Biomaterials for corneal bioengineering

To cite this article: Zhi Chen *et al* 2018 *Biomed. Mater.* **13** 032002

View the article online for updates and enhancements.

IOP ebooks™

Bringing you innovative digital publishing with leading voices to create your essential collection of books in STEM research.

Start exploring the collection - download the first chapter of every title for free.

Biomedical Materials

TOPICAL REVIEW

Biomaterials for corneal bioengineering

Zhi Chen[1], Jingjing You[2,3], Xiao Liu[1], Simon Cooper[2], Christopher Hodge[2,4], Gerard Sutton[2,3,4,5,8], Jeremy M Crook[1,6,7,8] and Gordon G Wallace[1,8]

[1] ARC Centre of Excellence for Electromaterials Science, Intelligent Polymer Research Institute, AIIM Facility, Innovation Campus, University of Wollongong, Squires Way, Fairy Meadow, New South Wales 2519, Australia
[2] Lions New South Wales Eye Bank and New South Wales Bone Bank, New South Wales Organ and Tissue Donation Service, GPO Box 1614, Sydney, 2001, New South Wales, Australia
[3] Save Sight Institute, University of Sydney, Sydney, New South Wales, Australia
[4] Vision Eye Institute, Sydney, New South Wales, Australia
[5] Sydney Medical School, University of Sydney, Sydney, New South Wales, Australia
[6] Illawarra Health and Medical Research Institute, University of Wollongong, Wollongong, New South Wales 2522, Australia
[7] Department of Surgery, St Vincent's Hospital, The University of Melbourne, Fitzroy, Victoria 3065, Australia
[8] Authors to whom any correspondence should be addressed.

E-mail: jcrook@uow.edu.au, gwallace@uow.edu.au and gerard.sutton@visioneyeinstitute.com.au

Abstract

Corneal transplantation is an important surgical treatment for many common corneal diseases. However, a worldwide shortage of tissue from suitable corneal donors has meant that many people are not able to receive sight-restoring operations. In addition, rejection is a major cause of corneal transplant failure. Bioengineering corneal tissue has recently gained widespread attention. In order to facilitate corneal regeneration, a range of materials is currently being investigated. The ideal substrate requires sufficient tectonic durability, biocompatibility with cultured cellular elements, transparency, and perhaps biodegradability and clinical compliance. This review considers the anatomy and function of the native cornea as a precursor to evaluating a variety of biomaterials for corneal regeneration including key characteristics for optimal material form and function. The integration of appropriate cells with the most appropriate biomaterials is also discussed. Taken together, the information provided offers insight into the requirements for fabricating synthetic and semisynthetic corneas for *in vitro* modeling of tissue development and disease, pharmaceutical screening, and *in vivo* application for regenerative medicine.

1. Introduction

The cornea is a transparent, avascular and highly innervated tissue located at the anterior part of the eye. Its primary functions are to transmit and refract light entering the eye and to protect the eye from mechanical damage, UV light, and infection. Corneal transplantation is an important surgical treatment for injury and disease [1]. The World Health Organization (WHO) 2010 global survey estimated 39 million people with blindness. 12% or 4.9 million have bilateral corneal blindness [2]. From regional surveys, 23 million people are estimated to suffer from unilateral corneal blindness globally [3]. The causes of corneal blindness are diverse and include various corneal dystrophies, infectious and inflammatory corneal disorders, and corneal surface damage.

Corneal transplantation remains an important treatment for corneal diseases/conditions, with 72% of recipients showing visual improvement [4]. However, several unfavorable factors impede the utilization of corneal transplantation. In particular, although allogeneic tissue from human donors remains the best option for corneal graft replacement, this approach is hindered by the shortage of donor tissue and by transplant rejection [5].

A global survey on corneal transplantation showed there were a total of 184 576 corneal transplantations conducted in 116 countries and 284 000 corneas procured in 2012 [6]. It also reported a substantial lack of tissue with only one cornea available for every 70 patients worldwide [6]. The incidence of immunologic corneal graft rejection has decreased compared with solid organ transplantation by virtue of the cornea

being avascular, an immunosuppressive ocular micro-environment, and the phenomenon of anterior chamber-associated immune deviation. Nevertheless, a recent study during a five year follow-up shows 23% of subjects experienced at least one rejection event after penetrating keratoplasty (PK) [7]. Normally, corneal immunologic rejection can be reversed with topical steroids, but severe rejection can lead to fast and permanent loss of endothelial cells and transplant failure. If no clinical improvement occurs, the only option is to surgically remove and replace the damaged cornea with a further corneal transplant [5, 8]. What is more, corneal rejection can result in permanent blindness [9]. Therefore, allogeneic corneal transplantation has limitations and a bioengineered cornea could potentially address the key issues of tissue availability and tissue rejection.

The first full-thickness PK was performed on 7 December 1905 by Dr Eduard Zirm [9]. The first case of lamellar keratoplasty (LK; removal of anterior layers of the cornea) was successfully conducted prior to this, but early outcomes were poor [10]. With the development of microsurgical instruments, and improved surgical techniques in the past ten years, selective lamellar keratoplasty (SLK; removal of diseased layers of corneas only), as the successor to LK, has significantly improved keratoplasty [1]. Specifically, SLK consists of anterior lamellar keratoplasty (ALK) and endothelial keratoplasty (EK). ALK replaces the epithelium and stroma, while retaining the uninfected healthy stroma and endothelium. Alternatively, a deep anterior lamellar keratoplasty (DALK) is performed if almost all the stroma has to be removed. DALK has been successfully used in treating keratoconus patients [11]. In contrast, EK replaces only Descemet's membrane and the endothelium, and is mainly used for corneal disease caused by endothelial disorders or failures. Compared to PK, ALK and DALK are less likely to cause loss of the host endothelial cells and thereby avoid endothelial rejection. EK can theoretically provide better visual outcomes due to improved astigmatism [1]. Recently, the frequency of lamellar procedures has been steadily increasing [12]. Nonetheless, all the above-mentioned techniques use donor corneas and therefore graft tissue availability and rejection remain challenging.

Since the discovery of limbal stem cells (LSCs), autologous or allogenic limbal tissue transplantation have been considered for reconstructing the injured ocular surface [13]. For patients with unilateral limbal stem cell deficiency (LSCD), a limbal autograft from the healthy contralateral eye is the best option for avoiding tissue rejection [14]. By contrast, the treatment for severe bilateral ocular surface disease is largely dependent on procuring allogeneic tissue from the limbal region of corneal tissue donors [13]. Benefits aside, it remains difficult to estimate the appropriate amount of limbal tissue from a donor eye for autologous transplantation. Inadequate volume may lead to the failure of eye surface-reconstruction and excess extraction tends to impair the healthy donor eye. Amniotic membrane (AM) has commonly been used as a natural substrate for limbal epithelial cell culture and delivery, but the potential for infection and the shortage of source tissue necessitate better alternatives [15]. Similarly, for reversible blindness caused by corneal endothelial cell failure, there is an urgent need for cell carriers to replace the failed endothelium with *ex vivo* expanded endothelium cells [16].

In this review, we provide an overview of the anatomy and function of the native cornea as the primer to a systematic summary of various biomaterials and methods for corneal engineering, including key characteristics for optimal material form and function, and suitable cells for material interfacing.

2. Human cornea anatomy and function

The human cornea is the first tissue through which light enters the eye and accounts for two thirds of the refractive power of the eye, directing the light rays towards the pupil [17]. Anatomically, the cornea is wider along the horizontal meridian compared to vertically. Population studies indicate the average horizontal diameter of the cornea in adults is around 11.80 mm [18, 19], and the vertical diameter is around 10.63 mm [20]. The radius of the corneal curvature is steepest at the center and flattens towards the periphery, with a mean central radius of curvature being approximately 7.7 ± 0.30 mm [21]. Until recently, the cornea was understood to comprise five layers, including anterior to posterior: epithelium, Bowman's layer, stroma, Descemet's membrane and endothelium (figure 1). In 2013, Dua *et al* reported a sixth layer termed pre-Descemet's layer that lies between the stroma and Descemet's membrane [22], although there is still controversy around its existence. Among these layers, the epithelium, stroma and endothelium are cellular layers, whereas Bowman's layer, pre-Descemet's layer and Descemet's membrane are acellular layers (figure 4).

The cornea is one of the most innervated tissues of the body, with the majority of nerves being sensory and derived from the ophthalmic division of the trigeminal nerve (5th cranial nerve) [23]. The nerve bundles enter the cornea through the limbus where they form the limbal plexus, then extend to the peripheral corneal stroma. The stromal nerve bundles start to branch at the mid-peripheral area, passing through Bowman's layer forming the sub-basal nerve plexus that locates in the basal epithelial layers. Individual nerve fibers arising from the sub-basal plexus move anteriorly and terminate in the superficial epithelium as free nerve endings [23]. Corneal nerves are important for both protection and maintaining the integrity of the ocular surface, and corneal sensation and nerve
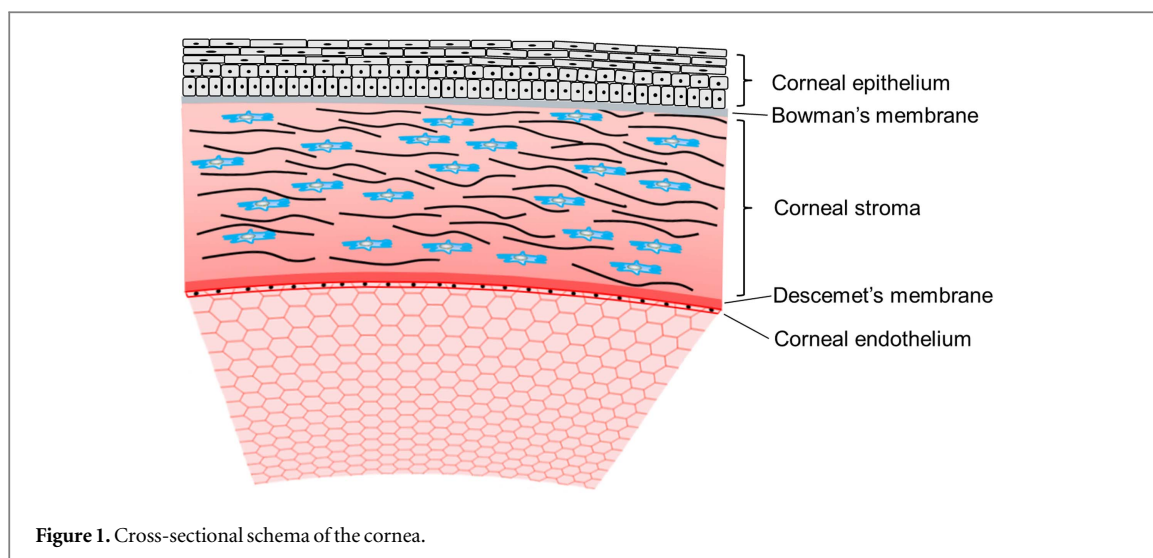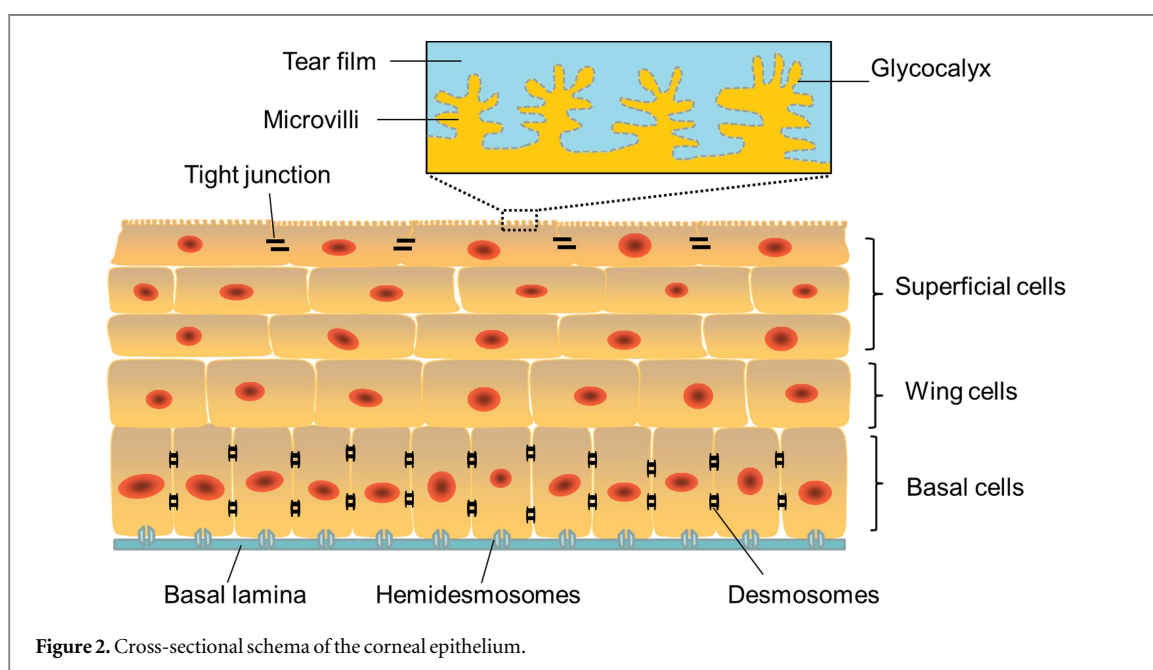
Figure 1. Cross-sectional schema of the cornea.

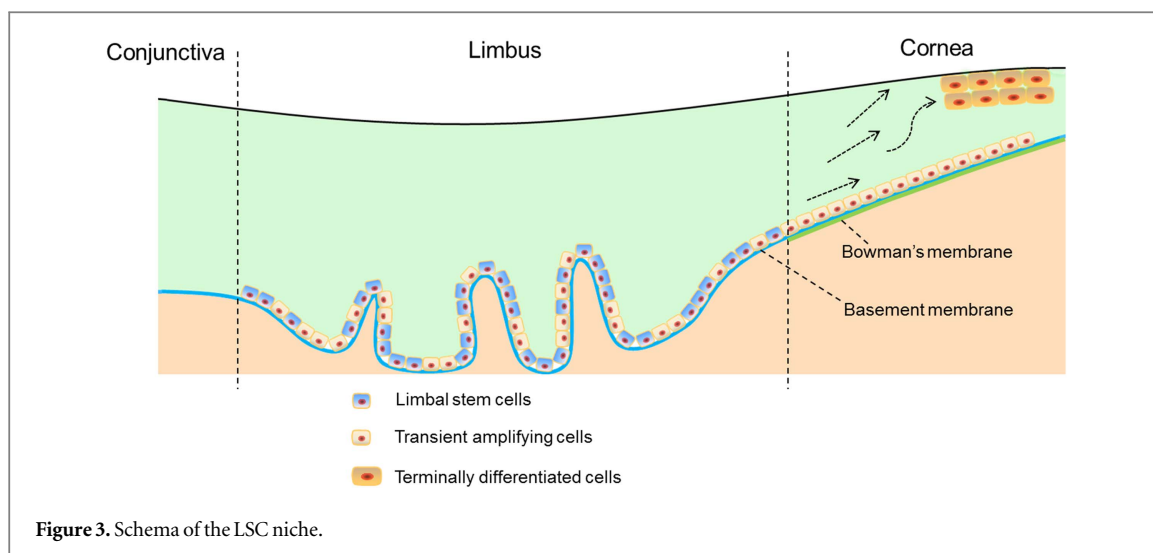**Figure 2.** Cross-sectional schema of the corneal epithelium.

density are important indicators for cornea recovery after surgery [23, 24].

2.1. Corneal epithelium

The corneal epithelium is the outermost layer of the cornea and the major refractive element of the eye. It is 40–50 μm thick and consists of 4–6 layers of stratified and nonkeratinized squamous epithelial cells [17] (figure 2). The most superficial corneal epithelial cells form 2 ∼ 3 flat layers with glycocalyx-covered microvilli on the top surface. The glycocalyx is indispensable for the stability of tear film, which in turn contributes to normal vision. These surface cells form tight junctions that prevent tears, toxins and microbes from entering the eye. Immediately below the superficial cells is the middle wing cell layer, and the inner basal columnar layer. The basal cell layer is approximately 20 mm thick and the source of wing and superficial cells and, apart from LSCs and transient amplifying cells, are the only corneal cells capable of mitosis [25]. Furthermore, the basal cells are firmly connected to each other by lateral gap junctions and zonulae adherens and strongly attached to underlying basal lamina by hemidesmosomes [25].

Corneal epithelial cells regenerate every 7–10 days through centripetal migration starting from the limbus, located on the corneal rim at the junction between the sclera and cornea (figure 3) [26]. The limbus contains corneal epithelial stem cells that are important for corneal epithelial homeostasis (figure 4(C)). The stem cells divide asymmetrically to produce transient amplifying cells, which migrate towards the central cornea to become basal central corneal epithelial cells. These cells then further differentiate and migrate towards the corneal surface to become the wing and superficial corneal epithelial cells (figure 3) [27]. In the normal resting state, p63 is totally absent in the corneal epithelium. When wound

Figure 3. Schema of the LSC niche.

healing occurs, the αisoform of ∇Np63 (N-terminally truncated transcripts generated by the p63 gene) was reported to be significant to identify the stem cells that reside in the basal layer of the limbus. ∇Np63α, is an indicator for the regenerative proliferation of the corneal epithelium and can also be detected in the central cornea due to the migration of LSCs once activated [28]. The main function of the corneal epithelium is to form a barrier to prevent toxins and microbes from entering the epithelium, integrate with tears to provide a smooth layer for light refraction, and transfer water and soluble components into or out of the stroma [29]. The most posterior layer of the epithelium is a basement membrane (BM), where the basal epithelial cells attach. The BM is about 40–60 nm thick and consists of type IV collagen, laminin, perlacan (a heparin sulfate proteoglycan), and nidogen (a sulfated glycoprotein) [30]. Together, the BM is essential for the adhesion and polarity of the epithelial cells, and to modulate cellular signaling and trafficking between the epithelium and stroma [30].

