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Experimental Eye Research

Development, structure and bioengineering of the human corneal stroma: a review

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Abstract:	<p>Bio-engineering technologies are currently developed to produce biomimetic artificial corneas which should present structural, chemical, optical and biomechanical properties close to the native tissue. These properties are mainly supported by the corneal stroma which accounts for 90% of corneal thickness and is mainly made of collagen type I. The stromal collagen fibrils are arranged in lamellae that have a plywood-like organization. The fibril diameter is between 25 and 35 nm and the interfibrillar space about 57 nm. The number of lamellae in the central stroma is estimated to be 300. In the anterior part their size is 10-40 μm. They appear to be larger in the posterior part of the stroma with a size of 60-120 μm. Their thicknesses also vary from 0.2 to 2.5 μm. During development, the acellular primary corneal stroma, which features a complex pattern of organization, serves as a scaffold for mesenchymal cells that invade and produce the secondary stroma. Several pathways including Bmp4, Wnt/β-catenin, Notch, retinoic acid, and TGF-β, in addition to EFTFs including the master gene Pax-6, are involved in corneal development. Besides, retinoic acid and TGF-β seem to have a crucial role for the neural crest cell migration in the stroma. Several technologies can be used to produce an artificial stroma. Taking advantage of the liquid-crystal properties of acid-soluble collagen, transparent stroma-like matrices with native-like collagen I fibrils and plywood-like organization can be produced and further colonized by epithelial cells. Other approaches include the use of recombinant collagen, cross-linkers, vitrification, plastically compressed collagen or magnetically aligned collagen, providing interesting optical and mechanical properties. These technologies can be classified according to collagen type and origin, presence of telopeptides and native-like fibrils, structure and transparency. Collagen matrices feature transparency >80% for the appropriate 500-μm thickness. Non-collagenous matrices made of biopolymers including gelatin, silk or fish scale have been developed which feature interesting properties but are less biomimetic. These bioengineered matrices still need to be colonized by stromal cells in order to fully reproduce the native stroma.</p>
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DEBORAH FERRINGTON, PHD
Reviews and Special Issues Editor
Experimental Eye Research

Paris, 06/08/2020

Dear Editor, dear Colleague

Following your confirmation mail, we submit our INVITED REVIEW ARTICLE entitled "**Development, structure and bioengineering of the human corneal stroma: a review**" to *Experimental Eye Research* for publication.

Our rationale for submitting this review paper is the following: Several research groups are developing bioengineering approaches to produce an artificial cornea that could be suitable for transplantation. As the aim is to synthesize the material the closest to the native tissue, we are interested in reviewing the current knowledge concerning artificial corneas with regards to the cornea organogenesis, the main signaling pathways involved in the stromal development and its structure. Our personal approach for the synthesis of an artificial cornea has already been described and relates on the liquid-crystal properties of acid-soluble collagen to induce a stroma-like 3D organization followed by an increase in pH collagen to mimic fibrillogenesis and stabilize the organization. Other approaches were developed including the use of recombinant collagen, cross-linkers, plastically compressed collagen or magnetically aligned collagen for instance. However, all these techniques failed to incorporate keratocytes inside the scaffold. That's why we intended to look closer to what happens *in vivo* during development, how the cornea is synthesized, what the major signaling pathways are, how the second wave of neural crest cells is able to colonize the stroma and what drives the stromal organization. This last question is a key issue for the development of artificial corneas: is it the role of the keratocytes to induce the characteristic plywood organization of the stroma, or does this organization precede the keratocytes invasion. Several papers underlined the role of the primary stroma acting as a template for the keratocytes that will further synthesize the collagen fibrils, and more recent studies reported an organization of the keratocytes prior to an organization of collagen fibrils. Also, it appears that several signaling pathways including TGF β and retinoic acid are crucial for appropriate neural crest cells migration. Thus, there is room for improvement in the artificial corneas field to be more biomimetic. It appears that there are still no artificial corneas that fit with all the requirements: mechanical properties, transparency, structure, inclusion of all cell types and particularly of the keratocytes. And a better understanding of the *in vivo* process may be needed for further developments.

With this, we strongly believe that this work would be of interest for the community reading *Experimental Eye Research* and we hope that this review will be favorably received.

Yours sincerely

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Highlights:

- During development, the acellular primary corneal stroma serves as a scaffold for mesenchymal cells that invade and produce the secondary stroma.
- Liquid-crystal properties of acid-soluble collagen can be used to produce transparent stroma-like matrices with native-like collagen I fibrils and plywood-like organization.
- Other approaches include the use of recombinant collagen, cross-linkers, plastically compressed collagen or magnetically aligned collagen.
- Bioengineered matrices still need to be colonized by stromal cells in order to fully reproduce the native stroma.

Development, structure and bioengineering of the human corneal stroma: a review

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Abstract

Bio-engineering technologies are currently developed to produce biomimetic artificial corneas which should present structural, chemical, optical and biomechanical properties close to the native tissue. These properties are mainly supported by the corneal stroma which accounts for 90% of corneal thickness and is mainly made of collagen type I. The stromal collagen fibrils are arranged in lamellae that have a plywood-like organization. The fibril diameter is between 25 and 35 nm and the interfibrillar space about 57 nm. The number of lamellae in the central stroma is estimated to be 300. In the anterior part their size is 10-40 μm . They appear to be larger in the posterior part of the stroma with a size of 60-120 μm . Their thicknesses also vary from 0.2 to 2.5 μm . During development, the acellular primary corneal stroma, which features a complex pattern of organization, serves as a scaffold for mesenchymal cells that invade and produce the secondary stroma. Several pathways including Bmp4, Wnt/ β -catenin, Notch, retinoic acid, and TGF- β , in addition to EFTFs including the master gene Pax-6, are involved in corneal development. Besides, retinoic acid and TGF- β seem to have a crucial role for the neural crest cell migration in the stroma. Several technologies can be used to produce an artificial stroma. Taking advantage of the liquid-crystal properties of acid-soluble collagen, transparent stroma-like matrices with native-like collagen I fibrils and plywood-like organization can be produced and further colonized by epithelial cells. Other approaches include the use of recombinant collagen, cross-linkers, vitrification, plastically compressed collagen or magnetically aligned collagen, providing interesting optical and mechanical properties. These technologies can be classified according to collagen type and origin, presence of telopeptides and native-like fibrils, structure and transparency. Collagen matrices feature transparency >80% for the appropriate 500- μm thickness. Non-collagenous matrices made of biopolymers including gelatin, silk or fish scale have been developed which feature interesting properties but are less biomimetic. These bioengineered matrices still need to be colonized by stromal cells in order to fully reproduce the native stroma.

