on collagen coated silk fibroin substrates thus demonstrating that these substrates may be used as tissue constructs for the repair of the corneal endothelium.<sup>73</sup> A dual layered silk fibroin scaffold consisting of a thin epithelial cell substrate layered onto a 3D stromal network was developed to provide an intact stem cell niche for long-term corneolimbic tissue regeneration.<sup>74</sup> Human limbal epithelial cells and limbal mesenchymal stromal cells maintain a similar phenotype (corneal and stromal, respectively) on separate layers and on the dual layered scaffold thus demonstrating the feasibility of the 3D scaffold for corneolimbic tissue regeneration applications.<sup>74</sup> Silk fibroin membranes functionalized with chitosan were reported to be biocompatible and support the growth of keratocytes.<sup>75</sup> While the mechanical properties of the silk membranes improved considerably with the addition of chitosan (1.3 MPa), they were still weaker compared to native cornea (3.8 MPa). Further, the transparency of silk-chitosan membranes (62%) was also lower than native cornea (>85%). Recent studies that focus on the development patterned silk substrates for corneal regeneration are elucidated in the microengineering corneal tissue section.<sup>76,77</sup>

#### 3.2.4. Other materials

A full thickness biological substitute of the rabbit cornea was developed using a sequential culturing technique of the endothelial, stromal and epithelial cells on porous cell culture inserts.<sup>78</sup> The endothelial cultured at the bottom of the insert were layered with keratocytes entrapped in a human fibrin and 0.1% agarose gel and the epithelial cells were grown on the surface of the gel. Microscopic evaluation revealed that the corneal epithelial cells form a stratified epithelium, keratocytes proliferate rapidly and endothelial cells exhibit a pattern similar to the corneal endothelium. These fibrin-agarose matrices mimic the native cornea and hence may be used as an *in vitro* model to understand the various biological mechanisms underlying the function of the cornea.

Due to the lack of fibrous structure, mechanical properties of most hydrogels are suboptimal. Tonsomboon *et al.*, significantly improved the mechanical properties of alginate hydrogels by reinforcing the hydrogels with electrospun gelatin nanofibers (450 KPa from 78 KPa).<sup>79</sup> Carbodiimide crosslinking further improved the stiffness of gelatin nanofiber reinforced alginate hydrogels to ~800 KPa but at the expense of the transparency of the hydrogel. These mechanically robust nanofiber reinforced hydrogels are promising materials for corneal transplantation.

## 4. Stem Cells in Corneal Regeneration

Regenerative medicine through stem cell transplantation is one of the promising treatments in ocular diseases.

### 4.1. Stem cells in corneal regeneration

Stem cells have been used in regenerative medicine since it was demonstrated that a laboratory grown human epidermis could be transplanted onto burnt patients to reconstitute a functional epidermis.<sup>80,81</sup> There are many different sources of stem cells used for corneal regeneration. Mesenchymal stem cells from different sources and limbal stem cells from the eye itself are the two main stem cell types used for cellular therapy.

### 4.2. Limbal stem cells

Limbal stem cells are found in the limbal corner of the cornea. Due to their capacity for self-renewal and proliferation, their role is accepted as to maintain the integrity of corneal epithelium. Even if there is no specific marker for the limbal stem cells, they can be distinguished by high nucleus to cytoplasm ratio, proliferative potential and capacity for self-renewal.<sup>82</sup> Injuries and several diseases can cause depletion of these stem cells which is called limbal stem cell deficiency. Deficiency of limbal stem cells causes visual loss and severe discomfort. To treat these deficiency, limbal stem cells have been used for more than 10 years.<sup>83</sup>