2.2. Bowman's layer
Between the epithelium and stroma layers lies the Bowman's layer (figure 1), which has a smooth anterior surface abutting the epithelial BM, with its posterior side merging with the stroma [31]. The Bowman's layer is acellular and has numerous pores allowing the passage of nerve bundles [31]. It consists mainly of collagen types I and III, as well as types V and VI [32–34], which form collagen fibrils with a diameter of 20–25 μm. The individual collagen fibrils interconnect to generate a sheet about 8–12 μm [35]. The thickness of the Bowman's layer decreases with age [35] and it does not regenerate following trauma or removal [31]. It is hypothesized that the Bowman's layer is formed early in the development of the cornea via the interaction between the corneal epithelial cells and keratocytes in the stroma [36]. Its absence does not appear to prevent re-epithelialization following

surgery or trauma [36]. However, the Bowman's layer has been reported to assist with stromal wound healing and restoration of the subepithelial nerve plexus [31].

2.3. Stroma
The corneal stroma accounts for roughly 80%–85% of the entire corneal thickness, and affords many biochemical properties of the cornea including tensile strength, stability and transparency [30]. The collagen fibrils in the stroma are bigger than in the Bowman's layer with diameters ranging from 25–35 nm [35]. Collagen type I is predominant in the stroma. Together with type III and V collagen they form collagen fibrils. Type VI collagen occurs as fine filaments in the inter-fibrillary matrix of the stroma [34]. The collagen fibrils form triple-helix bundles with diameters of 250–340 nm, which align to form a flat lamellar sheet [35]. The lamella sheets are enveloped by proteoglycans decorated with keratin sulfate or chondroitin sulfate, and preserve corneal hydration and transparency [37]. The stroma consists of 250 ∼ 300 collagen lamellae with a width of 10–320 μm and a thickness of 0.2–2.5 μm [38]. The arrangement of the lamellae in the stroma is heterogeneous, with the collagen fibers being interwoven in the anterior stroma and parallel in the mid to posterior stroma [38]. In addition, the intervals between lamellae are filled with keratocytes, which occupy more than 20% of the stroma. These cells are responsible for the stability of lamellar organization and regulation of the corneal stroma extracellular matrix (ECM) components via the synthesis of collagen and proteoglycan [39]. The framework of the corneal stroma is important for the transparency and mechanical property of the cornea.

Once the normal integrity or the interconnection with the Bowman's layer and Descemet's membrane is interrupted, the stromal wound-healing process is activated. Due to the distinct avascular nature of the cornea, a critical difference between stromal healing
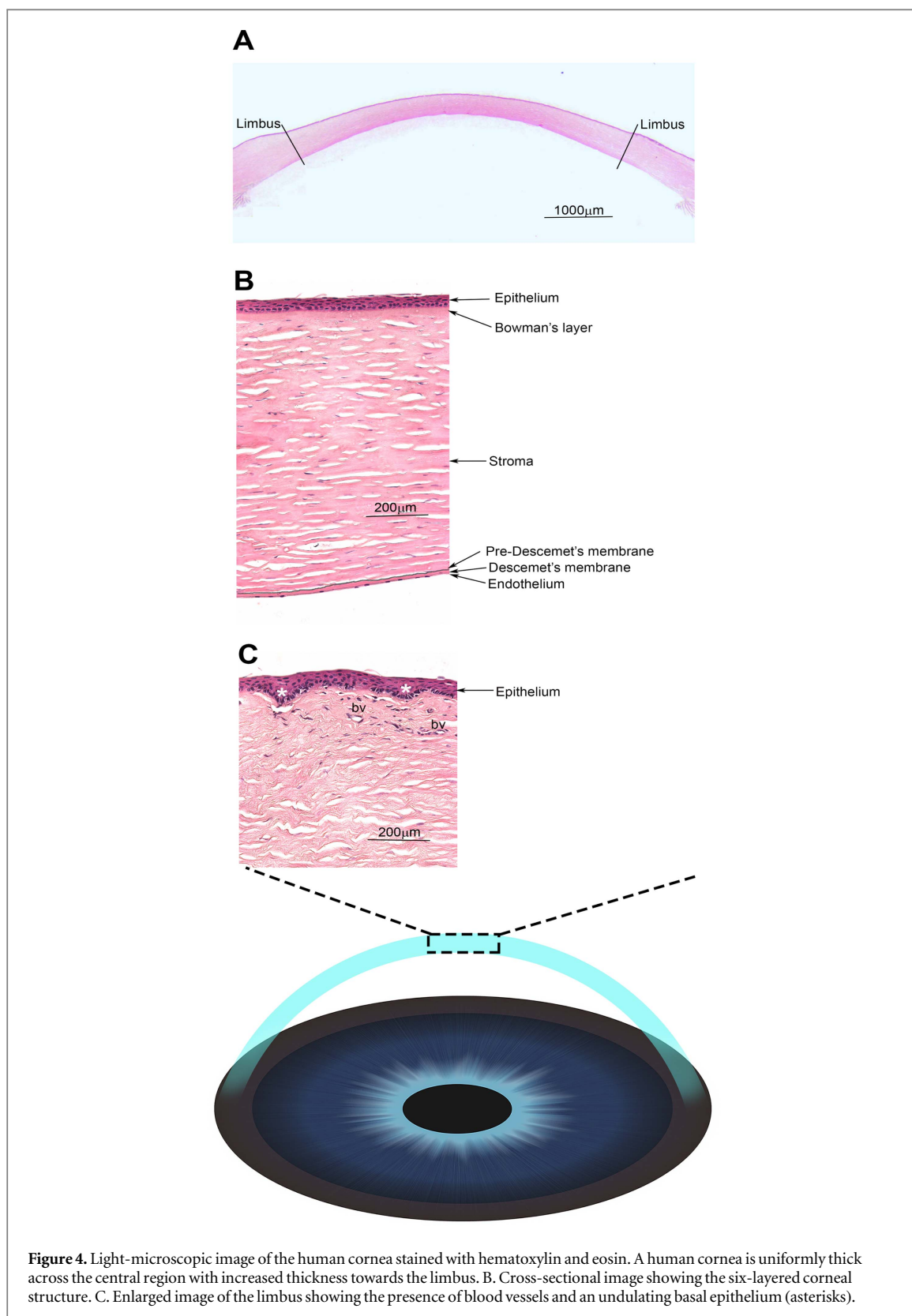
Figure 4. Light-microscopic image of the human cornea stained with hematoxylin and eosin. A human cornea is uniformly thick across the central region with increased thickness towards the limbus. B. Cross-sectional image showing the six-layered corneal structure. C. Enlarged image of the limbus showing the presence of blood vessels and an undulating basal epithelium (asterisks).

and that of other body tissues such as the skin is the absence of the vascular component [40].

2.4. Pre-Descemet's layer and Descemet's membrane

The pre-Descemet's layer is posterior to the stroma and anterior to the Descemet's membrane. The recent discovery of the pre-Descemet's layer remains controversial with many studies suggesting the layer to be part of the stroma [22, 41]. However, emerging evidence of the structure, constitution and clinical importance support its existence as a distinct layer. It is made up predominately of type I collagen but includes type VI collagen, which assembles into 5–8 thin

lamellae with an overall thickness of 10–15 μm [22]. A recent study suggests that it consists of high levels of elastic fibers that originate within the posterior limbus in the trabecular meshwork [42]. This elastic fiber network in the pre-Descemet's layer could be important for anchoring the cornea as well as the trabecular meshwork to the limbus and peripheral cornea [42]. The clinical importance of the pre-Descemet's layer is supported by the big bubble technique being unsuccessful if the layer is disrupted [43]. The predominant collagen in the Descemet's membrane is type IV, although it also includes collagen type VIII, XII, laminin, perlecan, nidogens, vitronection and fibronectin [44]. In contrast to the Bowman's layer, its thickness gradually increases from birth to adulthood, expanding to between 5–10 μm [45]. It is secreted by the endothelial cells to form the BM for the endothelial cells to anchor [45].

2.5. Endothelium

The human corneal endothelium (HCEN) is composed of a single layer of hexagonal-shaped cells with a thickness of around 5 μm. Similarly, the adjacent endothelial cells are characterized by a lateral gap and tight junctions and are vertically attached to the Descemet's membrane by numerous hemidesmosomes [46]. Once the endothelium layer is formed, the endothelial cells become mitotically inactive. The HCEN initially comprises approximately 4500 cells mm^{-2}, with the total endothelial cell number decreasing with age, while the cell surface area increases [47]. The main indicator for an eye bank to select corneas after storage for PK, is the endothelial cell counts [48]. Generally, donor corneas considered for transplantation need to have an endothelial cell density of $\geqslant$2000 cells mm^{-2}.

While acting as a barrier to restrain fluid into the cornea, the endothelial cells prevent excessive hydration (78% water content) of the stroma, maintaining stromal deturgescence by a passive pump-leak process that moves ions and water from the hypotonic stroma to the hypertonic aqueous humor [49]. The whole process has been shown to be associated with Na+, K+− adenosine triphosphatase (ATPase) pumps that reside in the basolateral membrane [50].

Thus, the endothelium is critical to maintaining the transparency and nutrition of the cornea via this 'pump-leak' mechanism. It is rich in ion and water channels for the transfer of nutrients from the aqueous humor to the cornea [51] and its function to nourish the cornea and inability to regenerate, renders it key to determining by assessment the suitability of the cornea for transplantation.

Due to their mitotically inactive nature, once the number of endothelial cells decreases, the surrounding cells gradually infiltrate the vacant region to maintain tissue integrity. The deformation of the

endothelium, however, tends to impair the pump function [52].

3. Biomaterials for corneal engineering

Biomaterials employed for engineering corneal substitutes must replicate the structural and functional requirements of the native cornea. Briefly, as scaffolds for cell support, materials must have suitable mechanical toughness, biocompatibility, transparency, appropriate biodegradability, and be clinically compliant. Commonly used biomaterials broadly include natural and synthetic polymers. While natural polymers tend to have excellent biocompatibility, synthetic polymers enable customization of the desired properties. The characteristics of extant materials determine their application (tables 1 and 2).

3.1. Collagen

Collagen is the most abundant component of ECM in most tissues [53, 54], with tripeptide arginine-glycine-aspartic acid (RGD) recognized by the cell surface integrin receptors, being important for cell adhesion, migration and proliferation [55]. The widespread application of collagen (especially collagen type I) in tissue engineering is partly due to its ease of production. Collagen type I is a key component to the tendon, ligament and dermis and is relatively cheap to derive. Collagen is therefore vital to fabricating biomimetic corneal stroma equivalents as the main component of human stromal tissue [56–58], and is compatible with all corneal cells and nerve regrowth *in vivo* [55, 59–62]. The primary drawback of collagen, however, is insufficient mechanical toughness and elasticity [63, 64]. Therefore, crosslinking collagen has been widely applied to considerably improve its mechanical strength and chemical stability without compromising the biological advantages. Importantly, the source of collagen impacts on the physical properties of the final product. For instance, the addition of soluble tropocollagen improves transparency and strength [65], and while both type I and type III collagen hydrogels have adequate tensile strength and elasticity for handling, type III collagen hydrogels tend to be mechanically and optically superior [66, 67].

The methods which have been investigated for collagen crosslinking for bioengineering mainly include chemical, physical and enzymatic approaches. Aldehydes such as formaldehyde and glutaraldehyde, the carbodiimide family (EDC), isocyanate chemical family (hexamethylene diisocyanate) and genipin are commonly used for the chemical crosslinking of collagen [68]. Physical crosslinking involves ultraviolet (UV) or dehydrothermal treatment and enzymatic crosslinking with transglutaminase can enhance the mechanical toughness of collagen without generating toxic by-products [68]. However, not all of these methods are commonly employed in corneal

Table 1. Biomaterials used for corneal bioengineering.

Material	N/S[l]	Processing method	Pros	Cons	Applications	Biocompatibility	Transparency	Mechanical toughness	Biodegradability	Permeability	Clinical status
Collagen	N	Crosslinking; Collagen-vitrigel; Compression	High biocompatibility from intrinsic RGD[m]	Inferior mechanical properties	Corneal stromal equivalents; Substrate for corneal epithelial and endothelial bioengineering	++++	+++	+	++++	+++	Clinical follow-up 48 months
Silk	N	Evaporation; Electrospinning	Inherent optical clarity; controllable degradation rates and mechanical properties	Surface modification or combination required	Corneal stromal equivalents; substrate for corneal epithelial cells; corneal fibroblasts and corneal endothelial cells	++	++++	+++	+++	+++	Animal model
Gelatin	N	Evaporation; Crosslinking	Natural biocompatibility; suitable biodegradability	Inferior mechanical properties	Substrate for corneal endothelial cell transplantation and corneal stromal cell growth	+++	+++	+	++++	+++	Animal model
Chitosan	N	Evaporation; Composition and crosslinking	Easily-biofunctionalized; favorable biocompatibility; controllable biodegradability	Composition of other materials (collagen / gelatin) required	Substrate for corneal epithelial regeneration and delivery and for endothelial cell growth	++	+++	++	++++	++++	Animal model
DC[a]	N	Decellularization	Similar mechanical and optical properties to natural cornea; low immunogenicity	Insufficient keratocyte infiltration and bioactivity	Substrate for corneal endothelial regeneration and epithelial and anterior stromal reconstruction	++	++++	+++	+++	None	Animal model
SS[b]	N	Coating; evaporation	High biocompatibility	Inferior mechanical property	Substrate for human corneal limbal epithelial cell	+++	None	++	None	None	*In vitro*
Fibrin/Agarose	N	Polymerization	Excellent optical properties	Complicated process	Keratocytes encapsulated corneal stroma equivalent	+++	++++	None	None	None	*In vitro*
PVA[c]	S	Crosslinking; Electrospinning	Sufficient mechanical properties	Surface modification required; unsatisfied light transmittance	Substrate for corneal epithelial and stromal regeneration and delivery	+	+	None	++	None	*In vitro*
PHEMA[d]	S	Crosslinking	Favorable transparency	Surface modification required	Substrate for limbal epithelial cells	+	++++	++++	+	None	*In vitro*
PEGDA[e]	S	Crosslinking	Controlled biochemical and topographical cues; low relaxed swelling ratios	Surface modification required	Substrate for corneal epithelial cells; epithelial wound healing	None	None	None	None	None	*In vitro*

Table 1. (Continued.)

Material	N/S[l]	Processing method	Pros	Cons	Applications	Biocompatibility	Transparency	Mechanical toughness	Biodegradability	Permeability	Clinical status
PLGA[f]	S	Evaporation	High transparency	Surface modification required	Substrate for corneal endothelial regeneration	+	++++	None	+++	None	*In vitro*
PEG[g]/PAA[h] hydrogel	S	Polymerization and crosslinking	High transparency and glucose permeability	Complicated process	Corneal stroma and epithelial reconstruction	++	++++	++	++	++++	Animal model
PGS[i]	S	Polycondensation	Favorable biodegradability	Complicated process	Substrate for human corneal epithelial cells	++	None	None	None	+++	None
PEUU[j]	S	Electrospinning	Excellent biodegradability	Complicated process	Substrate for corneal stromal cell growth and regeneration	++	None	None	++++	None	*In vitro*
Thermoresponsive materials	S	Graft; coating	Generate carrier-free cell sheet without enzymatic digestion	Generated cell layers are too fragile for surgical manipulation	Substrate for corneal endothelial sheets and epithelial sheets	++	None	None	None	None	Animal model (Cell sheets)
PA[k]	S	Self-assembly (in solution and coating)	Versatile and easily designed biocompatibility and structure	Complicated process	Design 2D and 3D structures for corneal cells and improve cell performance	++++	None	None	+++	None	Animal model

[a] Decellurized cornea.
[b] Silk sericin.
[c] Poly(vinyl alcohol).
[d] Poly(2-hydroxyethyl methacrylate).
[e] Polyethylene (glycol) diacrylate.
[f] Poly(lactic-co-glycolic acid).
[g] Poly(ethylene glycol).
[h] Poly(acrylic acid).
[i] Poly(glycerol sebacate).
[j] Poly(ester urethane) urea.
[k] Peptide amphiphiles.
[l] Naturally/synthetically derived.
[m] Arginine-glycine-aspartic acid.
'++++' stands for the highest level, while '+' is the lowest.

Table 2. Biomaterials and cell sources used for corneal bioengineering for each cellular layer.