Keywords

Artificial cornea, collagen, keratocytes, stromal structure, cornea development

1 Introduction

Approximately 10 million people are affected by corneal blindness worldwide. Corneal allograft (i.e., keratoplasty) still remains the best solution to treat such a disease. This is very efficient in short term but a rejection rate of 15% leads to 10% of failure within two years according to a study conducted in Sweden (Claesson and Armitage, 2009). At a longer term, the failure rate of the allograft increases (Williams et al., 2012). Indeed, 50% are still viable 10-15 years after the surgery. The lifespan of a penetrating keratoplasty is usually limited to 30 years (Borderie et al., 2009). In the case of patients with for instance Stevens-Johnson syndrome or those whose cornea was severely damaged by vascularization or alkali burn, the allograft is less well tolerated. The success rate is very low, close to zero, especially if a second graft is required (Khan et al., 2001). As donor corneal tissue shortage is a crucial issue, many research groups have developed corneal stroma substitutes, which do not simultaneously feature all the optical, biological and mechanical characteristics of the native tissue. In the aim of being close to what really happens *in vivo*, we reviewed the development of the corneal stroma in order to understand the role of each milestone in its resulting complex structure. One element that still raises questions is the origin of stroma structure and the role of the keratocytes in its organization. Also, it is important to identify the genes controlling stromal development and to understand the role and the time-specificity of molecules like proteoglycans acting in the formation of the stroma. A lot has already been made in order to replace the whole or a part of the cornea but only keratoplasty has remained the gold standard.

In this review we will focus on therapeutic approaches trying to be as biomimetic as possible, using biopolymers, incorporating or not cells, and we will not deal with synthetic polymers like the ones that have been used in the past to develop keratoprosthesis. Indeed, the use of very hydrophilic systems such as hydrogels of poly(2-hydroxymethylmethacrylate) (pHEMA) helped to avoid extrusion, inflammation and immune response issues but calcification problems were underlined (Vijayasekaran et al., 2000). Clinical trials were performed on the AlphaCor Kpro (Crawford et al., 2002) and showed that the device was well integrated in the cornea but there was no reepithelialization of its anterior surface. Several other studies (Hicks et al., 2002; Hicks et al., 2003; Hicks et al., 2004; Crawford et

al., 2005) showed limitations such as poor biointegration, formation of a retroprosthetic membrane, inability to measure the intraocular pressure and decrease in optic properties (Hicks et al., 2003). Another artificial synthetic cornea widely studied is the Boston Kpro developed by Dohlman-Doane (Doane et al., 1996). These devices do not have all the required properties to efficiently replace a corneal stroma. In addition, the Boston Kpro requires the use of a corneal graft, thus not reducing the impact of lack of donor tissues in the treatment of corneal blindness. Besides, we will not consider methods using extracellular matrix deposition by cells (Nishida et al., 2004; Kobayashi et al., 2013) and methods using decellularized materials (Lin et al., 2017; Hashimoto et al., 2010). Thus, we will present the works using either collagen (type I or type III) or other biopolymers to produce a mimetic scaffold. The review will underline the different steps of corneal development, focusing particularly on the stroma and its structural development. Different major signaling pathways will be described so as the role of several genes like *PAX6* and proteins like BMP4 and TGF β . The structure of the cornea and the properties of its major component, collagen I, are also described, in order to better understand the progresses already been made to replace a stroma and to provide an overview of the issues at stake and the improvements to make.

2 Cornea embryology and major signaling pathways

2.1 Overview of eye embryology

The eye development has been widely studied in frogs, zebrafish, chick, and mice. The eye is composed of three ectodermal derivatives of the embryo in the head region: the neuroectoderm (neural epithelium) forms the retina, the surface non-neural ectoderm is at the origin of the lens and part of the cornea and the neural crest yields mid and posterior parts of the cornea (Fig. 1) (Graw et al., 2010).

Following gastrulation, several patterning events occur for the neuroectoderm. Particularly, a patch of the neuroectoderm in the head region becomes the neural part of the eye that is called "eye field". During neurulation, two extensions from the eye field evaginate laterally to form the optic vesicles that then extend and contact the surface non-neural ectoderm. In the area of contact, the surface ectoderm

will develop into the lens placode and into primordia of other anterior structures such as the corneal and conjunctival epithelia (i.e., the ocular surface epithelium). The lens placodes and optic vesicles invaginate simultaneously to create the lens pit and the optic cup, respectively. The lens pit subsequently detaches from the adjacent progenitors of the overlying ectoderm to become the lens vesicle. Following the detachment of the lens vesicle, migratory mesenchymal cells of neural crest origin invade the space between the surface ectoderm and the lens. This layer, increasing in thickness, then separates into two parts, with the anterior one becoming the cornea, and the posterior one forming the anterior chamber of the eye. Neural crest cells of the periocular mesenchyme increase in number and condense between the lens vesicle and the corneal epithelium to give rise to the corneal endothelium in a first wave, and to the corneal stroma in a second wave. The neural crest cells are responsible for normal ocular development thanks to the lack of *Hox* gene function in the presumptive ocular neural crest area. When the neural crest cells migrate into the periocular region, they express different markers, such as the transcription factor Sox10 and the carbohydrate epitope HNK-1, which later are downregulated when the neural crest cells differentiate into different ocular tissues. The peripheral regions of the optic cup contain the progenitors, which, together with neural crest cells emanating from the ocular mesenchyme, form the auxiliary structures of the anterior segment: the ciliary body and the iris. It is here also important to underline the role of the lens in cornea development. Indeed, lens ablation experiments have shown that the presence of the lens is required for cornea formation. Without lens, neural crest cells are able to migrate but they fail to form the corneal endothelium and stroma (Beebe and Coats, 2000).