*Ex vivo* expansion of human LSCs for the treatment of LSCD was first described in 1997. Pellegrini *et al.* used limbal stem cells to restore the surface of the cornea of the patients who had limbal stem cell deficiency syndrome. In their study, biopsies from the limbus part of the eye were taken. The cells were plated on to 3T3-J2 cells. The cells were then mounted on a graft and implanted into the eyes of the patients. It was found that the limbal stem cells and patients' visions were restored.<sup>83</sup> At first, the original approach for *ex vivo* expansion of limbal stem cells were to seed the cells obtained from limbus of the healthy eye on 3T3 fibroblast cells. In time, different strategies were developed such as growing the cells on human amniotic membrane. Amniotic membrane can be used either alone or with 3T3 fibroblast cells. In one of the first studies using human amniotic membrane, 13 patients with severe limbal deficiency were treated. Healthy limbal tissue samples were taken from the donors. Tissue cells were then cultured on amniotic membrane. After a certain confluence, amniotic membrane with cells was transplanted into the eye of the patient and another amniotic membrane was used to cover the cells. At end of the surgical experiments, 6 of the 13 patients recovered.<sup>84</sup>

Use of thermo-responsive cell culture plates for culturing limbal stem cells is another novel technique which was firstly used by Nishida *et al.* In this study, human and rabbit limbal stem cells were co-cultured with 3T3 fibroblast cells on temperature sensitive culture plates. Cells were harvested from temperature responsive dishes after 2 week just by reducing the temperature to 200°C without using any proteases or EDTA. In this technique, multilayered corneal epithelial sheet were obtained intact simply by adjusting the temperature. The cell sheets were then transplanted to the eye directly without using any carrier and also reconstruction of the cornea of the rabbits were found very high.<sup>85</sup> In another study, fibrin cultured limbal stem cells were used in patients with limbal stem cell deficiency. Transplanted fibrin-cultured limbal stem cells were successful in 14 of 18 patients.<sup>86</sup> In summary, although there is still a lot of way to go and the studies that have been done so far are still experimental studies, it has been shown that limbal stem cells have the potential for treating limbal stem cell deficiency and treating the corneal dysfunctions.

### 4.3. Mesenchymal stem cells

Mesenchymal stem cells are multipotent progenitor cells found in many tissues including bone marrow, adipose tissue, heart, dental pulp, peripheral blood, cord blood and limbal stroma of the eye.<sup>87-93</sup> These cells can differentiate to form lineages of mesenchymal tissue.<sup>94</sup> Recently, researchers have started to use mesenchymal stem cells for corneal reconstruction. Mesenchymal stem cells can be administered by either intravenous injection or local administration. For corneal reconstruction, local administration is used. Mesenchymal stem cells can be administered to cornea directly or by carriers.<sup>95-97</sup> In a recent study done by Liu *et al.*,  $10^4$  mesenchymal stem cells was directly transplanted to mouse cornea which resulted formation of keratinocyte like cell formation.<sup>95</sup> In another *in vivo* study, healing of chemically burned rat cornea was accelerated with subconjunctival injection of mesenchymal stem cells.<sup>98</sup> In another study, human umbilical cord derived mesenchymal stem cells were used to treat thin and cloudy corneas of lumican null mouse. Intrastromal injection of mesenchymal stem cells resulted more transparent and thick cornea in mice.<sup>99</sup> In another *in vivo* study, adipose derived mesenchymal stem cells were used to treat post-traumatic persistent sterile corneal epithelial defect. It was observed that healing process started in 10 days after topical application of mesenchymal stem cells and after 1 month, complete healing was observed.<sup>100</sup> In mesenchymal stem cell therapies, carriers and fibrin gels can be also used to deliver the cells other than the direct injection of mesenchymal stem cells. Ma *et al.* showed that chemically burned rat cornea reconstructed by using bone marrow derived mesenchymal stem cells grown on amnion membrane. In this study, mesenchymal stem cells were isolated

from human bone marrow. Cells then were seeded on amnion membrane. After seven days of injury, the damaged corneal surfaces were carefully keratectomized. An amnion membrane with cells was sutured to cornea and a blank amnion membrane put on to cover them. It was found that cornea was reconstructed in 4 week time.<sup>101</sup> In another study, rat corneal injury induced by alkali treatment was treated by rat bone marrow derived mesenchymal stem cells. Stem cells were co-cultured with corneal stromal cells and then they were seeded on amniotic membrane. After keratectomization of the cornea, grafts were transplanted. The transplanted cells reconstructed the chemically burnt rat cornea.<sup>96</sup> Fibrin gels with mesenchymal stem cells are also used in corneal reconstruction. In a study, cornea of rabbits damaged by NaOH and the damage was reconstructed by transplantation of rabbit bone marrow derived mesenchymal stem cells seeded in fibrin gel.<sup>97</sup>