	Materials	Cell sources
Epithelium	Silk fibroin: Silk fibroin membrane [93, 95, 100] Porous silk fibroin membrane [104, 190] Patterned silk fibroin membrane [152] Silk sericin [107]	Primary human corneal limbal epithelial cells [56, 70, 72, 79, 87, 89, 90, 93, 95, 100, 104, 107, 121, 129, 141, 152, 160]
	Collagen: Type I collagen gel [55, 59, 83, 89] FNC[a] [54] CV[b] [56, 86] Collagen/silk gel [82]	Primary human corneal epithelial cells [78, 80, 89, 142, 143, 151, 154, 190] Immortalized human corneal epithelial cells [54, 59, 60, 67, 71, 82, 83, 129, 148, 150]
	Synthetic hydrogel PHEMA[c] [141] PEGDA[d] [142, 143, 159] Decellurized cornea [121, 125]	Immortalized human corneal limbal epithelial cells [153]
	Chitosan [128] Hydroxyethyl chitosan [133] Thermoresponsive polymer PNIPAAm[e] [134] Electrospun scaffold: PLGA[f] [163] PHBV[g]/gelatin [111] Chitosan/gollagen [129] PCL[h]/PVA[i] [166] Gelatin/PLLA[j] [165]	Primary rabbit corneal limbal epithelial cells [55, 117, 163, 166, 194] Primary rabbit cornea epithelial cells [133, 140, 165] Primary bovine corneal epithelial cells [128] Primary porcine corneal limbal epithelial cells [152]
Stroma	Silk fibroin: Porous silk fibroin membrane [105, 190] Patterned silk fibroin membrane [96, 97, 101, 158] RGD[k] functionalized silk film [97, 105] Collagen: Type I collagen [65, 89, 92] Insoluble type I collagen [65] CV [56] PEUU[l] [164, 180, 181]	Primary human corneal stromal stem cells [164, 170, 181] Primary human corneal fibroblasts [58, 62, 89, 121, 152, 173–175, 181] Primary human corneal keratocytes [59, 79, 90–92, 155, 158] Immortalized human corneal keratocytes [71, 96, 97, 101, 176]
	Gelatin hydrogel [111, 113] DC[m] [121, 122] PLDLA[n] [155] Electrospun scaffold: PHB[o]/PHBV/PCL [162] PEUU [164] Gelatin/PLLA [165]	Primary rabbit corneal stromal cells [106, 113, 117, 122, 140, 162, 165, 194] Primary rabbit corneal fibroblasts precursors [111] Primary rabbit corneal stromal fibroblasts [111] Other animal corneal stromal cells: goat [157]; bovine [148]
Endothelium	Gelatin hydrogel [110, 112, 114] Gelma [116] Thermoresponsive polymer: PNIPAAm [136] Poly (NiPAAm-co-DEGMA)[p] [138] PNIPAAm hybrids [157] Poly(NGMA)[q] [137] PVME[r] [139] Chitosan [131] HECTS[s] [132] PCL-chitosan [130] Silk fibroin [98] Aloe vera gel/SF [102] DC [123] Compressed collagen [64]	Primary human corneal endothelial cells [64, 90, 98, 110, 114, 116, 123, 136, 170] Immobilized human corneal endothelial cells [71, 79, 98, 138, 139] Primary rabbit corneal endothelial cells [56, 102, 112, 117, 131, 132, 137, 144, 194] Primary bovine corneal endothelial cells [130, 156]

Table 2. (Continued.)

	Materials	Cell sources
Full-thickness	Silk fibroin [38] Fibrin [192, 193] Collagen gel: Type I collagen [60] Type I and type III collagen [66] Collagen I and copolymer [74–77] [79] CV [57] DC [117, 118, 126, 127] PEG^t/PAA^u hydrogel [146, 147] PHEMA/PAA hydrogel [145]	Dorsal root ganglion (DRG) cells: from chick embryos [66, 76, 190] Human embryonic stem cells (hESCs) [127]

[a] Fibronectin-collagen.
[b] Collagen-vitrigel.
[c] Poly(2-hydroxyethyl methacrylate).
[d] Poly(ethylene glycol) diacrylate.
[e] Poly(N-isopropylacrylamide).
[f] Poly(lactide-co-glycolide).
[g] Poly(3-hydroxybutyrate-co-3-hydroxyvalerate).
[h] Poly(ε-caprolactone).
[i] Polyvinyl alcohol.
[j] Poly-L-lactic acid.
[k] Arg-Gly-Asp.
[l] Poly(ester urethane) urea.
[m] Decellurized cornea.
[n] Poly(L,D lactic acid).
[o] Polyhydroxybutyrate.
[p] Poly(N-isopropylacrylamide-co-diethyleneglycol methacrylate.
[q] Poly(N-isopropylacrylamide-co-glycidylmethacrylate).
[r] Poly(vinyl methyl ether).
[s] Hydroxyethyl chitosan.
[t] Polyethylene glycol.
[u] Poly(acrylic acid).

bioengineering, with some approaches generating toxic residues in the collagen-based biomaterial. Unlike glutaraldehyde, hexamethylene diisocyanate and acyl azide, which act as chemical 'bridges' between collagen molecules, with EDC, collagen molecules are linked directly. Genipin is a crosslinker with low toxicity [69]. A study of various crosslinking methods demonstrated the inclusion of generation 2 polypropyleneimine octamine dendrimers largely amplifies the quantity of amine groups in collagens available for EDC crosslinking. The membrane produced had superior optical and mechanical properties compared with dehydrothermally and glutaraldehyde crosslinked collagen gels [59]. Another study of collagen suggested that while UV light induced riboflavin, crosslinking or the use of genipin, or EDC crosslinking produced adequate tensile strength, superior strength was found using the EDC crosslinking group [70]. Another crosslinking method involving UV-induced glucose, results in collagen membranes with moduli and ultimate tensile strength comparable to that of the native human cornea [65].

Normally, crosslinked collagen gel can be produced in a membrane form that is suitable as a growth substrate for corneal cells [60] and biomimetic corneal equivalents [56, 57], or 3D structures with stromal cells encapsulated or scaffolds seeded with epithelial and endothelial cells [71, 72]. The latter provides for the development of full-thickness corneal tissue that is analogous to a native structure, but has relatively poor mechanical properties due to incomplete crosslinking and cell protection [71, 72]. The cytocompatibility of crosslinked collagen gels has been demonstrated in numerous studies. For example, dehydrothermally crosslinked, EDC and glutaraldehyde crosslinked gels are able to support the growth of human and rabbit corneal epithelial cells (rCECs), HCFs, and HCEN cells [55, 58, 60, 62, 71, 73]. In addition to pure collagen hydrogel, collagen-polymer composites have been reported to further enhance the mechanical strength and resistance to enzymatic degradation [74, 75]. For example, porcine type I collagen and 2-methacryloyloxyethyl phosphorylcholine (MPC) interpenetrating network hydrogels display improved mechanical strength, and stability, with comparable optical properties, and glucose as well as albumin permeability with those of the native human cornea [76, 77] enabling the regeneration of corneal cells and functional nerves in pig models. A combination of collagen with a copolymer from N-isopropylacrylamide, acrylic acid and acryloxysuccinimide resulted in a transparent, permeable and robust collagen gel that prevented excessive collagen fibrillogenesis and was well tolerated in dogs [74]. A composite collagen-

chitosan membrane demonstrated better mechanical toughness and optical properties and when transplanted into a pig cornea, allowed the regeneration of the epithelium, stroma and nerve in 12 months [63, 78]. The incorporation of chondroitin sulfate into collagen gels tends to lead to higher mechanical properties and moisture retention [73, 79]. Crosslinking tobramycin into collagen, a novel antibacterial collagen membrane with excellent antibacterial effect can be obtained [80]. The incorporation of cyclodextrin in collagen membrane is capable of improving the transparency as a result of reduced collagen fibrogenesis [81]. Enhanced mechanical properties can also be achieved by the integration of silk fibroin [82]. Duan *et al* reported that the incorporation of YIGSR (a model cell adhesion peptide) modified dendrimer into collagen gel promoted the adhesion and proliferation of human corneal epithelial cells (HCECs) as well as neurite extension from dorsal root ganglia [83]. While most collagen structures reported are flat, Zhang *et al* recently produced a natural corneal-shaped collagen membrane by placing dethermally crosslinked collagen in a rigid contact lens mold and then incubated it at 37 °C for complete dehydration [70]. By loading a compressive force on the dehydrothermally crosslinked collagen gel, plastic compression (PC) collagen with improved mechanical toughness has been explored as a carrier to expand the HCEN cell for transplantation [84]. The PC collagen films, however, are not easily manipulated. To solve this issue, electrospun PLGA films were then used to form a sandwich-like structure for transplantation. The light transmittance of the structure, however, is insufficient, being less than 80% under a 500 nm wavelength [85]. Other composites include collagen-vitrigel (CV), a collagen-based product with enhanced gel strength by vitrification [86]. Briefly, the mixture of collagen, FBS, N-2-hydroxyethylpiperadine-N0-2-ethansulfonic acid buffer and Dulbecco's modified eagle medium was incubated at 37 °C and 5% CO_2 for 2 h gelation. Under controlled humidity, the gel was vitrified into a glass-like material. Finally, the CVs were rehydrated to obtain a regenerated and stable CV membrane. During the whole process, the CV can be tailored for transparency and mechanical strength by optimizing the synthesis conditions [87]. Moreover, the CV has been shown to facilitate epithelial layer generation *in vitro* [56], and exhibits sustained tissue transparency and low inflammatory response [86].

To date, collagen is the most extensively used material in corneal bioengineering (table 3). Overall, collagen scaffolds, either pure or hybrid, provide excellent support of cells derived from different corneal tissue layers. The main challenge for tissue engineers using collagen for corneal bioengineering is the fabrication of collagen constructs that mimic the well-organized structure and cell-encapsulation ability of the corneal stroma [88]. Griffith *et al* created a multi-layered corneal matrix by seeding epithelial cells and

endothelial cells on the top and bottom of immobilized human corneal keratocyte-encapsulated glutaraldehyde crosslinked collagen-chondroitin sulfate substrate [71]. Similarly, German *et al* reconstructed a cornea by seeding primary HCECs on primary human fibroblasts encapsulated dehydrothermally into a crosslinked collagen gel. After 3 days in culture, 4–5 layers of the regenerated corneal epithelium and components of BM were detected [89]. Keratocytes have been incorporated into porous collagen sponges by crosslinking and lyophilisation [90]. The keratocytes migrated into the pores and evidence of matrix secretion and elongated cell morphology was observed. Controlling the organization of collagen fibrils to mimic the corneal stroma has long been of interest when utilizing collagen for corneal bioengineering. Builles *et al* reported that scaffolds of orthogonal lamellae composed of aligned collagen fibrils can be formed by the gelation of collagen in a horizontal magnetic field, mirroring the inner structure of the human corneal stroma (HCS) [91, 92]. Interestingly, keratocyte alignment is directed by the collagen orientation both on the surface and within the bulk of the scaffold [92]. Although the scaffold possesses similar structure to human cornea stroma, it is not transparent probably due to the relatively large diameter of collagen fibrils. Proteoglycans were thus incorporated into the scaffold to improve the transparency. Although the as-prepared scaffolds demonstrated great potential for the recovery of the anterior cornea in the rabbit model, the graft retention, however, still needed to be improved through the optimization of crosslinking [92].

3.2. Silk

Silk fibroin (SF) is a structural protein derived from the cocoon of the silkworm *bombyx mori* and has been widely used in tissue engineering and regenerative medicine, because of its non-immunogenic response, controllable degradation rates and mechanical properties. Normally, pure silk solution, the precursor of the diverse SF framework, is obtained by mixing SF with lithium bromide solution before dialysis. In the context of corneal engineering, the inherent optical clarity of SF undoubtedly makes it a promising candidate. SF membranes have largely been investigated as substrates for corneal epithelial cells. Pure and permeable SF membrane generated by simple evaporation [93] or electrospinning [94] is able to support the formation of a confluent multilayered epithelium and the growth of human corneal limbal epithelial (HCLE) cells similar to the AM, which is the current standard substrate used for corneal epithelial cell transplantation. Although pure SF membrane provides for similar cell attachment of HCLE cells to tissue culture plastics, it is inferior to AM due to the lack of natural ECM proteins [95]. Nevertheless, the RGD sequence present in collagen can be coupled on the

Table 3. Collagen used for corneal bioengineering.

Collagen Type	Processing method	References	Year	*In vitro* study	*In vivo* study
Bovine type I collagen	Dehydrothermal crosslinking	[89]	1999	Primary human corneal epithelial cells (limbus and center) and human corneal fibroblasts	None
Bovine type I collagen	Dehydrothermal crosslinking	[58]	2004	Primary human corneal stromal fibroblasts	None
Bovine collagen type I	Dehydrothermal crosslinking	[62]	2006	Primary human corneal stromal fibroblasts	None
Bovine type I collagen and type III collagen	Glutaraldehyde crosslinking; EDC[a]/NHS[ii] crosslinking	[59]	2006	Immortalized human corneal epithelial cells	None
Porcine type I collagen	EDC/NHS crosslinking	[61]	2006	None	Rabbits and mini-pigs by lamellar keratoplasty
Bovine collagen type I	EDC/NHS crosslinking	[83]	2007	Immortalized human corneal epithelial cells	None
Recombinant human collagens types I and III	EDC/NHS crosslinking	[66]	2008	Immortalized human corneal epithelial cells	Mini-pigs by deep lamellar keratoplasty
Recombinant human collagens types I and III	EDC/NHS crosslinking	[67]	2008	Immortalized human corneal epithelial cells	Rats by selective lamellar keratoplasty
Rat tail type I collagen	EDC/NHS crosslinking	[79]	2008	Primary cornea limbal epithelial cells; primary human kerato-cytes; immortalized human endothelial cells;	None
Type I porcine atelocollagen; recombinant human type III collagen	EDC/NHS crosslinking	[76]	2009	Immortalized human corneal epithelial cell line and dorsal root ganglia	Rabbits by deep lamellar keratoplasty
Type I collagen solution	Collagen-vitrigel	[56]	2009	Primary human limbal epithelial cells; primary bovine kerato-cytes; rabbit corneal endothelial cells	None
Recombinant human collagen, type III	EDC/NHS crosslinking	[177]	2010	None	24 month follow-up of a phase 1 clinical study
Type I porcine atelocollagen	EDC/NHS crosslinking	[77]	2010	Immortalized human epithelial cell line and dorsal root ganglia	Pigs by penetrating keratoplasty
Rat tail type I collagen	Dehydrothermal crosslinking	[87]	2012	Primary human limbal epithelial stem cells and stromal keratocytes	Rabbits by selective lamellar keratoplasty
Rat tail type I collagen solution	Dehydrothermal crosslinking	[55]	2011	Rabbit limbal epithelial cell	None
Rat tail type I collagen	Dehydrothermal crosslinking and plastic compression	[81]	2014	Primary human corneal endothelial cells; human corneal endothelial cell line	None
Fibronectin-collagen treatment		[54]	2013	Immortalized human corneal epithelial cells	None
Bovine type I collagen	EDC/NHS crosslinking	[154]	2014	Primary human corneal epithelial cells	None
Bovine type I collagen	EDC/NHS crosslinking	[78]	2014	Primary human corneal epithelial cells	None
Bovine type I collagen	EDC/NHS crosslinking	[82]	2015	Human corneal epithelial cells	Rabbits by lamellar keratoplasty
Bovine type I atelocollagen	Collagen-vitrigel	[86]	2015	None	Rabbits by lamellar keratoplasty and LSCD[iii] model induced by chemical injuries.
Rat collagen type I	Dehydrothermal crosslinking	[70]	2015	None	Pig keratocyte; primary human limbal epithelial cells

[a] -Ethyl-3-(3-dimethylaminopropyl)-carbodiimide.

[ii] N-hydroxysulfosuccinimide.

[iii] LSC deficiency.

surface of the SF membrane to enhance the attachment and proliferation of HCLE cells, human corneal fibroblasts (HCFs) [96, 97], and human corneal endothelial (HCEN) cells [98] by mediating cell-substrate adhesion interactions. Interestingly, poly-D-lysine (PDL) provides comparable support to modified RGD [99]. For corneal stromal bioengineering, porous silk film has been shown to support the construction of a two-layer structure using HCLE cells and stromal cells [100], and human corneal stromal stem cells (hCSSCs) while secreted ECM adhered to the material [101]. Moreover, enhanced cell viability and well-maintained morphology of rabbit corneal endothelial (rCEN) cells have been reported with the incorporation of natural aloe vera (AV) gel with SF [102]. To improve molecular permeability and biodegradability, porous SF structures were prepared using the porosity inducing agent poly(ethylene glycol) (PEG). The porous structure induced by PEG, however, tends to reduce the growth and stratification of human limbal epithelial cell (HLEC) growth. It is also noteworthy that by having high porosity the membranes tend to be more fragile [100, 103, 104]. Nonetheless, implanted RGD-functionalized SF films have been shown to keep their integrity and transparency for half a year without immunogenic and neovascular responses in rabbit stroma [105] and notably a faster degradation rate can be achieved by introducing porosity [101] or combining propionamide [106].

While fibroin has been extensively investigated in corneal engineering, another protein from the domesticated silkworm silk sericin (SS) has been consistently overlooked due to suspected allergenic activity. Recent interest, however, in the biocompatibility of SS has resulted in its consideration as a cell growth substrate. Pure or blended SS membrane showed inferior mechanical properties, but enhanced HCLE attachment compared with SF, suggesting a potential application for corneal epithelium regeneration [107]. Recently, Applegate *et al* innovatively published a silk photocrosslinking method using flavin-mononucleotide (FMN), which is a water-soluble variant of riboflavin, to transfer silk solution to a highly elastic and transparent hydrogel. The materials involved in the process are all biologically and environmentally friendly, which makes this technique very promising for corneal bioengineering [108].