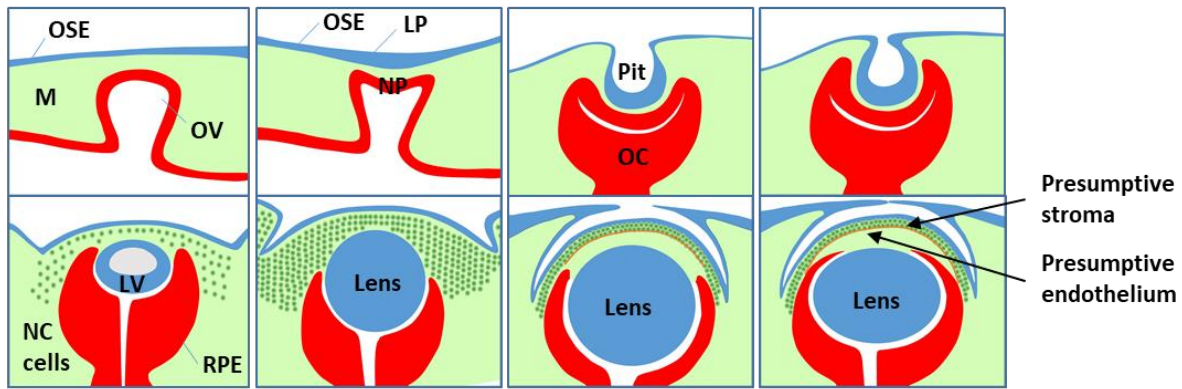


Fig. 1: Schematic representation of the events leading to the formation of the cornea. Extension from the eye field evaginates laterally to form the optic vesicle (OV) that then extends and contacts the surface non-neural ectoderm. In the area of contact, the surface ectoderm (OSE) will develop into the lens placode (LP) and into primordia of other anterior structures such as the corneal and conjunctival epithelia (i.e., the ocular surface epithelium, OSE). The lens placode and optic vesicle invaginate simultaneously to form the lens pit and the optic cup (OC), respectively. The lens pit subsequently detaches from the adjacent progenitors of the overlying ectoderm to become the lens vesicle. Following the detachment of the lens vesicle, migratory mesenchymal cells of neural crest origin (NC cells) invade the space between the surface ectoderm and the lens. This layer, increasing in thickness, then separates into two parts with the anterior one becoming the cornea and the posterior one forming the anterior chamber of the eye. M, mesenchyme; NP, neural plate; RPE retina pigment epithelium. Figure adapted from Lang et al., (2004).

2.2 Role of the master gene *PAX6* and major signaling pathways

Development of the cornea is mainly regulated by the master regulatory gene *PAX6* and its interactions with different major signaling pathways, including bone morphogenetic proteins (BMPs), retinoic acid (RA), transforming growth factor beta (TGF β) and members of the Wnt family of proteins. Their interactions and main roles are sum-up in Table 1.

2.2.1 *PAX6*

The ocular ectoderm is a multipotent region, forming in particular the lens, and the corneal and conjunctival epithelia. The corneal bud splits from the rest of the ectodermal buds. MicroRNA miR450b5p is responsible for maintaining the multipotent state of the surface ectoderm by controlling the activity of *PAX6* gene (Shalom-Feuerstein et al., 2012). Thanks to the contact with the neural plate expressing *PAX6*, this part of the ectoderm also starts to express *PAX6*, necessary for cornea and lens development. Then, there is a segregation of this pool of common progenitors into cells producing either the lens (in the central part) or the corneal and conjunctival epithelia (in the periphery) (Bailey

et al., 2006). Human PAX6 protein is expressed in the early neural plate during neurulation (Zhang et al., 2010) and was found to be necessary and sufficient for the differentiation of human embryonic stem cells to neuroectoderm. Specification of the neuroectoderm starts with the coordinated expression of eye-field transcription factors (EFTFs) at the anterior neural plate. The EFTF genes include *SIX3*, *LHX2*, *RAX (RX)*, *TBX3*, *OPTX2 (SIX6)*, *NR2E1 (TLX)* and *PAX6*, which can be expressed in conjunction with *OTX2*, the head-determination gene. EFTFs co-regulate each other. Indeed, in mice *PAX6* overexpression induces upregulation of *Six3*, *Lhx2*, *Tlx* and *Optx2*, while *Six3* expression provokes that of *PAX6*, *Tlx* and *Lhx2* (Zuber et al., 2003). *PAX6* is one of the first EFTFs to be expressed in a wide area of the neural plate that includes the eye field. Also, *PAX6* regulates human corneal epithelial cell identity (Kitazawa et al., 2016). *PAX6* knockdown causes a loss of epithelial cell identity by down-regulation of corneal epithelial-related genes (*KRT3* and *KRT12*) and up-regulation of epidermis- and keratinization-related genes such as *KRT10*. The loss of *PAX6* converts corneal epithelial stem cells to epidermal stem cells and *PAX6* is expressed in the primitive eye cup first, and later, in corneal tissue progenitors (Li G et al., 2015). Lens vesicle detachment is the stage the most sensitive to *PAX6* and requires a diploid level of *PAX6* expression. In both aniridia patients and the mouse heterozygous *Small eye (Sey)* model, the lens fails to detach from the overlying cornea (Shaham et al., 2012). Besides, corneal stroma formation is strongly related to the maintenance of *PAX6* signaling. A precise level of expression of *PAX6* is necessary for a complete and normal corneal development. Heterozygous *Sey* mice with *PAX6* mutations exhibit opaque cornea (Hogan et al., 1986; Hill et al., 1991). In humans, there are also correlations between heterozygous mutations in *PAX6* and Peters' anomaly, which includes opaque cornea, even if most cases of Peters' anomaly are not due to *PAX6* mutations. Progressive development of limbal stem cell deficiency associated with corneal stromal scarring after birth or in adults, is a feature of aniridia, a congenital disease related to *PAX6* mutations (Hanson et al., 1994).

2.2.2 Wnt and Notch pathways

Wnt/ β -catenin signaling pathway is of major importance in several aspects of corneal development through the regulation of various growth factors. The Wnt and Notch pathways are active in the

corneal epithelium and regulate cell proliferation, differentiation and stratification in the cornea. Notch plays a role in corneal epithelial differentiation. Notch proteins are transmembrane proteins and four *Notch* genes have up to date been identified, whose mutations induce severe abnormalities in many tissue development. The main ligands of Notch are Jagged proteins and Delta proteins. Notch1 and Jagged1 are expressed mainly in the basal and suprabasal layers of the corneal epithelium (Djalilian et al., 2008). When *Notch1* is deleted, mice develop a skin-like epithelium in the cornea (Nicolas et al., 2003). Notch1 regulates retinoid acid metabolism in the corneal epithelium by regulating cellular retinol binding protein 1 expression (Vauclair et al., 2007). During early stages of normal development, Wnt/ β -catenin modulates corneal epithelium stratification through inhibition of Bmp4. In the peripheral region, the inactivation of *Bmp4* down-regulates *PAX6*. Before eyelid opening, Wnt/ β -catenin signaling starts to be inactivated, causing an increase in Bmp4 expression. Bmp4 then stimulates p63 expression in the corneal epithelium, and corneal epithelial stratification is initiated (Zhang Y et al., 2015). Also, corneal epithelium and Bowman's layer are formed by ectodermal cells, which largely depend upon Wnt signaling (Chang et al., 2004). Loss of *Dkk2*, an antagonist of the canonical Wnt signaling pathway, can prevent corneal differentiation during mouse development (Gage et al., 2008; Mukhopadhyay et al., 2006). Concerning the stroma, Wnt signaling has a paracrine effect. Indeed, there is expression of Fzd receptors correlated with an activation of Wnt signaling in the stromal mesenchyme and corneal endothelium (Liu H et al., 2003). Particularly, *Fzd3* is involved in neural crest cells induction and migration (Yanfeng et al., 2006). A reduced expression of *Fzd3* and *Fzd4* coupled with an upregulation of *Fzd10* seems required for corneal cell differentiation and avascularity (Corda and Sala, 2017; Chesnutt et al., 2004; Panzica et al., 2019).