## 5. Microengineering Corneal Tissue

Corneal tissue exhibits a highly organized compositional layer structure along with specific nanotopographical features at each layer. An intricate relationship exists between the complex topographical structures of the corneal tissue and biophysical characteristics of the comprised cell types. Since these topographical structures are at the micro-nano scale levels, it is vital to approach corneal tissue engineering at the same order of scale. Although current state of the art microengineering methods have grown to be essential tools in engineering of other tissues, their applications are limited in corneal tissue engineering. Studies that focused on developing microengineering based approaches for corneal applications (Fig. 1) are described in this section.

One of the earliest studies is conducted by Teixeira *et al.*,<sup>102</sup> where they investigated the morphological and biophysical behavior of human corneal epithelial cells cultured on substrates with nano grooves and ridges with various spatial configuration. Topographically patterned substrate dimensions were ranging from as small as 70 nm to 2.1  $\mu\text{m}$  with feature pitch between 400 nm to 4  $\mu\text{m}$ , whereas the groove depths used were 150 nm and 600 nm. Elongated and aligned epithelial cells were observed on substrates with ridge and groove features whereas substrates with smooth surfaces resulted in spherical cell morphologies. Furthermore, focal adhesion and microfilament formation was not observed regularly, but when it did, alignment of these structures were in accordance with the alignment of the cell body.

In a follow-up study, Karuri *et al.*<sup>103</sup> studied the effect of surface topography on adhesion strength of corneal epithelial cells by using shear flow in a parallel flow chamber. Adhesion strength of cells cultured on smooth surfaces, and nano, and microscale grooved surfaces, with varying pitch size between 400 nm to 4  $\mu\text{m}$ , were compared. Increase in adhesion strength was observed with decreasing feature dimensions, which indicates cells exhibit a greater adherence to substrate topographies at the nanoscale that is also the native biological scale.

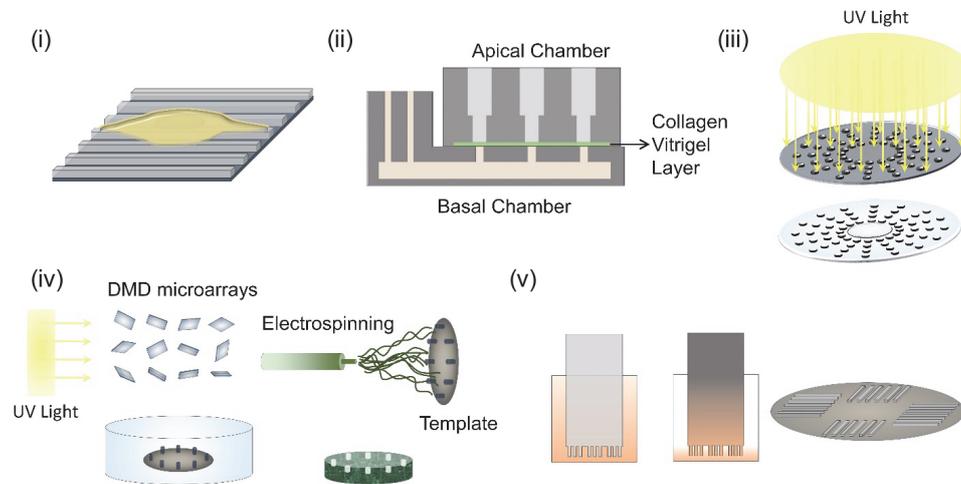


Fig. 1. Microengineering approaches in corneal tissue engineering. (i) Micro ridges/grooves for cell elongation and alignment.<sup>76,77,102,103,105,106</sup> (ii) Organ-on-a-chip devices for co-culture of different cell layers to mimic compositional structure of cornea.<sup>107</sup> (iii) Microfabrication of a 3D hydrogel construct for artificial cornea consisting of strong central optic part and resilient lateral part using photolithography.<sup>104</sup> (iv) Fabrication of biodegradable scaffold for limbus using stereolithography with digital microarrays and electrospinning.<sup>108</sup> (v) Microfabrication of limbal crypts from collagen hydrogel for artificial limbus via hydrophilic porous absorbers (HPAS) with microridge patterned topography.<sup>109</sup>