Overall, compared with collagen, the advantage of using silk is mainly due to the simplicity of production and modification, as well as the ease of patterning and preparing the porous structure, which profoundly impacts the cell behavior of all corneal cells. The depth and width of grooves, for instance, were found to affect HCF alignment. Moreover, building a multilayered corneal structure using stacked patterned porous silk films or sponge provides a useful 3D model, in spite of the technique requiring further improvement with the native cornea consisting of approximately 200 lamellae with a total thickness of 500 microns, while the stacked silk films have far fewer layers [109]. While chemical surface modification has been investigated to improve the biocompatibility, further research is necessary since the proteins in the natural cornea are varied and multifunctional. Most of the studies to date have focused on the correlation between surface pattern and porous structure, with further studies of *in vivo* performance (including the influence of porosity and patterning on biocompatibility) necessary.

3.3. Gelatin

Gelatin is a ubiquitous natural material derived through the hydrolysis of collagen. The application of gelatin for corneal engineering mainly involves constructing membranes for corneal cells through crosslinking. The applied methods to prepare gelatin hydrogel generally include dehydrothermal and chemical crosslinking. Being dehydrothermally crosslinked, gelatin is able to provide sheets with better transparency, elastic modulus, and albumin permeability compared with collagen. In addition, primary HCEN cells seeded on as-prepared gelatin showed normal expression levels of ZO-1, Na $+$/K$+$-ATPase, and N-cadherin, and achieved a continuous endothelial monolayer [110]. Mimura *et al* reported chemically crosslinked gelatin using glutaraldehyde, which was able to support rabbit fibroblast, fibroblast precursor adherence and ECM deposition, and was suitable for implantation into a rabbit stromal pocket [111]. Using EDC/NHS crosslinking and freeze-drying, gelatin membranes with varying porosity, Young's modulus and swelling ratio can be prepared by varying the concentration of gelatin and crosslinking parameters [112]. Compared with pure gelatin, the incorporation of chondroitin sulfate (CS) has been shown to promote the growth of primary rabbit corneal keratocytes (RCKs). The hybrid gelatin-CS gel, however, had lower Young's modulus and resistance against protease digestion [113]. By adding heparin during EDC crosslinking, Niu *et al* prepared an innovative transparent gelatin scaffold that has the capacity to enhance the basic fibroblast growth factor absorption and release kinetics [114]. Furthermore, the scaffold is sufficiently flexible to be folded for easier transplantation and HCEN cells can be grown on the scaffold, while maintaining their endothelial morphology and critical pumping function. Following transplantation in rabbits, the implanted scaffolds gradually fuse with the surrounding stroma [114]. Lai reported a 3D culture system for RCKs using hyaluronic acid (HA) functionlized gelatin microspheres that supported the large-scale growth of RCKs. Investigation of the biocompatibility of microcarriers indicated compatibility with rabbit corneal epithelial, stromal, and endothelial cells, and they were well tolerated in the anterior chamber of the rabbit eye [115]. Semisynthetic gelatin methacrylate (GelMA) has been increasingly used in tissue engineering due to

its biocompatibility and more importantly, tunable physical characteristics produced through radical polymerization in the presence of a photoinitiator. Rizwan *et al* developed a hybrid crosslinked GelMA hydrogel (GelMA+) by incubating a prepolymer solution at 4 °C for 1 h before UV crosslinking (figure 5). The GelMA+ showed superior mechanical properties due to the smaller and more uniform distribution of pores and excellent support of HCEN cells. GelMA+ films seeded with HCECs and transplanted into a rabbit model were associated with high cell viability following transplantation [116].
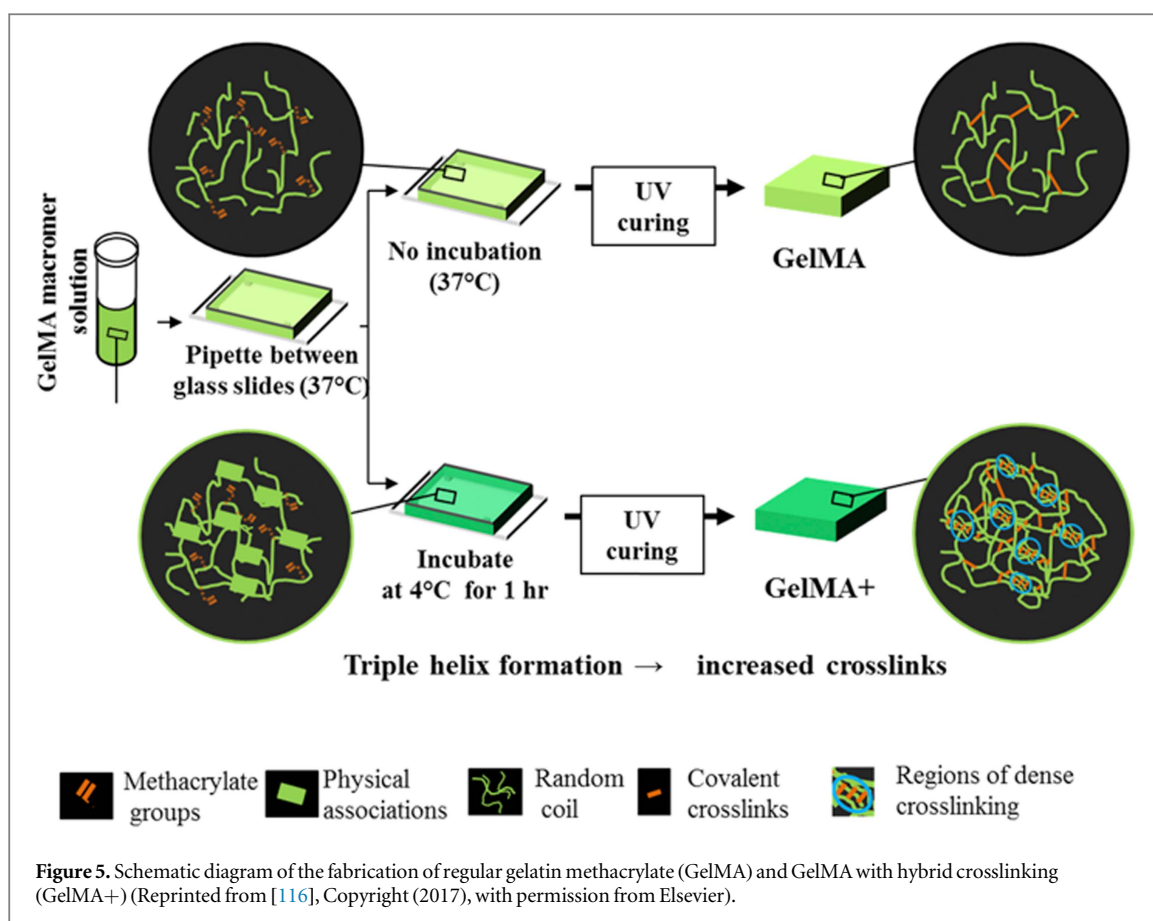
3.4. Decellularized cornea

Decellularized cornea (DC) has shown potential for corneal scaffold fabrication due to the similar mechanical and optical properties when compared to a natural cornea [117, 118] and low immunogenicity [119, 120]. The shortage of suitable corneal tissue as a requirement for a human DC remains a limiting factor and therefore most studies have employed porcine corneas or bovine corneas as the source material. Previous studies have revealed the feasibility of using a DC to support corneal cell growth *in vitro* and to facilitate tissue regeneration in animal models [121–123]. Conventional approaches to decellularize cornea tissue involve the removal of the cellular component using non-ionic detergent (e.g. Triton X-100, Sigma, St. Louis, MO, USA), ionic detergents (e.g. sodium dodecyl sulfate (SDS) and SD), zwitterionic detergent (e.g. CHAPS), freezing-thawing, and osmotic shock with hypotonic or hypertonic solutions [118, 123, 124]. Comparison between Triton X-100, SDS, SD and CHAPS revealed SDS to be the most effective for the removal of cellular components. Furthermore, 0.5% SDS was the most optimal concentration for cell removal, while preserving collagen ECM [118, 124]. Another study of decellularization using Triton X-100, liquid nitrogen, and poly(ethylene glycol), sodium dodecyl sulfate (SDS) and sodium chloride (NaCl) with nucleases found that only NaCl/nucleases completely removed the cellular component and supported the growth of fibroblasts and epithelial cells, while maintaining the integrity of the corneal epithelial BM [121]. Hashimoto *et al* describe a novel decellularization method using high-hydrostatic pressure (HHP) without detergents. The advantages of HHP include the elimination of bacteria and viruses, and the preservation of collagen that provides a relatively better mechanical support [125]. Similar to the HHP method, Huang *et al* reported a gentle physical decellularization method using supercritical carbon dioxide (SCCO$_2$), with less processing time, lower cost and the elimination of pathogens to prepare an acellular porcine cornea. Compared to the Triton treatment, the use of SCCO$_2$ enabled better removal of proteins with no immune response in a rabbit model

at 6 months follow-up. Importantly, the maximum load of the DC is reduced, because of the increased spaces between the collagen fibrils due to the decreased GAG contents [126]. A final decellularization method worth noting employs pancreatic phospholipase A2 (PLA2) and SD. This approach preserves proteoglycans and the structure of the native corneal stroma, while removing most of the xenogenetic cells [119].

By preserving the corneal structure, the DC can provide excellent functionality with similar optical and mechanical properties to a native cornea with the capacity to support the confluent growth of the corneal epithelium, keratocytes, and endothelium, as well as the ability to degrade slowly while merging with the surrounding stroma during regeneration of the corneal epithelium and nerve growth [117, 122]. By co-culturing limbal epithelial cell-like (LEC-like) cells and corneal endothelial cell-like (CEC-like) cells derived from hESCs on the two sides of a decellularized porcine scaffold, a full-thickness artificial cornea substitute with similar thickness and mechanical properties to a native rabbit cornea was successfully constructed, with multilayered epithelium-like cells and a uniform monolayer of CEC-like cells observed. The thickness, endothelial cell density, and mechanical properties of the construct were similar to that of native rabbit corneas. Scaffolds could be integrated in a rabbit model with the restoration of transparency at an 8 week follow-up, indicating the DC to be a promising substrate for full-thickness corneal bioengineering [127].

3.5. Chitosan

Chitosan is an abundant naturally derived polymer that is synthesized by deacetylation of chitin. For corneal bioengineering, chitosan has primarily been employed as a substitute for AM as a cell carrier to reconstruct the ocular surface or endothelium. It has been reported that primary bovine corneal epithelial cells (BCECs) seeded on pure chitosan membrane showed improved proliferation and attached more quickly than those on AM. Moreover, pure chitosan membrane is able to preserve the phenotypes of BCECs similar to AM [128]. Furthermore, chitosan surface modification can have a positive effect on generating a monolayer of HCECs, while non-modified substrates cannot [129]. For endothelial regeneration, a chitosan membrane with controllable biodegradation rates can be achieved by incorporating polycaprolactone (PCL), a slow degrading synthetic polymer. BCECs cultured on this blended membrane showed normal appearance, and proliferated well, forming a continuous monolayer [130]. In addition to serving as a potential substrate for HCECs, the incorporation of chitosan can promote the optical transparency and mechanical strength of collagen membranes [78]. Because of the poor solubility of

Figure 5. Schematic diagram of the fabrication of regular gelatin methacrylate (GelMA) and GelMA with hybrid crosslinking (GelMA+) (Reprinted from [116], Copyright (2017), with permission from Elsevier).

chitosan in physiological solvents, the research focus has been shifted to hydroxyethyl chitosan (HECTS), a water-soluble derivative of chitosan. With comparable optical transparency, water content, and higher glucose permeability compared to the natural human cornea [131, 132], membranes made up of hydroxypropyl chitosan, gelatin and chondroitin sulfate can improve the growth of HCECs and rCEN cells, promote the growth of primary rCEN cells and corneal epithelium restoration *in vitro* and are tolerated well in the anterior chamber of an *in vivo* rabbit model [131–133]. Although chitosan has demonstrated great potential for corneal bioengineering, it is more suitable to be utilized as blended, rather than pure scaffolds.

3.6. Thermal responsive polymers

Stimuli responsive polymers such as poly(N-isopropylacrylamide) (PNiPAAm) and PNiPAAm-based copolymers are being increasingly used in bioengineering, because of their thermoresponsive hydrophobic and hydrophilic properties, which enables the detachment of cell sheets without the use of enzymatic digestion. The ability to generate carrier-free cell sheets makes it a promising technique for corneal epithelium and endothelium engineering. While several publications support the use of both corneal epithelium [134] and endothelium cell sheets [135] derived from temperature-responsive substrates, endothelium construction

has attracted the most interest. Using rabbit limbal epithelial stem cells isolated from rabbit ocular surface, corneal epithelial sheets with compact multilayered cell sheet architecture were successfully generated from PNiPAAm grafted cell culture surfaces using poly(vinylidene difluoride) (PVDF) membrane and applied for autologous transplantation. The cell sheets attached to an exposed rabbit corneal stroma within minutes without suturing and successfully reconstructed the corneal epithelium [134]. The structure and function of HCEN cell sheets detached from pure PNiPAAm are able to restore corneal clarity without causing inflammation or rejection in rabbit models [135, 136]. The activity of Na+, K+-ATPase and existence of pump sites were confirmed *in vitro* and more importantly, proper stromal hydration was found and corneal clarity was gradually restored in the rabbit model [135, 136]. Apart from pure PNiPAAm, copolymerization with glycidylmethacrylate (NGMA) allows the manipulation of the critical temperature and comprises epoxy groups for the incorporation of biomolecules. The use of a copolymer of N-isopropylacrlamide (NiPAAm) and diethyleneglycol methacrylate (DEGMA) enables faster cell detachment with a critical temperature, closer to the physiological range [137, 138]. Teichmann *et al* reported a thermal responsive carrier using poly(vinyl methyl ether) (PVME) where layer thickness, stiffness, switching amplitude and bioactive modification can be

coordinated to regulate the initial immortalized HCEN cell adhesion and detachment of cell sheets [139]. Despite all the advantages, given the thermally detached cell monolayers are usually fragile, the use of compatible delivery methods is important for the surgical manipulation of sheet grafts. Varied carriers have thus been investigated for the delivery of constructed epithelial (PVDF) [134] and endothelial layers (stroma bed and gelatin) [135, 136].

3.7. Other synthetic polymer hydrogels

A variety of synthetic materials including poly(vinyl alcohol) (PVA) [140], poly(2-hydroxyethyl methacrylate) (PHEMA) [141], polyethylene (glycol) Diacrylate (PEGDA) [142, 143], poly(lactic-co-glycolic acid) (PLGA) [144] and poly(ethylene glycol)/poly(acrylic acid) (PEG/PAA)-based hydrogels [145–147] have also been applied in corneal bioengineering, as either substrates for corneal cells or artificial cornea substitutes. While exhibiting excellent permeability, controllable mechanical and optical properties, all of these materials display poor cell-adhesive function without surface modification [148]. For example, to improve the attachment and growth of primary rabbit and human corneal cells, electrospun PVA, PHEMA hydrogel and PEGDA hydrogel were modified with collagen type I, phosphate groups and RGD, respectively [140, 141, 149]. Moreover, hydrogels functionalized with amine can support epithelization using HCECs [148]. Similarly, collagen type I coated PLGA films can better serve as substratum for rCEN cells [144]. An artificial cornea fabricated using ZnS/ PHEMA/PAA hydrogel demonstrated high refractive index [145] and PEG/PAA hydrogel showed high glucose permeability, while remaining optically clear *in situ* for up to 2 weeks [146, 147]. Recently, poly (glycerol sebacate) (PGS), a novel biodegradable elastomer, was cured into films and capable of maintaining the viability of HCECs. However, there have been no reports of the mechanical or optical properties, or *in vivo* testing to date [150].

4. Material surface modification

4.1. Topographical modification

The influence of topographical cues and surface chemical modification are important for cell support regardless of the tissue type being engineered [151, 152]. For topographical modification, the most commonly used technique to create varied patterned features is soft lithography [101, 116, 151, 153–158]. Briefly, feature-patterned master molds (for instance, patterned silicon substrates), are covered with PDMS, cured on the patterned substrate and then removed to be used as patterned templates for cell seeding. Anatomically, for corneal epithelial engineering, the key findings are that HCEC contact guidance is not only modulated by the scale of the topographical cues

[151] and HCLE cells seeded on patterned silk created by lithographic techniques but suggest parallel line patterned surfaces promote initial cell attachment by 36%–54% and more that twofold increase in focal adhesion localization [153]. In addition, the adhesion and proliferation rate of HCECs on collagen film can be promoted by inducing a micro-rough surface using a freeze-drying technique [154]. Interestingly, for corneal stromal bioengineering, whilst both the depth and width of the grooves influence HCFs alignment, the former appears to play a more critical role for cell orientation [101]. On poly (L,D lactic acid) (PLDLA) electrospun nanofibrous substrates, HCS cell orientation and differentiation can be directed, with stiff and orthogonally arranged nanofiber layers resulting in keratocyte-like morphology other than those of HCFs [155]. Nara *et al* employed direct-write assembly and prepared parallel patterned thermos-responsive substrates that can guide goat corneal keratocyte alignment. However, these patterned substrates could not produce complete sheet restoration [157]. To compare the effect of different topography on endothelial cells, various kinds of patterns including channels, gratings, concentric circles, wells and pillars have been studied. Bovine corneal endothelial cells grown on pillars provide a lower coefficient of variation of area, enhanced Na+/K+-ATPase activity and a density of microvilli similar to the native cornea compared to other patterns [156]. A similar finding on HCECs reported by Rizwan *et al* showed that higher Na+/K +-ATPase expression was found on the substrates of square-array and hexagonal-array of 1 mm pillars with 6 mm spacing compared to flat substrate. Specifically, square-array topography increased ZO-1 and Na+/K +-ATPase expression higher, while hexagonal-array induced more favorable cell morphometry such as high cell densities, low cell areas and homogenous cell size, thus improving the quality of the regenerated HCEC monolayer [116].