2.2.3 Retinoic acid

Retinoic acid (RA) is synthesized by the optic cup and by the neuroepithelium and acts on the neural crest-derived periocular mesenchyme, which expresses several RA receptors proved to be major players in the formation of eye tissues in mice (Chambon et al., 1996). In humans, mutations in RA signaling pathway can induce corneal opacity and glaucoma associated with Axenfeldt/Rieger syndrome or congenital anterior dysgenesis (Canete et al., 2017). RA induces expression of the

transcription factors Foxc1 (forkhead box C1) and Pitx2 in the periocular mesenchyme (Kume, 2010; Kidson et al., 1999). Foxc2 and Foxc1 are together essential through their interactions for proper morphogenesis of the ocular surface by regulating Wnt signaling. Besides, the homeodomain transcription factor Pitx2 (and its target Dkk2) play a role in the specification and maintenance of cells in the corneal and conjunctival ectoderm. In corneas of *Foxc1*^{-/-} mice, the epithelium is thickened, the stroma appears disorganized and there is no differentiation of the endothelium. Also, the lens fails to separate from the cornea (Kidson et al., 1999). In human, mutations of *FOXCI* are associated with Peters 'anomaly.

2.2.4 TGFβ

TGFβ is essential for neural crest cell migration, proliferation and differentiation. It is expressed by the lens epithelium (Banh et al., 2006; Flugel-Koch et al., 2002) and lack of TGFβ is associated with impaired neural crest cell migration. TGFβ1 gain of function can prevent differentiation and formation of a transparent cornea. TGFβ (1, 2 and 3) also modulate or inhibit cell growth. TGFβ1 promotes proliferation of neural crest cells in the presence of FGF2, which is expressed in cornea and lens (Lovicu et al., 1997). The growth factor PDGF-A may also be induced by TGFβ1 to promote proliferation of corneal mesenchymal cells. PDGF-A is expressed in the endothelium and its overexpression leads to thickening of the stroma (Reneker and Overbeek, 1996). Fibronectin, which is localized in the extracellular matrix of the periocular mesenchyme and cornea (Doane et al., 1996), also has a critical role in neural crest cell migration (Perris and Perissinotto, 2000), as well as thrombospondin-1 (Tucker et al., 1999). The expression of these two matrix proteins is induced by TGFβ1. Besides, TGFβ has a role in corneal endothelium and stroma formation. The corneas of TGFβ2^{-/-} mice have reduced accumulation of extracellular matrix and are thinner with densely packed keratocytes. Saika et al. also exhibited the important role of TGFβ2 for a normal ocular morphogenesis, particularly regarding the thickness of the stroma (Saika et al., 2001). Besides, the corneal endothelium fails to differentiate (Saika et al., 2001). Also, Ma and Lwigale (Ma and Lwigale, 2019) showed that TGFβ is involved in the transition from highly proliferative to differentiating neural

crest cells in the developing mouse cornea, which involves a TGF β -induced upregulation of several regulators of ECM remodeling and collagen synthesis (Tumelty et al., 2014).

Table 1 Summary of the main actions of the major actors in corneal development. The last line underlines the interactions between them.

Events	Pax6	Wnt/Notch	Retinoic acid	TGFβ
Differentiation of human embryonic stem cells to neuroectoderm	+	Not found in literature	Not found in literature	Not found in literature
Eye Field Transcription Factors expression	+	Not found in literature	Not found in literature	Not found in literature
Lens vesicle detachment	+	Not found in literature	Not found in literature	+
Corneal epithelial cell differentiation	+	+ (stratification)	+ (role in the specification and maintenance of cells in the corneal and conjunctival ectoderm)	Not found in literature
Neural crest cells migration, proliferation and differentiation	+ (?)	Fzd expression: - Neural crest cells migration - avascularity	+ (migration)	+ (migration, proliferation and differentiation)
Bowman's layer formation	Not found in literature	+	Not found in literature	Not found in literature
Stroma development	Not found in literature	Not found in literature	Not found in literature	+ (stroma thickness, upregulation of Aebp1 and Creb3l1, important for collagen synthesis)
Interactions		Role on Bmp4 expression -regulating PAX6, -increasing p63 α	Role on Foxc1 & pitx2 expression: regulates Wnt	

3 Description of the corneal stromal structure during development and in adulthood

In attempt to replicate *in vitro* the process of stromal formation, we focus on the works that described stroma deposition. A major issue for bioengineering is to understand what drives the stromal organization.

To study corneal development and particularly the stroma, the chick embryo was often used as a model (Coulombre AJ and Coulombre JL, 1958; Coulombre AJ and Coulombre JL, 1961; O’Rahilly and Meyer, 1959; Anseth A, 1961; Hay and Revel, 1969; Conrad GW, 1970; Trelstad and Coulombre AJ, 1971; Toole and Trelstad, 1971). The organization of the stroma likely depends on the secretion of the acellular primary stroma followed by invasion of the neural crest cells and deposition of the secondary stroma by neural crest-derived keratocytes. Trelstad and Coulombre (Trelstad and Coulombre AJ, 1971) further studied corneal stromal development in chick. The corneal stroma in the adult chicken is mainly composed of water, collagen, glycosaminoglycans and stromal fibroblasts. The stroma is approximately 200 μm thick and formed by layers lying roughly parallel to the corneal surface. These layers are about 2-4 μm in thickness. The collagen fibrils within each layer seem oriented in the same direction and at approximately right angles to the collagen fibrils in two adjacent layers, forming a plywood-like structure. There is also a gradual angular shift of the layers in a clockwise direction proceeding from the outer to inner layers (Coulombre AJ, 1965). In the chick embryo, the corneal stroma develops within the framework of an acellular collagenous matrix, called the post-epithelial layer (O’Rahilly and Meyer, 1959) or primary corneal stroma (Hay and Revel, 1969). The primary corneal stroma, produced by the corneal epithelium (Hay and Revel, 1969; Goodfellow and Revel, 1969), is deposited in an orthogonal pattern beneath the epithelial surface (Laguesse, 1926, Hay and Revel, 1969). The acellular primary corneal stroma is believed to serve as a scaffold for mesenchymal cells that invade and produce the adult or secondary corneal stroma, and it has been suggested that the organization of the primary corneal stroma possesses the complex pattern of organization found in the adult (Coulombre AJ, 1965).