Myung *et al.*<sup>104</sup> designed and fabricated a 3D hydrogel construct for an artificial cornea, consisting of a mechanically strong and rich in water central optic part and a resilient and microperforated lateral skirt part by using photolithography. Poly(ethylene glycol)/poly(acrylic acid) (PEG/PAA) was utilized for the central optic part, whereas poly(hydroxyethyl acrylate) (PHEA) hydrogel was used for the peripheral skirt. Both parts were functionalized with 5-azidinitrobenzoyloxy N-hydroxysuccinimide to covalently bind the collagen type 1 for cell adhesion. Furthermore, adhesion of endothelial cells and fibroblast cells onto the central and peripheral part, respectively, was demonstrated to further support the cellular compatibility of the implant.

Crabb *et al.*<sup>105</sup> studied effects of collagen processing methods (isolation and crosslinking) and film topography on optical and biomechanical properties of collagen film-based stroma. To micropattern collagen films, a dispersion of soluble and insoluble collagen was cast onto holographic diffraction grating replicas with 1  $\mu\text{m}$  and 2  $\mu\text{m}$  groove spacing and 1  $\mu\text{m}$  depth. On microgrooved substrates flattened stellate cells were observed in a tightly packed form, on the other hand, cells on the smooth substrates were spindle shaped and in a formation presenting greater gaps between cells. After a week in culture, cells were aligned along the grooves

with 2  $\mu\text{m}$  spacing, whereas no alignment was noted for the grooves with 1  $\mu\text{m}$  spacing. There were no significant differences between smooth and microgroove substrates, but transparency decreased after cell culture for both substrates. Ultimate tensile strength was similar for both smooth and micropatterned substrates with or without cells at any point during the 3 week culture period.

In a similar study,<sup>76</sup> microgrooves were utilized for cell alignment on silk protein films. Silk films used in the study were 2  $\mu\text{m}$  thick as the corneal collagen lamellae and 0.5  $\mu\text{m}$  to 5  $\mu\text{m}$  pores were introduced into the film to facilitate trans-lamellar nutrient diffusion and cell–cell interactions. Furthermore, corneal fibroblast proliferation, alignment and corneal ECM expression were observed in 2D and 3D cultures using micropatterned silk protein films. In a follow-up study, Gil *et al.*<sup>77</sup> investigated the effect of micropatterned grooves with depth and spacing ranging from 37 to 342 nm and 445 to 3,580 nm, respectively. Greater alignment of human corneal fibroblasts was observed with deeper and narrower microgrooves. However, there was not any significant difference in DNA content between cells seeded on different substrates after 4 day in culture.

In an organ-on-a-chip study, Puleo *et al.*<sup>107</sup> fabricated a co-culture bilayer microfluidic device with collagen vitrigel between the layers as a barrier. The device consisted of two separate culture chambers that were bonded with vacuum with vitrified collagen between. First, epithelial cells were seeded into apical culture wells and after culture of an epithelium layer, collagen vitrigel, which structurally supported epithelium initially, was sacrificially degraded through injection of a collagenase etching media. Afterwards, keratocytes, as stromal layer, were seeded through the other layer onto the epithelium. Since vacuum provides a reversible sealing, micro corneal patches were able to be removed from the chip for further analysis or use. After viability analysis and culture of bilayered corneal micropatches, sacrificial etching was found to be biocompatible. Furthermore, results of laser-induced fluorescence epithelial permeability assays and histological examinations validated the development of bilayer corneal tissue micropatch.