Apart from stereo lithography, the effect of topographical modification on corneal cell growth and subsequently corneal tissue regeneration can be explored via a noteworthy fabrication technique, namely electrospinning. Electrospinning is a widely investigated fiber fabrication method that applies a voltage between a syringe and a collector to draw fibers from polymer solutions dispensed by the syringe [159]. Electrospun scaffolds are increasingly being investigated to develop biomimetic bioengineered corneas as a result of the bionic reconstruction of ECM, which showed the promotion of corneal cell adhesion, proliferation, migration and differentiation [129, 160]. In many instances natural materials are used including SF [94], gelatin [161], collagen [129], HA [129]) and synthetic polymers PCL [162], PLGA [163], RGD (PEUU) [164], poly-L/D-lactide (PLDLA) [155], poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) [162], with the latter characterized by outstanding mechanical properties. While the use of

natural materials is limited by inferior mechanical toughness and therefore handling for surgery, synthetic polymers such as FDA approved PLA, PLGA, and PCL have been employed extensively. The application of electrospun scaffolds is primarily limited to serve as substrates for the growth and delivery of corneal cells and the exploration of cell-substrate interactions. Electrospun substrate as a replacement for AM to support the regeneration and delivery of corneal epithelium has been widely investigated. For instance, (PHBV)/gelatin [160], collagen/chitosan/hyaluronate(HA)/PEO [129], PLGA [163] electrospun substrates have been shown to be suitable substitutes for AM. Gelatin/PLLA-based substrates have been used to investigate the relationship between HCECs and keratocyte cell behaviors and fiber arrangement [165]. HA/PEO electrospun film was characterized with excellent mechanical properties, and comparable optical and biological performance *in vivo* for rabbit ocular restoration [129]. Ortega *et al* electrospun biodegradable PLGA fibers onto PEGDA ring with microfeatures to mimic corneal limbus with the rabbit corneal limbal epithelial cells well attached and proliferated on the designed microstructure [159]. Furthermore, PCL electrospun nanofibers support HCLE cell phenotype to facilitate cornea epithelium construction and promote the cell attachment of rabbit LSCs and rabbit keratocytes [162]. Notably, PLGA electrospun scaffold is not only biocompatible with HCS cells but capable of maintaining a keratocyte phenotype [155] and multilayered rabbit limbal epithelial cells [163]. Apart from the choice of material, the ease of manipulating mechanical properties, transparency and biological properties is also determined by polymer components, special arrangement and functionalization [165–167]. For example, the degree of alignment of fibers effects the mechanical properties of scaffolds [165]. Aligned fibers mirror the natural configuration necessary for cell support and differentiation [161, 168]. For instance, HCS cells cultured on electrospun PEUU aligned scaffolds tend to secrete collagenous matrix with similar alignment to natural corneal stroma [164]. Similar results have shown that aligned electrospun collagen scaffolds tend to dedifferentiate rabbit corneal fibroblasts from the myofibroblast phenotype [167]. Salehi *et al* reported electrospun nanofibrous PGS/PCL semitransparent substrate that is able to support the growth of HCEN cells with hexagonal morphology and direct human conjunctival epithelial cells growing along the aligned fibers with the incorporation of PGS improve the cell proliferation and viability [169]. Earlier studies demonstrated the potential of electrospun scaffolds in corneal tissue formation as a result of the favorable mechanical properties, transparency and the biocompatibility with various corneal cells. However, few *in vivo* studies have been performed to date.

4.2. Chemical modification

Modifications with proteins, peptide and chemical groups are commonly utilized to promote corneal cell growth. Apart from the widespread modification of RGD or collagen on synthetic polymers, carbon chain length of amine modifications are able to impact the attachment of epithelial cells and stromal cells [148]. A study of collagen type I, collagen type IV, fibronectin (FN), FNC coating mix and laminin modifications indicate all these coatings facilitate cell adhesion, with FN coating being the most favorable for HCEN cell growth and optimum compact cellular morphology [170]. Interestingly, chitosan coating was also found to improve the growth of HCEC line [129].

Peptide amphiphiles (PAs) are peptide-based molecules typically composed of hydrophilic and hydrophobic domains and of great interest in tissue engineering by virtue of their ability to self-assemble into high-aspect-ratio and highly bioactive nanostructures under certain physical conditions (pH, temperature and ionic strength) [171]. PA generally comprises three components: a hydrophobic tail, a beta-sheet forming amino acid, and a hydrophilic head that promotes water-solubility and biocompatibility. Self-assembly of PA can be guided by the hydrophobic collapse of the tail and achieved by hydrogen bonding between beta-sheet forming amino acids (figure 6). More specifically, hydrophobic tails of PAs were internalized and the hydrophilic heads exposed as bioactive interacting surfaces [172]. For tissue engineering, PAs are commonly applied in different formats, including coating, solution and hydrogel formats. For corneal bioengineering, PA has primarily been used for coating or in solution due to the inferior mechanical toughness of hydrogels, able to support a wide range of corneal cells [171]. As previously mentioned, RGD has been widely coated on various biomaterials to promote corneal cell interaction and adhesion. In this context, Gouveia *at al* employed PA consisting of RGD-containing peptides as a film coating that was able to enhance the adhesion, proliferation and alignment of HCFs and enable the construction of 3D lamellar-like stromal tissue [173]. By also adding a matrix metalloprotease (MMP)-cleavable sequence, the PA not only acted as a substrate for the attachment and growth of HCS cells in a quiescent phenotype but enabled self-release after adding retinotic acid to the culture medium to facilitate the detachment of regenerated stromal matrix from the base [174]. In addition, Jones *et al* [175] report a special PA: C16-KTTKS, which self-assembled into nanotape structures being able to stimulate collagen I production from HCS cells. Finally, Uzunalli *et al* [176] applied a laminin-derived sequence YIGSR into PA to regulate cell adhesion for corneal stroma regeneration. Compared to RGD-PA, as-prepared PA demonstrated enhanced cell proliferation, keratocyte migration, and collagen I synthesis both *in vitro* and *in vivo*.

5. *In vivo* performance

Due to the excellent intrinsic biocompatibility, collagen gel, both pure and blended developed by various kinds of methods, have shown promising performances *in vivo*. From 24 months follow-up of a phase 1 clinical study on the EDC crosslinked collagen gel scaffold, the fabricated implants remain stable and avascular, with corneal re-epithelialisation, restored tear film, recruited stromal cells, and nerve regeneration [177]. Varied hybrid collagen gels, such as collagen/chitosan [63], glutaraldehyde crosslinked collagen/TERP [75] were well tolerated in pig corneas for 12 months and dogs for 16 weeks, respectively. Being applied as full-thickness implants in guinea pigs, collagen–MPC hydrogels integrated into host corneas and promoted regeneration of corneal tissue and nerves. Electrical activity recorded from the regenerating corneal nerves suggested that the regeneration of corneal nerves into the implant started at 3 months post-surgery and the nerves were functionally active within the implant by 8 months [77].

Although surface modifications are normally necessary to promote corneal cell growth, silk-based films have demonstrated favorable *in vivo* responses. RGD-functionalized five layers of stacked silk implanted in the lamellar pocket at the limbus in the rabbit models can maintain their integrity and transparency over 180 days without causing immunogenic and neovascular responses or degradation of the rabbit corneal stroma. The regenerated ECM deposited and adhered well to the surface of the silk films [105]. Porous silk films prepared using PEG were inserted into stromal pockets at the limbus of rabbits and were gradually replaced by corneal stromal tissue, while stromal cells infiltrated into the silk films without causing inflammatory reaction 6 months later. Notably, unlike porous silk films, non-porous ones did not degrade at 6 months [93]. Transparent blended films with aloe vera gel and SF as a carrier for corneal endothelial cells was developed by Kim *et al* by seeding rCECs on the films to reach confluency. Transplanted into the anterior chamber of the rabbits' eyes together, the constructs attached well to the surface of the corneal stroma and 4 weeks later, started to integrate with the surrounding corneal tissue with the rCECs firmly adhered and functional. Moreover, rabbit cornea transplanted using hybrid AV/SF scaffolds with rCECs revealed a higher corneal transparency compared to that of pure SF films with rCECs, indicating the improvement of clarity brought by the AV. However, many constructs were difficult to insert during the transplantation surgery, because of insufficient mechanical properties [178]. An investigation on how transplantation techniques affect the biological responses of silk films in rabbits is stimulating and worthwhile noting. Porous silk films transplanted into different locations of the ocular surface (peripheral-median P-M, central-superficial C-S, central-deep C-D) were used to assess the *in vivo* response. The *in vivo* response of the films was found to be dependent on the method to create the corneal pocket, the position of the sample and sutures. To be more specific, with the use of flushing air (instead of using a surgical a blade), the C-D position (compared to P-M and C-S) and a lack of sutures resulted in less injury and inflammatory responses. Two months after the surgery, a comparison of the degradation of silk films with low, medium and high beta-sheet (crystalline) content showed that low beta-sheet samples lost structural integrity, while medium and high beta-sheet content films remained at the initial stage [179]. Although fibroin film is designed as a transplantable carrier, improvement in physical properties is needed to graft the sheet onto a curved surface such as the cornea.

One common application of gelatin for corneal bioengineering is to serve as carriers for endothelial regeneration and delivery. For instance, EDC cross-linked intact and porous gelatin carriers have been inserted into the anterior chamber of rabbit eyes and no change was observed in the cell morphology of the rCECs by day 3 [112, 115]. Being implanted into the anterior chamber of the endothelium-removed rabbit model, EDC crosslinked heparin-modified gelatin remained transparent by week 3 with little surrounding intraocular inflammation [114]. Glutaraldehyde crosslinked gelatin seeded with rabbit fibroblast and fibroblast precursors were transplanted into rabbit eyes with lamellar dissection. While both carriers were well tolerated during the 4 weeks follow-up, the gelatin/precursor group showed more intense expression of ECM molecules [111]. Longer-term observations of the biological responses of gelatin used *in vivo* are however limited.

Due to the well-maintained integrity of ECM, DC has demonstrated excellent biocompatibility *in vivo*. Porcine corneas, decellularized using Tris-HCL [117], SDS [118], PLA2/SD [119], HHP method [125], SCCO₂ [126], have been transplanted into rabbit corneal stroma pockets to investigate the biological responses. The implants were normally capable of becoming completely transparent within 2 weeks, being maintained for at least 12 months after transplantation with the implants being able to support a stratified epithelium with infiltrated keratocytes. Zhang *et al* developed a full-thickness cornea substitute using LEC-like and CEC-like cells derived from human embryonic stem cells and decellularized bovine corneas. Being transplanted into a rabbit model using PK, no significantly high intraocular pressure was found in the follow-up period. By 8 weeks, the substitutes were well integrated with corneal tissue. LEC-like cells were completely replaced by host cells and the endothelial cell density of tumor endothelial cells was similar to that of native rabbit corneas. Compared with a decellularized bovine cornea without cells, the full-thickness graft showed less neovascularization with new blood vessels only in the periphery, while its counterpart was completed invaded [122].
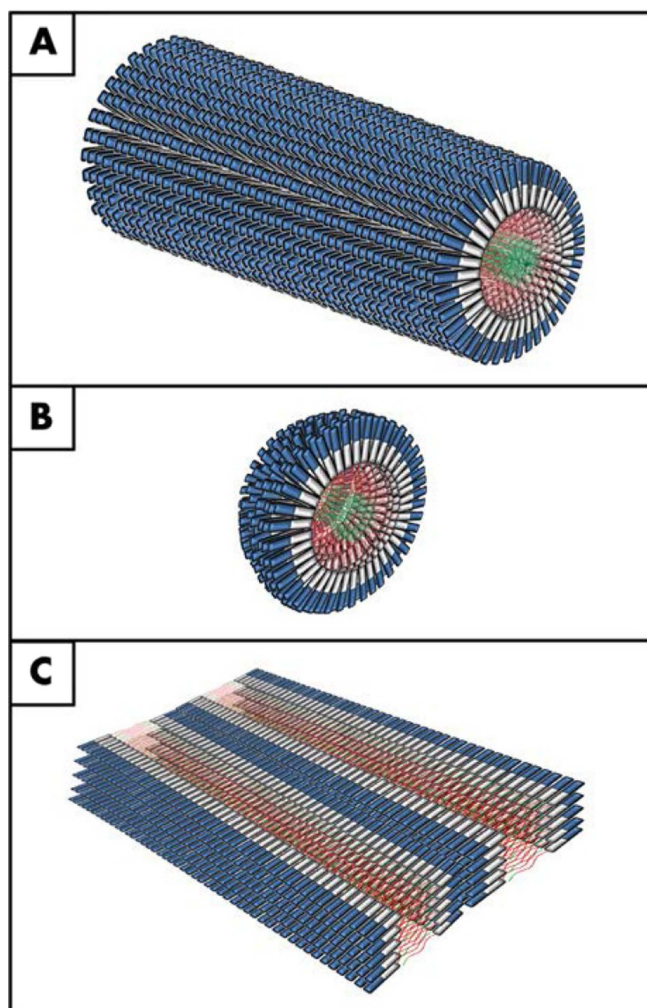
Figure 6. Examples of supramolecular self-assembled PA nanostructures: (A) nanofibers; (B) micelles; and (C) multilayered nanotapes. All three structures have a hydrophilic outer corona consisting of bioactive peptide (blue), self-assembly-inducing/spacer sequence (white), and a hydrophobic inner core with organized and/or non-organized PA tails (red and green, respectively). Reprinted from [171] CC BY 4.0.

Research relating to the *in vivo* performance of chitosan is limited. It is reported that after HECTS/CS blended membranes were transplanted into the anterior chamber of rabbits, the cornea still retained clarity and no apparent symptoms could be detected [132]. For corneal epithelial bioengineering, rCEC-seeded chitosan membranes were transplanted onto the ocular surface in rabbits, promoting corneal recovery [133] and that chitosan coatings were able to promote corneal epithelial recovery in a rabbit corneal defect model [129].

While most synthetic hydrogels are applied as substrates to investigate fundamental cell behaviors, most of them did not receive *in vivo* evaluation. As an artificial cornea, PEG/PAA hydrogel was able to remain optically clear and show excellent tolerance after being sutured into the stroma of a rabbit for 2 weeks [146]. A comparison between poly(ethylene glycol) diacrylate (PEGDA) and poly(ethylene glycol) diacrylamide (PEG-dAAm) hydrogel showed that in terms of *in vivo* biocompatibility in rabbits, the PEG-dAAm-based hydrogel was superior to the PEGDA-based hydrogel,

with far longer maintenance of corneal clarity up to 6 months, while the PEGDA-based gel implanted ones showed haze and opacity within 10 weeks [147].

6. The importance of cell biology

Considering the different cell types throughout the cornea and the likely requirement of more than one type of cell support substrate with a composite of biomaterials, there is a need for extensive optimization studies towards merging the components for a complete and functional cornea. For example, electrospun aligned PEUU substrate [164], combined with growth factors [180] and the selection between hCSSCs and HCFs will result in different collagen fibril matrices and therefore different tissue constructs. Specifically, multilayered lamellae with orthogonally oriented collagen fibrils that accurately mimic the HCS tissue will likely require the combination of fibroblast growth factor (FGF-2) and transforming growth factor-beta3 (TGF-b3). Both of these growth factors