More recently, a process called keratopodia was studied thanks to scanning block face electron microscopy. It shows the involvement of a keratocyte process allowing the synchronized deposition of a collagen matrix along a cellular protrusion (Young et al., 2014). This process was proposed to explain the collagen fibrils organization, giving to the keratocytes a much more active role in the stromal organization, similarly to the role of fibroblasts in tendon organization for instance (Canty et al., 2004). This view is supported by Koudouna et al. (Koudouna et al., 2018) after studying the morphogenesis of the chick cornea by non-linear optical SHG imaging and confocal microscopy. These authors underlined that the organization, contrary to what was mentioned before, seemed to appear at embryonic day 10 (E10), following keratocyte invasion and deposition of the primary stroma. Keratocyte rotation preceding that of collagen further supports a role for the keratocytes in the stromal arrangement. It seems that keratocytes develop a new stroma and not just use the primary stroma as a scaffold. Wang et al. (Wang F et al., 2004) have also underlined the role of cellular orientation in matrix deposition.

4 Structure

As previously mentioned in this review, the cornea is a connective tissue, containing collagen forming fibrils in a particular architecture. The cornea is curved and the reasons why are still not known, but the collagen organization might have a role. The air-tear film interface is the main focusing component, and 80 to 95% of the visible light passing through the cornea is transmitted. Concerning the macroscopic structure, the human cornea is about 520 μm thick (Fig. 2) at the center but can increase to 700 μm at the periphery, keeping in mind that all the dimensions can vary between individuals.

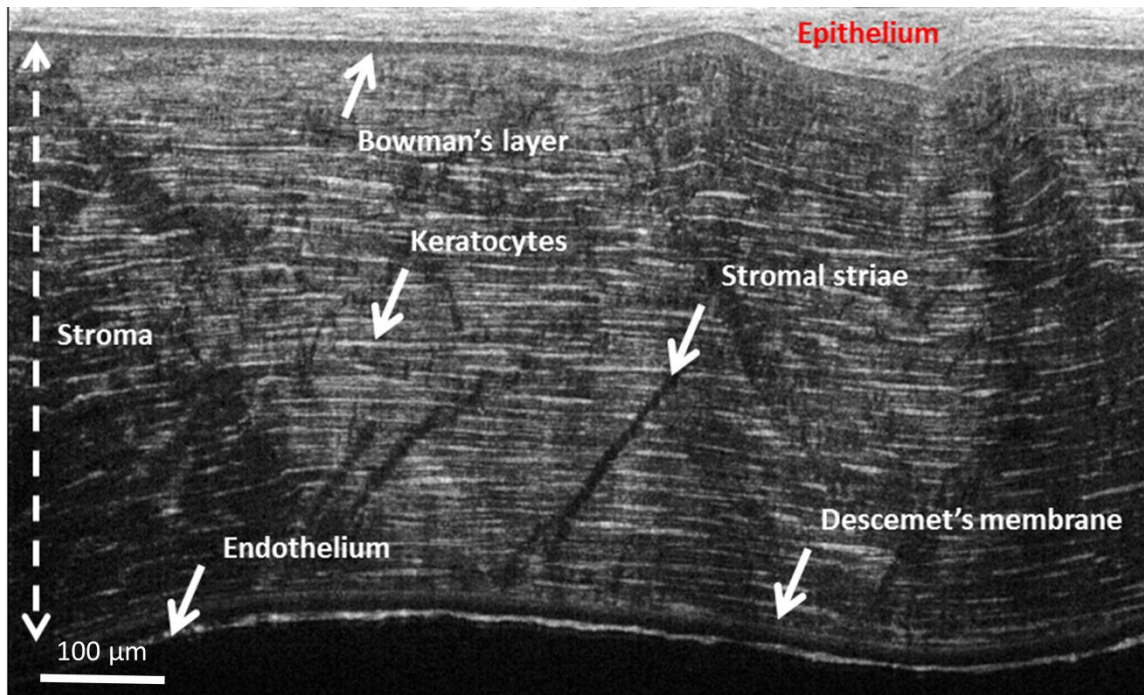


Fig. 2: Full-field optical coherence tomography view of a whole human cornea. The different layers can be seen: the epithelium, Bowman's layer, the stroma with the keratocytes (the density varies from the anterior stroma with a higher density, to the posterior stroma), Descemet's membrane and the endothelium. The stromal striae can also be seen, exiting from Descemet's membrane and running obliquely. The stroma is approximately 470 μm thick.

The anterior surface of cornea appears to be smaller in the vertical dimension with a diameter of about 11mm while it is 12mm for the horizontal dimension (in front view). Also, it is more curved than the rest of the eyeball with a radius of curvature of 7.7 mm (at the front surface).

From the anterior side to the posterior side, there is the epithelium, Bowman's layer, the stroma, Descemet's membrane and the endothelium. We will mainly focus on the stromal structure. The stroma is approximately 470 μm thick, representing 90% of the corneal thickness. It is essentially formed by an extracellular matrix, collagen fibrils and keratocytes. It is usually separated in three main regions, i.e. the anterior, mid and posterior stroma, which differ by organization of the collagen lamellae, hydration rate, biomechanical properties and cell density. The very anterior stroma, beneath Bowman's layer features a very high density of keratocytes.

The stromal collagen fibrils are arranged in lamellae that have a plywood-like organization (Figure 3).

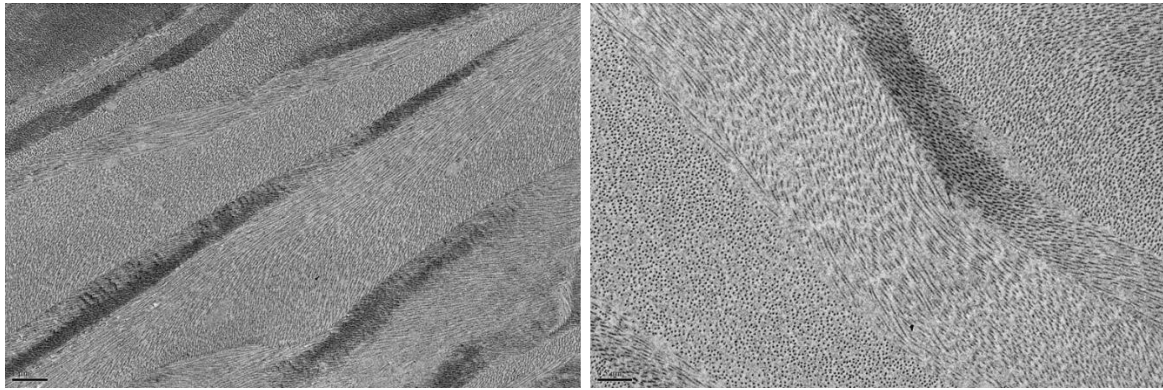


Figure 3: Transmission Electron Microscopy pictures of the anterior stroma (left) and the posterior stroma (right) showing the lamellar organization of the collagen fibrils. In a lamella the fibrils have the same orientation and in an adjacent lamella the fibrils have a different orientation, approximately perpendicular. Scale bar: 0.5 μm .

