the chapter is concluded with potential future directions that need to be pursued

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

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.[16]

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.[22] 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.[26] Chelating agents such as EDTA bind to metallic ions (divalent cations) and disrupt cell adhesion causing dislocation of cells.[22,26]

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.[27] 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.[16] 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

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°C.[34] 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.[35] 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.[112]

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.[5] 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.[36] 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.[36]

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.[26] 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

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 developement 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 collagenÐchondroitin sulfate substrate.[37] 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 eptihelial 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.[38,39] 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-coacrylicacid-coacryloxysuccinimide) (TERP).[40] 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[41] 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.[42,43] 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.[42] 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.[44]

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.[45] 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.[46] 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.[46] A recent assessment four

years post-implantation revealed that the RHC III collagen substitutes outperformed donor human corneas in many realms.[47] 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.[48] The transparency of the collagen sponge matrix was five times greater than conventional collagen gels but only 50Ð60% of the native cornea. Highly porous collagen-based foams were developed as biode(eat)ueded t

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 transpar-

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.[61] 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.[62] 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.[62] 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. *K*

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.[63] 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.[64] 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. *C*

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%.[65] 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.[66] The transparency of ultrathin chitosan films (~50 μm thick) crosslinked with diepoxy-polyethylene glycol (PEG) and cystamine was reported to be on par with human cornea.[67] 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.[67] Grolik *et al*, reported that the genipin crosslinked chitosan-collagen hydrogels were significantly stronger (46.93 MPa) than amniotic membrane (2.3 MPa).[68] 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. *F b*

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

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.[80,81] 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.[82] 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

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. LSCs were harvested from temperature responsive dishes after 2 week just by reducing the temperature to 20°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.[85] 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.[86] 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.

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

Myung *et al*[104] 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(acrylicacid) (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-azidonitrobenzoyloxy 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.*,[105] 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 μm and 2 μm groove spacing and 1 μm 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

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with 2 μm spacing, whereas no alignment was noted for the grooves with 1 μm 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,[76] microgrooves were utilized for cell alignment on silk protein films. Silk films used in the study were 2 μm thick as the corneal collagen lamellae and 0.5 μm to 5 μm pores were introduced into the film to facilitate translamellar nutrient diffusion and cell–cell interactions. Furthermore, corneal fibro-

In a similar study, Levis et al[109] 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

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

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