can stimulate the secretion of collagen from cells resulting in the directional growth of collagen fibrils and orthogonal fibril orientation of a collagenous layer [180]. Regarding cell choice, hCSSCs should differentiate into keratocytes and secrete multilayered lamellae with well-organized collagen fibrils, while HCF cells are more likely to differentiate into fibroblasts and generate a less-organized collagen construct [181]. Similar results can be expected for porous gelatin hydrogel-based constructs, with fibroblast precursors likely better candidates for corneal stroma engineering compared with fibroblasts, because of higher collagen I expression *in vivo* after implantation [111]. Moreover, the use of serum tends to induce hCSSCs to become fibroblastic. In contrast, a keratocyte-like morphology arises in the absence of serum [155]. Compared to culturing HCECs and stromal cells alone, co-culturing both cells demonstrated improved differentiation and growth for both of the cells [182]. A study on the influence of serum concentration, oxygen tension for incubation and macromolecules (carrageenan)-supplemented media on ECM deposition in HCF culture interestingly found that the low oxygen tension coupled with supplemented media in the culture significantly promoted ECM deposition at day 14 [183].
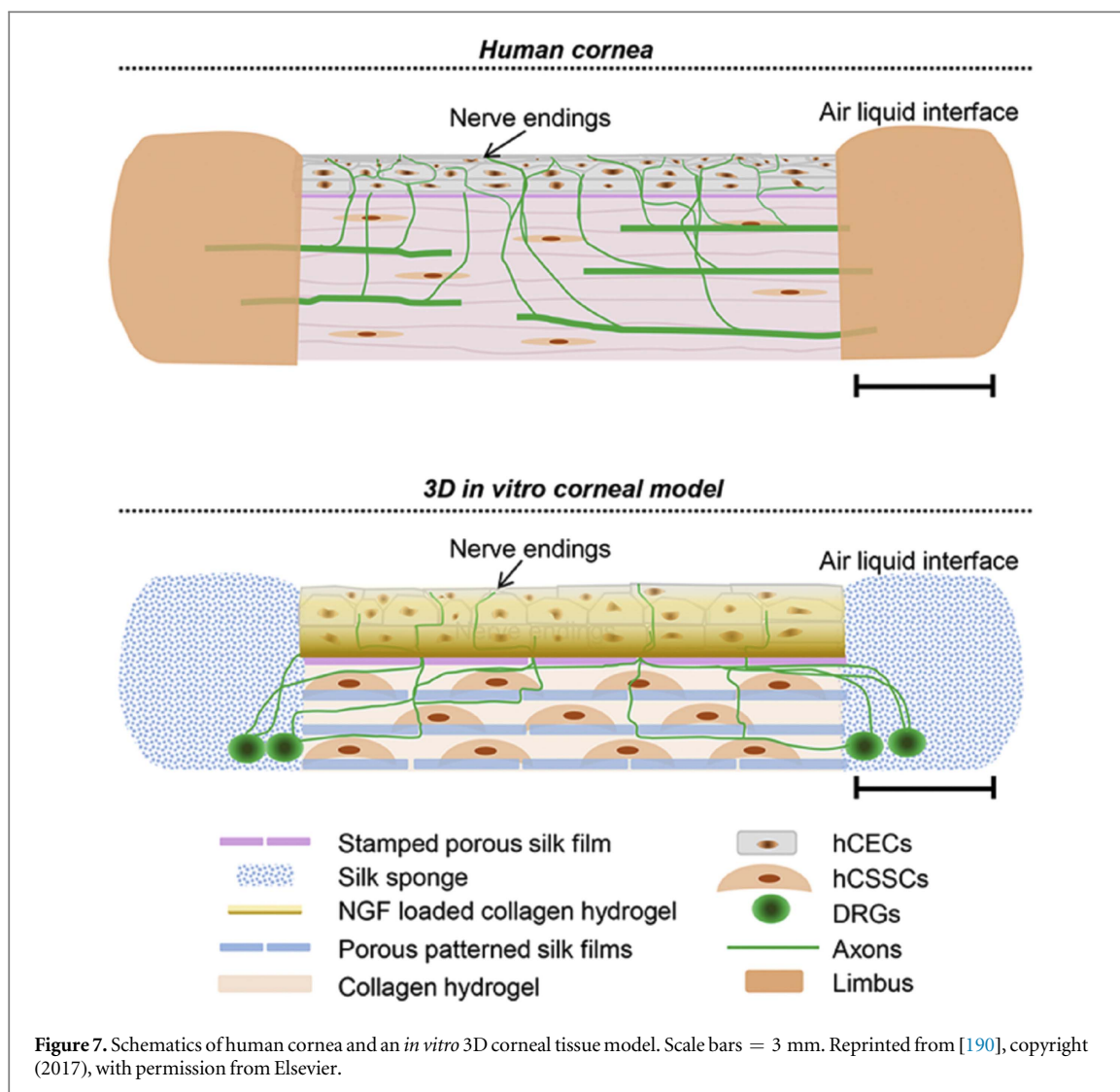
Practically, the use of seeding cells to differentiate or modulate the local tissue environment thereby promoting regeneration and repair remains a logical progression for research. A breakthrough on corneal epithelial regeneration using tissue-engineered cell sheets composed of autologous oral mucosal epithelium offered a new treatment for ocular trauma or disease. After scar tissues on the ocular surface were removed, cell sheets constructed by culturing oral mucosal tissue from patients on temperature-responsive polymer poly(N-isopropylacrylamide) were transplanted into the patients' eyes. Excitingly, complete re-epithelialization was detected within 1 week in all treated eyes and corneal transparency was restored and well maintained through long-term follow-up (mean period of 14 months) [184]. Yang *et al* previously showed that human amniotic epithelial cells could differentiate into conjunctival epithelium-like cells and goblet cells with partially physiological function thereby providing successful restoration of ocular surface integrity in a rabbit model of conjunctival disease [185]. More directly, Mathan *et al* successfully isolated and transplanted sphere-forming cells from the peripheral cornea for the potential use of targeting *in vivo* ocular surface regeneration and stem cell repopulation [186]. Yet challenges remain. Understanding the detailed mechanism of differentiation induced by the niche, the method of immortalization of differentiated cells and the physiological functions of tissue-engineered surfaces is essential to long-term success. The microenvironment appears to play an important role in the directional differentiation and fate of the implanted cells [187]. As evidence, Mathan and co-

authors found that corneal repopulation differed from scleral cell repopulation. The authors hypothesized that inherent characteristics within cells may lead to preferential changes (i.e. a cell derived from the limbus would like to reform a limbus or limbal niche). Separately, the authors found sphere implantation indicated a similar reactive biological response to the wounding process. Regardless, current findings provide tangible evidence to support long-held theoretical beliefs. The concurrent use of biomaterials may provide additional benefits and further research is essential to optimizing an effective approach to corneal tissue regeneration.
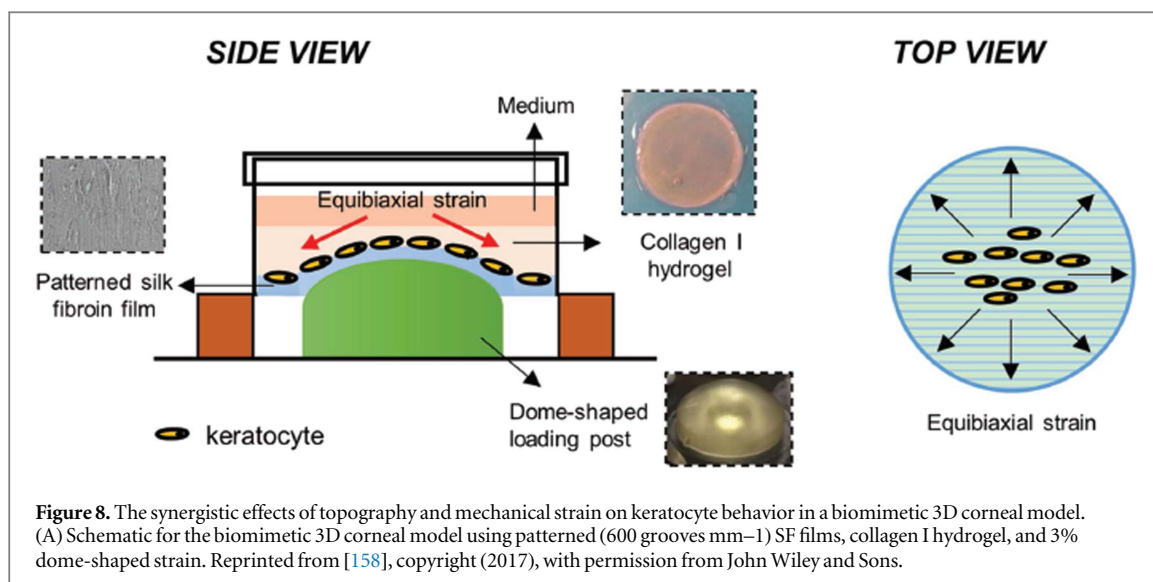
7. Conclusion and future challenges

Over the past few years, many different types of bioengineered substitutes have been developed to address the shortage of qualified corneal donors. Biomaterials used are largely limited to natural polymers able to support cell growth and function *in vitro*, and tissue regeneration *in vivo*. Collagen remains the best candidate in part due to its excellent biocompatibility from intrinsic RGD, with the main challenges in the biomimetic fabrication of aligned collagen fibrils mirroring corneal stromal inner structure. Silk has shown excellent optical clarity and more importantly, controllable degradation rates and mechanical properties, unlike other natural polymers. In terms of pursuing cell-biomaterial interactions, the ease of patterning and chemical modification enables silk to outweigh other materials. Derived from collagen, gelatin does not show any better overall performance. Alternatively, chitosan is easily functionalized and highly biodegradable and could be more useful when acting as an incorporated component in blended scaffolds. Despite their excellent physiological properties, the modification of synthetic polymers (e.g. RGD, collagen addition) is required to achieve satisfactory biological performance. Notwithstanding, they have significant potential as artificial corneas or substrates for various kinds of corneal cells, with most of the candidates mentioned above showing favorable ability to support the growth and delivery of diverse cell types.

In spite of the progress and promise of a bioengineered cornea, there are many challenges to overcome. Firstly, human trials are necessary to definitively determine the efficacy of corneal substitutes. Currently, clinical trials of bioengineered corneal substitutes are by-and-large limited to collagen-based materials with other materials rarely reported [177, 188]. A 12 month follow-up of LSC therapy and long-term corneal regeneration conducted on 113 eyes of 112 patients with burn related LSC deficiency showed that the total success rate by one year, as characterized by a transparent, avascular, and stable corneal surface being restored, was 76.6% [189]. A significant finding is that a minimum number of 3000 p63-bright holoclone-forming LSCs is

Figure 7. Schematics of human cornea and an *in vitro* 3D corneal tissue model. Scale bars = 3 mm. Reprinted from [190], copyright (2017), with permission from Elsevier.

essential and suggested for clinical success of autologous LSC transplantation [189]. Most bioengineered substitutes are merely designed to fulfill the requirements of transparency, biocompatibility and biodegradability, with other important properties such as innervation and regeneration often overlooked. Corneal innervation is critical to corneal sensation and protection, with optimal nerve density important for wound healing. Notwithstanding, some studies have demonstrated the reinnervation of collagen-based grafts *in vivo* [66, 74–77, 177], but reports on neural regrowth of other materials remain limited. Notably, Wang *et al* recently developed an innervated silk-based multilayered corneal epithelium and stroma including innervation induced and directed by nerve growth factor *in vitro*. Increased IVL, GJA4, ALDH3A1 and KERA expression of innervated models indicated better corneal epithelial maturity and corneal stromal transparency compared to non-innervated ones, which further demonstrated the key role of innervation in the maintenance of the corneal epithelium and stroma (figure 7) [190]. Then, while a great number of studies have reported the effect of the characteristics of scaffolds

(composition, structure, pattern, etc) on *in vitro* response, few were carried out on the influence of those on the *in vivo* response, which might be more significant when pursuing the best substitute for corneal tissue. The use of a porous structure [101] or combining propionamide [106], for instance, tends to induce a faster biodegradation rate in an animal model. Finally, recognition of the structural distinction of different cornea tissue layers has underscored the need for a variety of strategies to develop substitutes amenable to generating each layer. Despite the challenges, progress is being made towards identifying efficacious and accessible substrates capable of supporting cell growth and delivery, and tissue restoration towards recapitulating *bona fide* corneal tissue. Most challenging, has always been the fabrication of a stroma consisting of parallel lamellar sheets and a highly ordered microstructure of collagen fibrils with functional keratocytes and proteins to mimic the cornea stroma, as a result of the anatomical and functional importance of the stroma. Once it is achieved, bioengineering a whole-thickness cornea should be much easier, since substantial progress has been claimed on the regeneration of the cornea

Figure 8. The synergistic effects of topography and mechanical strain on keratocyte behavior in a biomimetic 3D corneal model. (A) Schematic for the biomimetic 3D corneal model using patterned (600 grooves mm−1) SF films, collagen I hydrogel, and 3% dome-shaped strain. Reprinted from [158], copyright (2017), with permission from John Wiley and Sons.

epithelium and endothelium, which are mainly cell layers. Engineering corneal substitutes with all layers for full-thickness PKP procedure would be preferable. Currently, protocols to fabricate a whole-thickness cornea are cell-free and cell-encapsulated membranes. Recently, electrochemically compacted collagen matrices with improved strength and modulus after being crosslinked by EDC/NHS or genipin demonstrated highly dense structure and comparable transparency to the native cornea, suggesting great potential for corneal tissue engineering [191]. Apart from collagen gel, human keratocytes encapsulated into fibrin-agarose scaffolds with similar transparency, and UV absorption characteristics to the native human cornea [192, 193], have been built into a whole cornea by seeding corneal epithelial cells and endothelial cells on each side [194]. Zhang *et al* recently developed a biomimetic 3D corneal model by loading patterned silk films and collagen gel onto a dome-shaped post and more interestingly, the effect of mechanical strain on human keratocyte phenotype and ECM formation was investigated (figure 8). The keratocyte alignment and ECM arrangement were directed by the topography of the silk films and compared with traditional 2D culture, and higher expression of keratocyte marker and expression of ECM were detected in the 3D model. Moreover, higher expression of keratocyte marker was also observed on a 3% dome-shaped mechanical strain cornea model compared with flat-shaped strain [158]. Gouveia *et al* successfully fabricated auto-generated scaffold-free 3D corneal equivalents by stacking HCS cell sheets collected from PA coatings and subsequently culturing the multilayered cell sheets for 21 days. The corneal equivalent was not only transparent but well integrated into rabbit models after 9 months. Other interesting findings included the alignment of stromal cells and secreted collagen fibrils were directed by the topography of growth substrates with different anisotropies, with tissues comprising aligned collagen fibrils tending to be thicker, denser and more resistant to proteolytic degradation compared to those comprising randomly oriented fibrils. This work is definitely significant for fabricating a scaffold-free human tissue that mimics the corneal stroma with controlled structure and functional properties [195].

In spite of the challenges, there is little doubt that corneal implants will be partly or completely engineered *in vitro* as qualified alternatives to current treatments, thereby addressing the present shortfall in donor corneas. The rapid advancement in biomaterials, cell biology and tissue engineering methods such as bioprinting will facilitate the formation of durable biomimetic structures that are customized to the individual needs of recipients, improving transplant durability and functionality, and ultimately the quality of life for the large numbers of patients worldwide suffering from corneal blindness.

Acknowledgments

ORCID iDs

Jeremy M Crook ⓘ https://orcid.org/0000-0002-6415-1639

References

[1] Tan D T, Dart J K, Holland E J and Kinoshita S 2012 Corneal transplantation *Lancet* **379** 1749–61
[2] Pascolini D and Mariotti S P 2012 Global estimates of visual impairment: 2010 *Br. J. Ophthalmol.* **96** 614–8
[3] Oliva M S, Schottman T and Gulati M 2012 Turning the tide of corneal blindness *Indian J. Ophthalmol.* **60** 423–7

[4] Williams K A, Lowe M, Bartlett C, Kelly T L, Coster D J and All C 2008 Risk factors for human corneal graft failure within the Australian corneal graft registry *Transplantation* **86** 1720–4

[5] Panda A, Vanathi M, Kumar A, Dash Y and Priya S 2007 Corneal graft rejection *Surv. Ophthalmol.* **52** 375–96

[6] Gain P *et al* 2016 Global survey of corneal transplantation and eye banking *JAMA Ophthalmol.* **134** 167–73

[7] Stulting R D *et al* 2012 Effect of donor and recipient factors on corneal graft rejection *Cornea* **31** 1141

[8] Claerhout I, Beele H and Kestelyn P 2008 Graft failure: I. Endothelial cell loss *Int. Ophthalmol.* **28** 165–73

[9] Boisjoly H M *et al* 1993 Risk factors of corneal graft failure *Ophthalmology* **100** 1728–35

[10] Moffatt S L, Cartwright V A and Stumpf T H 2005 Centennial review of corneal transplantation *Clin. Exp. Ophthalmol.* **33** 642–57

[11] Ilango B 2007 Comparative cohort study of the outcomes of deep lamellar keratoplasty and penetrating keratoplasty for keratoconus *Eye* **21** 447

[12] Wagoner M D, Ba-Abbad R, Al-Mohaimeed M, Al-Swailem S, Zimmerman M B and Group KKESHCTS 2009 Postoperative complications after primary adult optical penetrating keratoplasty: prevalence and impact on graft survival *Cornea* **28** 385–94

[13] Tsubota K *et al* 1999 Treatment of severe ocular-surface disorders with corneal epithelial stem-cell transplantation *New Engl. J. Med.* **340** 1697–703

[14] Keivyon K R and Tseng S C Limbal autograft transplantation for ocular surface disorders *Ophthalmology* 1989 **96** 709–23

[15] Ma D H-K, Lai J-Y, Cheng H-Y, Tsai C-C and Yeh L-K 2010 Carbodiimide cross-linked amniotic membranes for cultivation of limbal epithelial cells *Biomaterials* **31** 6647–58

[16] Zavala J, Jaime G L, Barrientos C R and Valdez-Garcia J 2013 Corneal endothelium: developmental strategies for regeneration *Eye* **27** 579–88

[17] DelMonte D W and Kim T 2011 Anatomy and physiology of the cornea *J. Cataract Refract. Surg.* **37** 588–98

[18] Hashemi H, Khabazkhoob M, Emamian M H, Shariati M, Yekta A and Fotouhi A 2015 White-to-white corneal diameter distribution in an adult population *J. Curr. Ophthalmol.* **27** 21–4

[19] Gharaee H, Abrishami M, Shafiee M and Ehsaei A 2014 White-to-white corneal diameter: normal values in healthy Iranian population obtained with the Orbscan II *Int. J. Ophthalmol.* **7** 309–12

[20] Khng C and Osher R H 2008 Evaluation of the relationship between corneal diameter and lens diameter *J. Cataract Refract. Surg.* **34** 475–9

[21] Hoffmann P C and Hutz W W 2010 Analysis of biometry and prevalence data for corneal astigmatism in 23 239 eyes *J. Cataract Refract. Surg.* **36** 1479–85

[22] Dua H S, Faraj L A, Said D G, Gray T and Lowe J 2013 Human corneal anatomy redefined: a novel pre-Descemet's layer (Dua's layer) *Ophthalmology* **120** 1778–85

[23] Al-Aqaba M A, Fares U, Suleman H, Lowe J and Dua H S 2010 Architecture and distribution of human corneal nerves *Br. J. Ophthalmol.* **94** 784–9

[24] Parissi M *et al* 2016 Corneal nerve regeneration after collagen cross-linking treatment of keratoconus: A 5-year longitudinal study *JAMA Ophthalmol.* **134** 70–8