It means that in a lamella, the fibrils are parallel. And in the adjacent lamella, the fibrils are oriented in a perpendicular direction, or at least, there is a large rupture angle between two lamellae. The angle between adjacent lamellae is close to 90° . These lamellae are composed of heterofibrils formed by collagen I and collagen V (Birk et al., 1990). Collagen type V is at the center and surrounded by collagen type I. The lamellae are not all parallel to the corneal curvature. Some lamellae are obliquely inserted in Bowman's layer. Also, the plywood-organized domains appear to be larger in the posterior part of the stroma with a size of 60-120 μm (Komai and Ushiki, 1991). In the anterior part their size is 10-40 μm . Their thicknesses also vary from 0.2 to 2.5 μm (Komai and Ushiki, 1991). The number of lamellae in the central stroma is estimated to be 300 (Birk et al., 2001). The fibrils diameter is between 25 and 35 nm (Meek and Leonard, 1993; Daxer et al., 1998) and the interfibrillar space of about 57 nm (center to center) (Meek and Knupp, 2015). The diameter increases dramatically to 50 nm at the limbus (Boote et al., 2003). The posterior stroma is more hydrated (Turss et al., 1971) and has a more important swelling capacity than the anterior stroma. Also, in the posterior stroma, lamellae with the same fibril direction are more parallel and regularly arranged. The arrangement is less precise in the anterior stroma (Freund et al., 1995). Freund et al. (Freund et al., 1995) has also shown that the fibril diameter does not vary with depth. The keratocyte density also differs, with a higher density in the anterior stroma (Borderie M et al., 2017).

The corneal stroma also features presence of striae, which correspond to undulations of the lamellae. Striae generally depart from Descemet's membrane in the posterior stroma and run obliquely in a V-

shape toward Bowman's layer (Fig. 2). They are associated with discontinuity of rigidity and they play a role in the biomechanical behavior of cornea; i.e., to absorb increases of intraocular pressure and external shocks. They predominantly contain collagen VI, lumican and keratocan (Grieve et al., 2017).

As for all collagens, collagen I is formed by three polypeptide chains called α chains. Collagen I, which accounts for 90% of the extracellular matrix, is heterotypic since it is composed of two different α chains called $\alpha 1$ and $\alpha 2$. There are two $\alpha 1$ chains. The primary sequences of $\alpha 1$ and $\alpha 2$ are similar, made of approximately a thousand amino acids with a repetitive central sequence $(\text{Gly-X-Y})_n$ where X and Y are often proline or hydroxyproline. These imino acids (amino acids containing a carboxyl and an imine group) account for 30% of the α -chain residues. Also, they are cyclic molecules, provoking a steric hindrance and increasing the chain's stiffness by reducing the number of stable conformations (Silver et al., 2002). To minimize the constraints related to these imino acids, the $(\text{Gly-X-Y})_n$ domain is structured in a left helix called PPII since its structure is close to the polyproline helix (Ramachandran, 1988; Berisio et al., 2009). The helicoidal pitch is 8.68 nm. A single α -chain is not stabilized by interchain hydrogen bonds. The folding of the α -chains into left helix is mainly due to the steric repulsion between proline residues in position "X" and the 4-Hydroxyproline in "Y" position. The regular repetition of the glycine allows the trimerization of the helicoidal- α chains to create a triple right helix that is stabilized by several interchain hydrogen bonds. All the glycine residues are oriented towards the interior of the helix thus exposing at the surface the other residues that favor an interaction with other molecules, like collagen (Ramachandran, 1988, Hulmes et al., 1995; Hulmes, 2002). At the N-terminal and C-terminal parts of the α -chain, there are two domains called telopeptides composed of approximately 20 amino acids. These domains are not organized in a helicoidal manner but they have an important role in the formation of collagen fibrils. The distribution of the amino acids Lysine, Glutamine and Arginine has a periodicity of 18 residues. The agglomeration of hydrophobic residues has a periodicity of 234 residues which corresponds to the characteristic distance $D=67$ nm found in the fibrils (Ottani et al., 2002). *In vivo*, the collagen is synthesized by the cells in the procollagen form, which is soluble in the physiological medium. Collagen molecules form microfibrils with a diameter of 4 nm (Holmes et al., 2001), which are tilted by about 15° to the fibril axis (Baldock et al., 2002). Then, it is the enzymatic cleavage of the C and N

propeptides that leads to an insoluble collagen molecule in the physiological medium and to self-association into fibrils outside the cell. The cleavage begins during the carrying of procollagen in vesicles with metalloproteases from the Golgi apparatus and ends in the extracellular space (Figure 4).