Ortega *et al.*<sup>108</sup> combined stereolithography with electrospinning to produce a biodegradable scaffold that incorporates artificial stem cell niches imitating the palisades of Vogt in the limbus, for cell delivery application in corneal regeneration. A digital micromirror device was utilized in the stereolithography part, to fabricate a template for the scaffold from a photocurable polymer PEG diacrylate. Afterwards, a biodegradable membrane of poly(lactic-co-glycolic acid) 50:50 was deposited on this template to obtain the scaffold with specific microstructure features. To demonstrate the functionality of the scaffold for corneal regeneration, the authors cultured rabbit limbal fibroblasts and rabbit limbal epithelial cells on the electrospun scaffold.

In a similar study, Levis *et al.*<sup>109</sup> designed and fabricated bioengineered limbal crypts from collagen hydrogel to mimic the native limbal stem cell niche. Bioengineered limbal crypts were produced by incubating HPAs with ridged base topography on top of the collagen hydrogels with or without human limbal fibroblast cells. During 15 min incubation of HPA, water content of hydrogels was depleted by capillary wicking. After removal of HPA, topography of the thin collagen substrate resembles the native limbal crypts. Next, human limbal cells were seeded on the collagen construct and formed several layers. The results demonstrated that cells lining the limbal crypts showed expression of stem cell marker p63 $\alpha$ .

In a recent study, Kilic *et al.*<sup>106</sup> produced different combinations of stacked 3D collagen and/or collagen–elastin like recombinamer (Col:ELR) with or without ridge and groove patterns as a substitute for native corneal tissue. The effect of several parameters, such as ridge and groove patterns, scaffold material (collagen or Col:ELR), and various levels of dehydrothermal treatment for crosslinking, on cell alignment, proliferation, and transparency were investigated. The results showed that dehydrothermal treatment at 150°C produced the best results in film fabrication with respect to durability and transparency. Moreover, both collagen and Col:ELR showed sufficient support for cell adhesion and proliferation and cells seeded on micropatterned films showed elongation as soon as culture day 1. Also, transparency of micropatterned films with seeded keratocytes showed improvement over the course of the study. It was shown that while seeding keratocytes increased the transparency, seeding 3T3 fibroblasts decreased the transparency. Overall, after the 30 days of incubation period, transparency of the Col:ELR films were better than the collagen films and were close to the transparency of native cornea.

## 6. Conclusions and Future Directions

As summarized in this chapter, researchers over the past two decades have focused on the development of bioartificial corneas to be used as an alternative to donor corneas for the treatment of corneal disease. Efforts have mainly focused on hydrogel based approaches for the development of bioscaffolds mimicking the physiochemical properties of the native cornea. Although much work has been done, a fully tissue engineered cornea has not yet been realized. While many studies in the literature report novel fabrication methodologies and processing protocols to synthesize materials that mimic the physical (transparency, stability etc.), mechanical (strength and stiffness) and biological (cellular compatibility) properties of native cornea, very few studies have focused on mimicking the lamellae structure of the corneal stroma. Each lamellae of the corneal stroma is composed of highly aligned collagen fibrils and the fibrils of adjacent lamellae are arranged in an orthogonal manner. This

organized structure of the corneal stroma imparts a high level of transparency and robust biomechanical properties to the native cornea. Development of functional bioscaffolds that mimic the orthogonal lamellae structure of the native corneal stroma will help gain significant headway towards realizing a bioartificial cornea for corneal tissue engineering applications.

Further, limited work has been done in the direction of stem cell based approaches for the development of a fully tissue engineered cornea. Since the corneal tissue is multicellular, understanding cell differentiation pathways will be a key step in devising regenerative approaches using different cell sources such as corneal limbal stem cells, mesenchymal stem cells and induced pluripotent stem cells for corneal regeneration. In conclusion, employing a combinatory approach of developing functional bioscaffolds that mimic the native cornea and fostering stem cell based regenerative therapies will help expedite the development of bioartificial corneas that can be used in the clinic for corneal repair and regeneration.

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