[25] Wiley L, SundarRaj N, Sun T and Thoft R 1991 Regional heterogeneity in human corneal and limbal epithelia: an immunohistochemical evaluation *Investigative Ophthalmol. Vis. Sci.* **32** 594–602

[26] Osei-Bempong C, Figueiredo F C and Lako M 2012 The limbal epithelium of the eye—A review of limbal stem cell biology, disease and treatment *BioEssays* **35** 211–9

[27] Dua H S and Azuara-Blanco A 2000 Limbal stem cells of the corneal epithelium *Surv. Opthamol.* **44** 415–25

[28] Di Iorio E, Barbaro V, Ruzza A, Ponzin D, Pellegrini G and De Luca M 2005 Isoforms of ΔNp63 and the migration of ocular limbal cells in human corneal regeneration *Proc. Natl Acad. Sci. USA* **102** 9523–8

[29] Ghezzi C E, Rnjak-Kovacina J and Kaplan D L 2015 Corneal tissue engineering: recent advances and future perspectives *Tissue Eng. Part B Rev.* **21** 278–87

[30] Eghrari A O, Riazuddin S A and Gottsch J D 2015 Overview of the cornea: structure, function, and development *Prog. Mol. Biol. Transl. Sci.* **134** 7–23

[31] Lagali N, Germundsson J and Fagerholm P 2009 The role of Bowman's layer in corneal regeneration after phototherapeutic keratectomy: a prospective study using *in vivo* confocal microscopy *Investigative Ophthalmol. Vis. Sci.* **50** 4192–8

[32] Delaigue O, Arbeille B, Rossazza C, Lemesle M and Roingeard P 1995 Quantitative analysis of immunogold labellings of collagen types I, III, IV and VI in healthy and pathological human corneas *Graefes Arch. Clin. Exp. Ophthalmol.* **233** 331–8

[33] Marshall G E, Konstas A G and Lee W R 1991 Immunogold fine structural localization of extracellular matrix components in aged human cornea. I. Types I-IV collagen and laminin *Graefes Arch. Clin. Exp. Ophthalmol.* **229** 157–63

[34] Marshall G E, Konstas A G and Lee W R 1991 Immunogold fine structural localization of extracellular matrix components in aged human cornea. II. Collagen types V and VI *Graefes Arch. Clin. Exp. Ophthalmol.* **229** 164–71

[35] Meek K M and Boote C 2004 The organization of collagen in the corneal stroma *Exp. Eye Res.* **78** 503–12

[36] Wilson S E and Hong J W 2000 Bowman's layer structure and function: critical or dispensable to corneal function? A hypothesis *Cornea* **19** 417–20

[37] Ruberti J W, Sinha Roy A and Roberts C J 2011 Corneal biomechanics and biomaterials *Annu. Rev. Biomed. Eng.* **13** 269–95

[38] Morishige N, Takagi Y, Chikama T, Takahara A and Nishida T 2011 Three-dimensional analysis of collagen lamellae in the anterior stroma of the human cornea visualized by second harmonic generation imaging microscopy *Invest. Ophthalmol. Vis. Sci.* **52** 911–5

[39] Chen S, Mienaltowski M J and Birk D E 2015 Regulation of corneal stroma extracellular matrix assembly *Exp. Eye Res.* **133** 69–80

[40] Netto M V, Mohan R R, Ambrósio R Jr, Hutcheon A E, Zieske J D and Wilson S E 2005 Wound healing in the cornea: a review of refractive surgery complications and new prospects for therapy *Cornea* **24** 509–22

[41] McKee H D *et al* 2014 Re: Dua *et al*: Human corneal anatomy redefined: a novel pre-Descemet layer (Dua's layer) (ophthalmology 2013;120:1778-85) *Ophthalmology* **121** e24–5

[42] Lewis P N, White T L, Young R D, Bell J S, Winlove C P and Meek K M 2016 Three-dimensional arrangement of elastic fibers in the human corneal stroma *Exp. Eye Res.* **146** 43–53

[43] Dua H S *et al* 2015 Big bubble deep anterior lamellar keratoplasty: the collagen layer in the wall of the big bubble is unique *Acta Ophthalmol.* **93** 427–30

[44] Kabosova A *et al* 2007 Compositional differences between infant and adult human corneal basement membranes *Investigative Ophthalmol. Vis. Sci.* **48** 4989–99

[45] Johnson D H, Bourne W M and Campbell R J 1982 The ultrastructure of Descemet's membrane. I. Changes with age in normal corneas *Arch. Ophthalmol. (Chicago, Ill: 1960)* **100** 1942–7

[46] Stiemke M M, Edelhauser H F and Geroski D H 1991 The developing corneal endothelium: correlation of morphology, hydration and Na/K ATPase pump site density *Curr. Eye Res.* **10** 145–56

[47] Laule A, Cable M K, Hoffman C E and Hanna C 1978 Endothelial cell population changes of human cornea during life *Arch. Ophthalmol. (Chicago, Ill: 1960)* **96** 2031–5

[48] EEBA. European Eye Bank Association Directory 2002 (https://www.eeba.eu)

[49] Geroski D H, Matsuda M, Yee R W and Edelhauser H F 1985 Pump function of the human corneal endothelium: effects of age and cornea guttata *Ophthalmology* **92** 759–63

[50] Watsky M A, McDermott M L and Edelhauser H F 1989 *In vitro* corneal endothelial permeability in rabbit and human: the effects of age, cataract surgery and diabetes *Exp. Eye Res.* **49** 751–67

[51] Bonanno J A 2012 Molecular mechanisms underlying the corneal endothelial pump *Exp. Eye Res.* **95** 2–7

[52] Joyce N C 2003 Proliferative capacity of the corneal endothelium *Prog. Retinal Eye Res.* **22** 359–89

[53] Abbenante G and Fairlie D P 2005 Protease inhibitors in the clinic *J. Med. Chem.* **1** 71–104

[54] Raghunathan V *et al* 2013 Influence of extracellular matrix proteins and substratum topography on corneal epithelial cell alignment and migration *Tissue Eng. Part* A **19** 1713–22

[55] Ke Q *et al* 2011 Carrier-free epithelial cell sheets prepared by enzymatic degradation of collagen gel *J. Tissue Eng. Regen. Med.* **5** 138–45

[56] McIntosh Ambrose W *et al* 2009 Collagen vitrigel membranes for the *in vitro* reconstruction of separate corneal epithelial, stromal, and endothelial cell layers *J. Biomed. Mater. Res. Part B: Appl. Biomater.* **90** 818–31

[57] Karkhaneh A *et al* 2011 Novel materials to enhance corneal epithelial cell migration on keratoprosthesis *Br. J. Ophthalmol.* **95** 405–9

[58] Borene M L, Barocas V H and Hubel A 2004 Mechanical and cellular changes during compaction of a collagen-sponge-based corneal stromal equivalent *Ann. Biomed. Eng.* **32** 274–83

[59] Duan X and Sheardown H Dendrimer crosslinked collagen as a corneal tissue engineering scaffold: mechanical properties and corneal epithelial cell interactions *Biomaterials* 2006 **27** 4608–17

[60] Liu L *et al* 2007 Immunological responses in mice to full-thickness corneal grafts engineered from porcine collagen *Biomaterials* **28** 3807–14

[61] Liu Y *et al* 2006 A simple, cross-linked collagen tissue substitute for corneal implantation *Investigative Ophthalmol. Vis. Sci.* **47** 1869–75

[62] Crabb R A, Chau E P, Evans M C, Barocas V H and Hubel A 2006 Biomechanical and microstructural characteristics of a collagen film-based corneal stroma equivalent *Tissue Eng.* **12** 1565–75

[63] Rafat M *et al* 2008 PEG-stabilized carbodiimide crosslinked collagen–chitosan hydrogels for corneal tissue engineering *Biomaterials* **29** 3960–72

[64] Levis H J *et al* 2012 Plastic compressed collagen as a novel carrier for expanded human corneal endothelial cells for transplantation *PLoS One* **7** e50993

[65] Crabb R A and Hubel A 2008 Influence of matrix processing on the optical and biomechanical properties of a corneal stroma equivalent *Tissue Eng. Part* A **14** 173–82

[66] Liu W *et al* 2008 Recombinant human collagen for tissue engineered corneal substitutes *Biomaterials* **29** 1147–58

[67] Merrett K *et al* 2008 Tissue-engineered recombinant human collagen-based corneal substitutes for implantation: performance of type I versus type III collagen *Investigative Ophthalmol. Vis. Sci.* **49** 3887–94

[68] Parenteau-Bareil R, Gauvin R and Berthod F 2010 Collagen-based biomaterials for tissue engineering applications *Materials* **3** 1863–87

[69] Chattopadhyay S and Raines R T 2014 Review collagen-based biomaterials for wound healing *Biopolymers* **101** 821–33

[70] Zhang J, Sisley A M, Anderson A J, Taberner A J, McGhee C N and Patel D V 2015 Characterization of a novel collagen scaffold for corneal tissue engineering *Tissue Eng. Part C: Methods* **22** 165–72

[71] Griffith M *et al* 1999 Functional human corneal equivalents constructed from cell lines *Science* **286** 2169–72

[72] Giasson C J, Deschambeault A, Carrier P and Germain L 2014 Adherens junction proteins are expressed in collagen corneal equivalents produced *in vitro* with human cells *Mol. Vis.* **20** 386 PMCID: PMC3976688

[73] Liu Y, Lv H, Ren L, Xue G and Wang Y 2016 Improving the moisturizing properties of collagen film by surface grafting of chondroitin sulfate for corneal tissue engineering *J. Biomater. Sci. Polym. Ed.* **27** 758–72

[74] Li F *et al* 2005 Recruitment of multiple cell lines by collagen-synthetic copolymer matrices in corneal regeneration *Biomaterials* **26** 3093–104

[75] Bentley E, Murphy C J, Li F, Carlsson D J and Griffith M 2010 Biosynthetic corneal substitute implantation in dogs *Cornea* **29**

[76] Liu W *et al* 2009 Collagen–phosphorylcholine interpenetrating network hydrogels as corneal substitutes *Biomaterials* **30** 1551–9

[77] McLaughlin C R *et al* 2010 Regeneration of functional nerves within full thickness collagen–phosphorylcholine corneal substitute implants in guinea pigs *Biomaterials* **31** 2770–8

[78] Li W *et al* 2014 Fabrication and characterization of chitosan–collagen crosslinked membranes for corneal tissue engineering *J. Biomater. Sci. Polym. Ed.* **25** 1962–72

[79] Vrana N E *et al* 2008 Development of a reconstructed cornea from collagen–chondroitin sulfate foams and human cell cultures *Investigative Ophthalmol. Vis. Sci.* **49** 5325–31

[80] Liu Y, Ren L, Long K, Wang L and Wang Y 2014 Preparation and characterization of a novel tobramycin-containing antibacterial collagen film for corneal tissue engineering *Acta Biomater.* **10** 289–99

[81] Guo Q, Shores L, Schein O, Trexler M M and Elisseeff J H (ed) 2014 Developing biomimetic collagen-based matrix using cyclodextrin for corneal repair *2014 40th Annual Northeast Bioengineering Conf. (NEBEC)* 1–2 IEEE

[82] Long K *et al* 2015 Improving the mechanical properties of collagen-based membranes using silk fibroin for corneal tissue engineering *J. Biomed. Mater. Res. Part* A **103** 1159–68

[83] Duan X, McLaughlin C, Griffith M and Sheardown H 2007 Biofunctionalization of collagen for improved biological response: scaffolds for corneal tissue engineering *Biomaterials* **28** 78–88

[84] Levis H J *et al* 2012 Plastic compressed collagen as a novel carrier for expanded human corneal endothelial cells for transplantation *PLoS One* **7** e50993

[85] Kong B *et al* 2017 Tissue-engineered cornea constructed with compressed collagen and laser-perforated electrospun mat *Sci. Rep.* **7** 970

[86] Chae J J *et al* 2015 Regeneration of corneal epithelium utilizing a collagen vitrigel membrane in rabbit models for corneal stromal wound and limbal stem cell deficiency *Acta Ophthalmol.* **93** e57–66

[87] Calderón-Colón X *et al* 2012 Structure and properties of collagen vitrigel membranes for ocular repair and regeneration applications *Biomaterials* **33** 8286–95

[88] Ruberti J W and Zieske J D 2008 Prelude to corneal tissue engineering–gaining control of collagen organization *Prog. Retin. Eye Res.* **27** 549–77

[89] Germain L *et al* 1999 Reconstructed human cornea produced *in vitro* by tissue engineering *Pathobiology* **67** 140–7

[90] Orwin E J and Hubel A 2000 *In vitro* culture characteristics of corneal epithelial, endothelial, and keratocyte cells in a native collagen matrix *Tissue Eng.* **6** 307–19

[91] Torbet J *et al* 2007 Orthogonal scaffold of magnetically aligned collagen lamellae for corneal stroma reconstruction *Biomaterials* **28** 4268–76

[92] Builles N *et al* 2010 Use of magnetically oriented orthogonal collagen scaffolds for hemi-corneal reconstruction and regeneration *Biomaterials* **31** 8313–22

[93] Chirila T V, Barnard Z, Harkin D G, Schwab I R and Hirst L W 2008 Bombyx mori silk fibroin membranes as potential substrata for epithelial constructs used in the management of ocular surface disorders *Tissue Eng. Part* A **14** 1203–11

[94] Biazar E, Baradaran-Rafii A, Heidari-keshel S and Tavakolifard S 2015 Oriented nanofibrous silk as a natural

scaffold for ocular epithelial regeneration *J. Biomater. Sci. Polym. Ed.* **26** 1139–51

[95] Bray L J, George K A, Ainscough S L, Hutmacher D W, Chirila T V and Harkin D G 2011 Human corneal epithelial equivalents constructed on Bombyx mori silk fibroin membranes *Biomaterials* **32** 5086–91

[96] Lawrence B D, Marchant J K, Pindrus M A, Omenetto F G and Kaplan D L 2009 Silk film biomaterials for cornea tissue engineering *Biomaterials* **30** 1299–308

[97] Gil E S, Mandal B B, Park S-H, Marchant J K, Omenetto F G and Kaplan D L 2010 Helicoidal multi-lamellar features of RGD-functionalized silk biomaterials for corneal tissue engineering *Biomaterials* **31** 8953–63

[98] Madden P W, Lai J N, George K A, Giovenco T, Harkin D G and Chirila T V 2011 Human corneal endothelial cell growth on a silk fibroin membrane *Biomaterials* **32** 4076–84

[99] Jia L, Ghezzi C E and Kaplan D L 2016 Optimization of silk films as substrate for functional corneal epithelium growth *J. Biomed. Mater. Res. Part B: Appl. Biomater.* **104** 431–41

[100] Bray L J, George K A, Hutmacher D W, Chirila T V and Harkin D G 2012 A dual-layer silk fibroin scaffold for reconstructing the human corneal limbus *Biomaterials* **33** 3529–38

[101] Gil E S, Park S H, Marchant J, Omenetto F and Kaplan D L 2010 Response of human corneal fibroblasts on silk film surface patterns *Macromol. Biosci.* **10** 664–73

[102] Kim D K, Sim B R and Khang G 2016 Nature-derived aloe vera gel blended silk fibroin film scaffolds for cornea endothelial cell regeneration and transplantation *ACS Appl. Mater. Interfaces* **8** 15160–8

[103] Suzuki S *et al* 2015 Treatment of silk fibroin with poly (ethylene glycol) for the enhancement of corneal epithelial cell growth *J. Funct. Biomater.* **6** 345–66

[104] Higa K *et al* 2011 Porous silk fibroin film as a transparent carrier for cultivated corneal epithelial sheets *J. Biomater. Sci. Polym. Ed.* **22** 2261–76

[105] Wang L, Ma R, Du G, Guo H and Huang Y 2015 Biocompatibility of helicoidal multilamellar arginine-glycine-aspartic acid-functionalized silk biomaterials in a rabbit corneal model *J. Biomed. Mater. Res. Part B: Appl. Biomater.* **103** 204–11

[106] Zhang S, Li J, Yin Z, Zhang X, Kundu S C and Lu S 2015 Silk fibroin composite membranes for application in corneal regeneration *J. Appl. Polym. Sci.* **132**

[107] Chirila T V, Suzuki S, Bray L J, Barnett N L and Harkin D G 2013 Evaluation of silk sericin as a biomaterial: *in vitro* growth of human corneal limbal epithelial cells on Bombyx mori sericin membranes *Prog. Biomater.* **2** 1–10

[108] Applegate M B *et al* 2016 Photocrosslinking of silk fibroin using riboflavin for ocular prostheses *Adv. Mater.* **28** 2417–20

[109] Ghezzi C E, Marelli B, Omenetto F G, Funderburgh J L and Kaplan D L 2017 3D functional corneal stromal tissue equivalent based on corneal stromal stem cells and multi-layered silk film architecture *PloS one* **12** e0169504

[110] Watanabe R *et al* 2011 A novel gelatin hydrogel carrier sheet for corneal endothelial transplantation *Tissue Eng. Part A* **17** 2213–9

[111] Mimura T *et al* 2008 Tissue engineering of corneal stroma with rabbit fibroblast precursors and gelatin hydrogels *Mol. Vis.* **14** 1819 PMCID: PMC2566587

[112] Lai J-Y, Ma D H-K, Lai M-H, Li Y-T, Chang R-J and Chen L-M 2013 Characterization of cross-linked porous gelatin carriers and their interaction with corneal endothelium: biopolymer concentration effect *PLoS One* **8** e54058