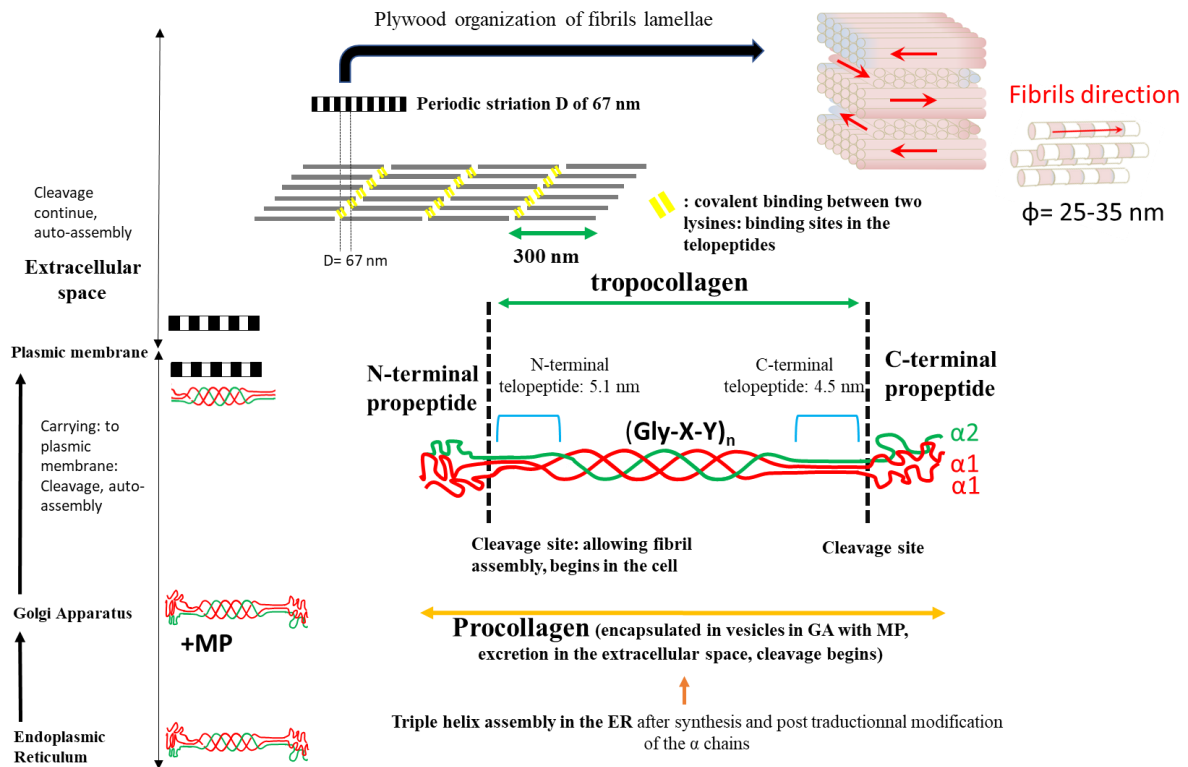


Figure 4: from procollagen to domains formation. Procollagen (with propeptides) is assembled in the endoplasmic reticulum (ER) after the synthesis of the three α chains (two of them are identical) and their post-translational modifications. Then the procollagen is encapsulated with metalloproteases (MP) in vesicles in the Golgi Apparatus (GA). The cleavage of the propeptides starts during the carrying of the vesicles to the plasmic membrane and ends in the extracellular space to form tropocollagen. The three chains are mainly formed by the repetition of a Gly-X-Y sequence adopting a left helix structure called PPII, where X and Y are respectively Proline and Hydroxyproline. The trimerization of the three chains induces the formation of a right triple helix. The telopeptides contain about 20 amino acids, containing the binding sites for fibrils formation. Excreted as tropocollagen in the extracellular matrix, the molecules self-assemble into fibrils, stabilized by electrostatic interactions and covalent bindings. The fibrils exhibit an axial periodicity of 67 nm with two types of regions: regions with poor electron density (gaps) and regions with a high density of electrons. In the cornea, the fibrils assemble in lamellae and there is a large angle rupture between two lamellae (approximately 90°) forming domains that vary in size, with a plywood-like organization. In each lamella the fibrils are aligned in the same direction. In the cornea, the fibrils diameter is very regular (≈ 25 nm).

The fibrils are thus a unidirectional assembly of tropocollagen whose diameter varies with the tissue. There is an axial periodicity of 67 nm called D period (Fig. 5), which can be observed in transmission electron microscopy. This period is due to the axial disposition of collagen in the fibril according to

the model of Petruska et al (Petruska and Hodge, 1964). The collagen molecules are aligned along the fibril axis with a periodic shift δ where $\delta = k \times 67 \text{ nm}$ with k nonzero integer. The collagen molecule has a size of 4.4D, which means 300 nm. Thus, there is a space between two consecutive molecules. Two regions called “gap” and “overlap” appear corresponding to respectively poor and high electron density zone. Several authors differently described the transverse organization of the fibril. According to Hulmes et al., there is a quasi-hexagonal disposition of tropocollagen molecules in a cylindrical fibril. There are perfectly organized zones, radially disposed and separated by more amorphous regions. The proportion of amorphous and crystalline regions varies from one tissue to another. Orgel et al. (Orgel et al., 2006) propose that tropocollagen molecules are associated into microfibrils that are a substructure of the fibril. These microfibrils are an assembly of five tropocollagen molecules into a super-right helix. This assembly is disposed in a quasi-hexagonal network.

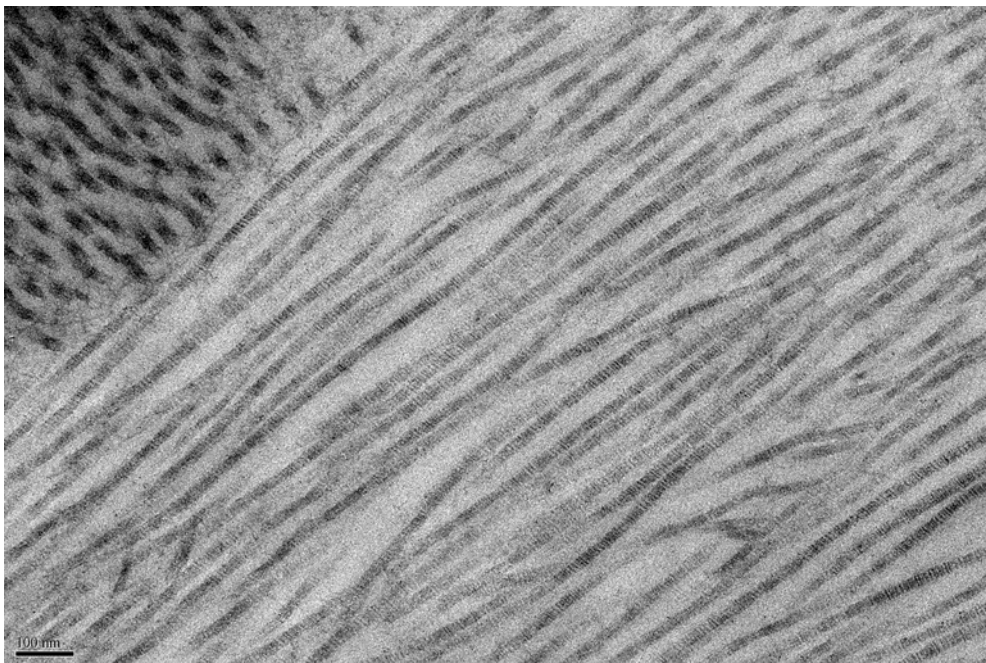


Fig. 5: fibrils of human corneal stroma exhibiting the 67 nm striation. As it appears in this transmission electron microscopy picture, the fibrils in a lamella are mainly aligned around a principal direction. Scale bar: 100 nm

In the extracellular matrix are also found collagens type V (in the heterofibrils), type VI (which is also found in Bowman’s layer), type VII, small amount of type III (also in Bowman’s layer and

Descemet's membrane), type XII, and proteoglycans such as keratan sulfate, chondroitin sulfate, dermatan sulfate or lumican. The proteoglycans have a critical role in maintaining the interfibrillar space constant. They also act in hydration mechanisms since they are highly hydrophilic and hyper hydrated. The proteoglycans are negatively charged, insuring the swelling pressure of the stroma. The regular interfibrillar space is important for corneal transparency since it prevents light from scattering. Concerning the keratocytes, they are responsible for the synthesis of collagen and other elements of the extracellular matrix. They are found between collagen lamellae and are connected in the stroma by cytoplasmic extensions.

The intra-fibril covalent cross-links helping collagen molecules to stabilize increase with age. They increase swelling and allow the refractive index of the fibrils to decrease.