[113] Lai J-Y 2013 Corneal stromal cell growth on gelatin/chondroitin sulfate scaffolds modified at different NHS/EDC molar ratios *Int. J. Mol. Sci.* **14** 2036–55

[114] Niu G, Choi J-S, Wang Z, Skardal A, Giegengack M and Soker S 2014 Heparin-modified gelatin scaffolds for human corneal endothelial cell transplantation *Biomaterials* **35** 4005–14

[115] Lai J-Y and Ma D H-K 2017 Ocular biocompatibility of gelatin microcarriers functionalized with oxidized hyaluronic acid *Mater. Sci. Eng.: C* **72** 150–9

[116] Rizwan M *et al* 2017 Sequentially-crosslinked bioactive hydrogels as nano-patterned substrates with customizable stiffness and degradation for corneal tissue engineering applications *Biomaterials* **120** 139–54

[117] Xu Y-G, Xu Y-S, Huang C, Feng Y, Li Y and Wang W 2008 Development of a rabbit corneal equivalent using an acellular corneal matrix of a porcine substrate *Mol. Vis.* **14** 2180 PMCID: PMC2592998

[118] Du L and Wu X 2011 Development and characterization of a full-thickness acellular porcine cornea matrix for tissue engineering *Artif. Organs* **35** 691–705

[119] Wu Z *et al* 2009 The use of phospholipase A 2 to prepare acellular porcine corneal stroma as a tissue engineering scaffold *Biomaterials* **30** 3513–22

[120] Márquez S P *et al* 2009 Decellularization of bovine corneas for tissue engineering applications *Acta Biomater.* **5** 1839–47

[121] Shafiq M A, Gemeinhart R A, Yue B Y and Djalilian A R 2011 Decellularized human cornea for reconstructing the corneal epithelium and anterior stroma *Tissue Eng. Part C: Methods* **18** 340–8

[122] Zhang C *et al* 2007 Survival and integration of tissue-engineered corneal stroma in a model of corneal ulcer *Cell Tissue Res.* **329** 249–57

[123] San Choi J *et al* 2010 Bioengineering endothelialized neo-corneas using donor-derived corneal endothelial cells and decellularized corneal stroma *Biomaterials* **31** 6738–45

[124] Du L, Wu X, Pang K and Yang Y 2011 Histological evaluation and biomechanical characterisation of an acellular porcine cornea scaffold *Br. J. Ophthalmol.* **95** 410–4

[125] Hashimoto Y *et al* 2010 Preparation and characterization of decellularized cornea using high-hydrostatic pressurization for corneal tissue engineering *Biomaterials* **31** 3941–8

[126] Huang Y-H *et al* 2017 Preparation of acellular scaffold for corneal tissue engineering by supercritical carbon dioxide extraction technology *Acta Biomater.* **58** 238–43

[127] Zhang C *et al* 2017 Construction of tissue-engineered full-thickness cornea substitute using limbal epithelial cell-like and corneal endothelial cell-like cells derived from human embryonic stem cells *Biomaterials* **124** 180–94

[128] Yeh L K, Chen Y H, Chiu C S, Hu F R, Young T H and Wang I 2009 The phenotype of bovine corneal epithelial cells on chitosan membrane *J. Biomed. Mater. Res. Part A* **90** 18–26

[129] Ye J *et al* 2014 Chitosan-modified, collagen-based biomimetic nanofibrous membranes as selective cell adhering wound dressings in the treatment of chemically burned corneas *J. Mater. Chem. B* **2** 4226–36

[130] Young T-H, Wang I-J, Hu F-R and Wang T-J 2014 Fabrication of a bioengineered corneal endothelial cell sheet using chitosan/polycaprolactone blend membranes *Colloids Surf. B: Biointerfaces* **116** 403–10

[131] Gao X, Liu W, Han B, Wei X and Yang C 2008 Preparation and properties of a chitosan-based carrier of corneal endothelial cells *J. Mater. Sci., Mater. Med.* **19** 3611–9

[132] Liang Y *et al* 2011 Fabrication and characters of a corneal endothelial cells scaffold based on chitosan *J. Mater. Sci., Mater. Med.* **22** 175–83

[133] Liang Y, Xu W, Han B, Li N, Zhao W and Liu W 2014 Tissue-engineered membrane based on chitosan for repair of mechanically damaged corneal epithelium *J. Mater. Sci., Mater. Med.* **25** 2163–71

[134] Nishida K *et al* 2004 Functional bioengineered corneal epithelial sheet grafts from corneal stem cells expanded *ex vivo* on a temperature-responsive cell culture surface *Transplantation* **77** 379–85

[135] Sumide T *et al* 2006 Functional human corneal endothelial cell sheets harvested from temperature-responsive culture surfaces *FASEB J.* **20** 392–4

[136] Lai J-Y, Chen K-H and Hsiue G-H 2007 Tissue-engineered human corneal endothelial cell sheet transplantation in a

rabbit model using functional biomaterials *Transplantation* **84** 1222–32

[137] Madathil B K, Anil Kumar P R and Kumary T V 2014 N-isopropylacrylamide-co-glycidylmethacrylate as a thermoresponsive substrate for corneal endothelial cell sheet engineering *BioMed Res. Int.* **2014**

[138] Nitschke M *et al* 2007 Thermo-responsive poly (NiPAAm-co-DEGMA) substrates for gentle harvest of human corneal endothelial cell sheets *J. Biomed. Mater. Res. Part A* **80** 1003–10

[139] Teichmann J *et al* 2013 Human corneal endothelial cell sheets for transplantation: Thermo-responsive cell culture carriers to meet cell-specific requirements *Acta Biomater.* **9** 5031–9

[140] Kobayashi H 2007 Surface modified poly (vinyl alcohol) nanofiber for the artificial corneal stroma *Key Eng. Mater.* **342–3** 209–12

[141] Zainuddin, Barnard Z, Keen I, Hill D J, Chirila T V and Harkin D G 2008 PHEMA hydrogels modified through the grafting of phosphate groups by ATRP support the attachment and growth of human corneal epithelial cells *J. Biomater. Appl.* **23** 147–68

[142] Yañez-Soto B, Liliensiek S, Murphy C J and Nealey P 2013 Biochemically and topographically engineered poly (ethylene glycol) diacrylate hydrogels with biomimetic characteristics as substrates for human corneal epithelial cells *J. Biomed. Mater. Res. Part A* **101** 1184–94

[143] Yanez-Soto B, Liliensiek S J, Gasiorowski J Z, Murphy C J and Nealey P F 2013 The influence of substrate topography on the migration of corneal epithelial wound borders *Biomaterials* **34** 9244–51

[144] Kim E Y, Tripathy N, Cho S A, Lee D and Khang G 2016 Collagen type I–PLGA film as an efficient substratum for corneal endothelial cells regeneration *J. Tissue Eng. Regen. Med.* **11** 2471–8

[145] Zhang Q *et al* 2012 High refractive index inorganic–organic interpenetrating polymer network (IPN) hydrogel nanocomposite toward artificial cornea implants *ACS Macro Lett.* **1** 876–81

[146] Myung D *et al* 2008 Glucose-permeable interpenetrating polymer network hydrogels for corneal implant applications: a pilot study *Curr. Eye Res.* **33** 29–43

[147] Hartmann L *et al* 2011 Toward the development of an artificial cornea: improved stability of interpenetrating polymer networks *J. Biomed. Mater. Res. Part B: Appl. Biomater.* **98** 8–17

[148] Rimmer S *et al* 2007 Epithelialization of hydrogels achieved by amine functionalization and co-culture with stromal cells *Biomaterials* **28** 5319–31

[149] Yañez-Soto B, Liliensiek S, Murphy C and Nealey P 2013 Biochemically and topographically engineered poly (ethylene glycol) diacrylate hydrogels with biomimetic characteristics as substrates for human corneal epithelial cells *J. Biomed. Mater. Res. Part A* **101** 1184–94

[150] Salehi S, Fathi M, Javanmard S H, Barneh F and Moshayedi M 2015 Fabrication and characterization of biodegradable polymeric films as a corneal stroma substitute *Adv. Biomed. Res.* **4**

[151] Tocce E *et al* 2013 The influence of biomimetic topographical features and the extracellular matrix peptide RGD on human corneal epithelial contact guidance *Acta Biomater.* **9** 5040–51

[152] Massie I, Levis H J and Daniels J T 2014 Response of human limbal epithelial cells to wounding on 3D raft tissue equivalents: Effect of airlifting and human limbal fibroblasts *Exp. Eye Res.* **127** 196–205

[153] Lawrence B D, Pan Z, Liu A, Kaplan D L and Rosenblatt M I 2012 Human corneal limbal epithelial cell response to varying silk film geometric topography *in vitro Acta Biomater.* **8** 3732–43

[154] Liu Y, Ren L and Wang Y 2014 A novel collagen film with micro-rough surface structure for corneal epithelial repair fabricated by freeze drying technique *Appl. Surf. Sci.* **301** 396–400

[155] Wilson S L, Wimpenny I, Ahearne M, Rauz S, El Haj A J and Yang Y 2012 Chemical and topographical effects on cell differentiation and matrix elasticity in a corneal stromal layer model *Adv. Funct. Mater.* **22** 3641–9

[156] Teo B K K, Goh K J, Ng Z J, Koo S and Yim E K F 2012 Functional reconstruction of corneal endothelium using nanotopography for tissue-engineering applications *Acta Biomater.* **8** 2941–52

[157] Nara S *et al* 2015 Strategies for faster detachment of corneal cell sheet using micropatterned thermoresponsive matrices *J. Mater. Chem.* B **3** 4155–69

[158] Zhang W, Chen J, Backman L J, Malm A D and Danielson P 2017 Surface topography and mechanical strain promote keratocyte phenotype and extracellular matrix formation in a biomimetic 3D corneal model *Adv. Healthcare Mater.* **6**

[159] Ortega Í, Ryan A J, Deshpande P, MacNeil S and Claeyssens F 2013 Combined microfabrication and electrospinning to produce 3D architectures for corneal repair *Acta Biomater.* **9** 5511–20

[160] Baradaran-Rafii A, Biazar E and Heidari-Keshel S 2015 Cellular response of limbal stem cells on PHBV/gelatin nanofibrous scaffold for ocular epithelial regeneration *Int. J. Polym. Mater. Polym. Biomater.* **64** 879–87

[161] Tonsomboon K and Oyen M L 2013 Composite electrospun gelatin fiber-alginate gel scaffolds for mechanically robust tissue engineered cornea *J. Mech. Behav. Biomed. Mater.* **21** 185–94

[162] Azari P *et al* 2015 Electrospun biopolyesters as drug screening platforms for corneal keratocytes *Int. J. Polym. Mater. Polym. Biomater.* **64** 785–91

[163] Deshpande P *et al* 2010 Using poly (lactide-co-glycolide) electrospun scaffolds to deliver cultured epithelial cells to the cornea *Regen. Med.* **5** 395–401

[164] Wu J, Du Y, Watkins S C, Funderburgh J L and Wagner W R 2012 The engineering of organized human corneal tissue through the spatial guidance of corneal stromal stem cells *Biomaterials* **33** 1343–52

[165] Yan J *et al* 2012 Effect of fiber alignment in electrospun scaffolds on keratocytes and corneal epithelial cells behavior *J. Biomed. Mater. Res. Part A* **100** 527–35

[166] Bakhshandeh H *et al* 2011 Poly (ε-caprolactone) nanofibrous ring surrounding a polyvinyl alcohol hydrogel for the development of a biocompatible two-part artificial cornea *Int. J. Nanomed.* **2011** 1509–15

[167] Phu D, Wray L S, Warren R V, Haskell R C and Orwin E J 2010 Effect of substrate composition and alignment on corneal cell phenotype *Tissue Eng. Part A* **17** 799–807

[168] Ravichandran R *et al* 2013 Mimicking native extracellular matrix with phytic acid-crosslinked protein nanofibers for cardiac tissue engineering *Macromol. Biosci.* **13** 366–75

[169] Salehi S *et al* 2017 Poly (glycerol sebacate)-poly (ε-caprolactone) blend nanofibrous scaffold as intrinsic bio-and immunocompatible system for corneal repair *Acta Biomater.* **50** 370–80

[170] San Choi J *et al* 2013 *In vitro* evaluation of the interactions between human corneal endothelial cells and extracellular matrix proteins *Biomed. Mater.* **8** 014108

[171] Miotto M, Gouveia R M and Connon C J 2015 Peptide amphiphiles in corneal tissue engineering *J. Funct. Biomater.* **6** 687–707

[172] Kokkoli E, Mardilovich A, Wedekind A, Rexeisen E L, Garg A and Craig J A 2006 Self-assembly and applications of biomimetic and bioactive peptide-amphiphiles *Soft Matter* **2** 1015–24

[173] Gouveia R M, Castelletto V, Alcock S G, Hamley I W and Connon C J 2013 Bioactive films produced from self-assembling peptide amphiphiles as versatile substrates for tuning cell adhesion and tissue architecture in serum-free conditions *J. Mater. Chem.* B **1** 6157–69

[174] Gouveia R M, Castelletto V, Hamley I W and Connon C J 2015 New self-assembling multifunctional templates for the biofabrication and controlled self-release of cultured tissue *Tissue Eng. Part A* **21** 1772–84

[175] Jones R R, Castelletto V, Connon C J and Hamley I W 2013 Collagen stimulating effect of peptide amphiphile C16–KTTKS on human fibroblasts *Mol. Pharm.* **10** 1063–9

[176] Uzunalli G *et al* 2014 Bioactive self-assembled peptide nanofibers for corneal stroma regeneration *Acta Biomater.* **10** 1156–66

[177] Fagerholm P *et al* 2010 A biosynthetic alternative to human donor tissue for inducing corneal regeneration: 24-month follow-up of a phase 1 clinical study *Sci. Transl. Med.* **2** 46ra61

[178] Kim D K, Sim B R and Khang G 2016 Nature-derived aloe vera gel blended silk fibroin film scaffolds for cornea endothelial cell regeneration and transplantation *ACS Appl. Mater. Interfaces* **8** 15160–8

[179] Ghezzi C E *et al* 2016 Degradation of silk films in multipocket corneal stromal rabbit models *J. Appl. Biomater. Funct. Mater.* **14** e266–76

[180] Wu J, Du Y, Mann M M, Yang E, Funderburgh J L and Wagner W R 2013 Bioengineering organized, multilamellar human corneal stromal tissue by growth factor supplementation on highly aligned synthetic substrates *Tissue Eng. Part A* **19** 2063–75

[181] Wu J, Du Y, Mann M M, Funderburgh J L and Wagner W R 2014 Corneal stromal stem cells versus corneal fibroblasts in generating structurally appropriate corneal stromal tissue *Exp. Eye Res.* **120** 71–81

[182] Gosselin E A *et al* 2017 Multi-layered silk film co-culture system for human corneal epithelial and stromal stem cells *J. Tissue Eng. Regen. Med.* (https://doi.org/10.1002/term.2499)

[183] Kumar P, Satyam A, Cigognini D, Pandit A and Zeugolis D I 2018 Low oxygen tension and macromolecular crowding accelerate extracellular matrix deposition in human corneal fibroblast culture *J. Tissue Eng. Regen. Med.* **12** 6–18

[184] Nishida K *et al* 2004 Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium *New Engl. J. Med.* **351** 1187–96

[185] Yang S, Yang X and Cao G 2015 Conjunctiva reconstruction by induced differentiation of human amniotic epithelial cells *Genet. Mol. Res.* **14** 13823–34

[186] Mathan J J, Ismail S, McGhee J J, McGhee C N J and Sherwin T 2016 Sphere-forming cells from peripheral cornea demonstrate the ability to repopulate the ocular surface *J. Stem Cell Res. Ther.* **7** 1

[187] Dua H S, Shanmuganathan V, Powell-Richards A, Tighe P and Joseph A 2005 Limbal epithelial crypts: a novel anatomical structure and a putative limbal stem cell niche *Br. J. Ophthalmol.* **89** 529–32

[188] Fagerholm P *et al* 2014 Stable corneal regeneration four years after implantation of a cell-free recombinant human collagen scaffold *Biomaterials* **35** 2420–7

[189] Rama P, Matuska S, Paganoni G, Spinelli A, De Luca M and Pellegrini G 2010 Limbal stem-cell therapy and long-term corneal regeneration *New Engl. J. Med.* **363** 147–55

[190] Wang S, Ghezzi C E, Gomes R, Pollard R E, Funderburgh J L and Kaplan D L 2017 *In vitro* 3D corneal tissue model with epithelium, stroma, and innervation *Biomaterials* **112** 1–9

[191] Kishore V, Iyer R, Frandsen A and Nguyen T-U 2016 *In vitro* characterization of electrochemically compacted collagen matrices for corneal applications *Biomed. Mater.* **11** 055008

[192] de la Cruz Cardona J *et al* 2011 Transparency in a fibrin and fibrin–agarose corneal stroma substitute generated by tissue engineering *Cornea* **30** 1428–35

[193] Ionescu A-M *et al* 2010 UV absorbance of a bioengineered corneal stroma substitute in the 240–400 nm range *Cornea* **29** 895–8

[194] Alaminos M *et al* 2006 Construction of a complete rabbit cornea substitute using a fibrin-agarose scaffold *Investigative Ophthalmol. Vis. Sci.* **47** 3311–7

[195] Gouveia R M *et al* 2017 Controlling the 3D architecture of self-lifting auto-generated tissue equivalents (SLATEs) for optimized corneal graft composition and stability *Biomaterials* **121** 205–19