Also, type VI collagen forms 100 nm periodic filaments throughout the extracellular matrix, and is able to associate with type I collagen fibrils. Types XII and XIV are FACIT (fibril associated collagen with interrupted triple helix) collagens. Type XII is aligned on the surface of type I fibrils and this confers stability (Wessel et al., 1997). Type XIV has been located throughout the avian stroma associated with the surfaces of types I/V collagens, where it is thought to play a role in the compaction of the cornea, critical to its transparency (Gordon et al., 1996).

5 Bioengineering

5.1 Introduction

Over the last few years, huge progresses were made to replace partially or totally the diseased cornea. Hicks et al. (Hicks et al., 2002) and Griffith et al. (Griffith et al., 2002) mentioned the importance, in addition to appropriate transparency, of adequate refraction index, mechanical properties acting as a barrier, and of nutriment and oxygen permeability, all necessary for corneal cell survival. Also, the ability to be colonized by host stromal stem cells is fundamental for a proper integration and to avoid inflammation. It is also necessary to avoid vascularization and opacification, which result from a strong immune response leading to biodegradation or calcification of the corneal substitute. In 2001, Chirila et al. developed a system based on a flexible belt with incorporated pores around a transparent central part (Chirila, 2001). The porosity (pore diameter was comprised between 10 and 30 μm) allows the fibroblasts to grow and the extracellular matrix to deposit in a manner similar to what happens in a scarring process. This helped the integration of this system to the autologous tissue (Khan et al., 2001). The posterior part of the implant inhibits cellular attachment to prevent the formation of a fibrous opaque membrane (Griffith et al., 2002). The central part has a refractive index close to that of the cornea.

Sweeney et al. (Sweeney et al., 2003) put in evidence the necessity to have a developing epithelium on the system to avoid extrusion or infections. Materials used for artificial corneas such as PMMA, PVA, pHEMA do not allow cellular adhesion, so it was necessary to improve the systems to enhance cell migration and adhesion. Thus, collagen, fibronectin, laminin or peptides like RGD or IKVAV, were grafted at the surface of artificial corneas (Sweeney et al., 2003; Kobayashi and Ikada, 1991; Merrett et al., 2001). These proteins, *in vitro*, induce the secretion of extracellular matrix proteins by the cells and the formation of adhesion complexes to create a new basement membrane (Sweeney et al., 2003).

The next parts will focus on biomimetic scaffolds using collagen or other biomaterials such as silk presenting the best promising results. Scaffolds implying the use of synthetic polymers or decellularized materials will not be discussed here.

5.2 Biomimetic materials as scaffolds for regeneration

5.2.1 Using collagen

The bioengineering processes used to produce collagen scaffolds can be classified according to presence or absence of collagen fibrils in the biomimetic material, collagen type and origin, presence or absence of telopeptides and triple helix assembly process (i.e., electrostatic interactions, vitrification, cross-linking with EDC/NHS or with glutaraldehyde). The biomimetic materials that are closest to the native tissue feature presence of fibrils made of collagen type I with telopeptides assembled through electrostatic interactions.

The 3D-culture of cells was not frequently obtained for synthetic polymers. It was successful for polyethylene oxide, polypropylene oxide and poly (N-isopropylacrylamide) (PNiPAAm) (Wallace et al., 2005, Lee and Mooney, 2001). However, natural hydrogels made of alginate, chitosan, agarose, albumin or collagen, are widely used for encapsulation of cells. Collagen type I is very interesting for the elaboration of a scaffold matrix because first, it is the major component of the cornea but also, it provides the RGD sequence allowing cellular attachment (Hoffman, 2002). At low collagen concentration, it is however necessary to cross-link (Lee and Mooney, 2001) because of inadequate mechanical stiffness. Also, a NIPAAm polymer modified with the YIGSR peptide copolymerized with bovine collagen I allowed to obtain a transparent hydrogel that can be molded into cornea shape (Pierschbacher and Ruoslathi, 1987).

Multilayers systems were elaborated. Griffith et al. have developed a system with three cellular layers (Griffith et al., 1999). It was made of a collagen hydrogel associated with chondroitin sulfate

and immortalized corneal cells. This system was able to respond to a chemical stimulus by a modification of gene expression and/or of the transparency. However, it had poor mechanical properties.

Besides, collagen hydrogels associated with TERP5 (poly(N-isopropylacrylamide-coacrylic acid-coacryloxysuccinimide) have been grafted on dwarf pigs. The second eye of each pig was untreated while allografts were used as controls. This was the first reported case of stromal and epithelial cells growth in an implant to recreate the corneal tissue. Cell regeneration was observed and touch sensitivity of the animals recovered after 6 weeks, which was not the case for the allografts used as controls. Duan and Sheardown have then shown that, when replacing TERP5 by multifunctional dendrimers, mechanical properties were improved. Moreover, these dendrimers allow to adding functional groups of biological interest to the hydrogels (Duan and Sheardown, 2005; Duan and Sheardown, 2006).

Liu et al. (Liu et al., 2009) have created an artificial cornea based on collagen and a synthetic lipid called phosphorylcholine. It consisted in a collagen network reticulated with 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide and N-hydroxysuccinimide. A second network comprised polyethylene glycodiacrylate reticulated with 2-methacryloyloxyethyl phosphorylcholine (MPC). The final hydrogel had appropriate mechanical properties with elastic modulus from 2.09 to 11.42 MPa depending on the hydrogel formulation and a tensile strength from 0.69 to 1.86 MPa (3.81 MPa for human cornea). It has also good resistance to enzymatic digestion by collagenase. The MPC displayed poor cellular adhesion properties but the hydrogels provided efficient corneal and nervous cell growth when implanted in animals with and without corneal injury. Besides, these hydrogels showed high transparency and a good permeability to glucose and albumin. McLaughlin et al. developed a collagen-MPC implant, which was implanted as full-thickness transplant in guinea pig cornea. Nervous regeneration occurred within 8 months following surgery and the corneal tissue was reconstructed thanks to cell growth in the implant (McLaughlin et al, 2010).

Artificial cornea based on recombinant collagen type III reticulated with EDC and NHS has been tested in human. In Sweden, a lamellar graft study was conducted on 10 patients and after 24 months,

a regeneration of the epithelium and growth of stromal cells in the implant occurred. Partial growth of nerves was also seen (Fagerholm et al., 2010).

Chen et al. have synthesized collagen-chitosan sodium hyaluronate complexes with native corneal tissue and cells, which were implanted in rabbits. Light transmittance of the implants reached 95% and they showed an increase in transparency with 0.5 to 0.9% concentration of sodium hyaluronate. These scaffolds allowed the corneal cells to attach, migrate, and proliferate (Chen et al., 2005).

Another possibility is to form collagen sponges that present the interest to be porous, allowing the corneal cells to invade the scaffold. Orwin et al. have added chondroitin sulfate to collagen sponges (Orwin et al., 2003). They succeeded in increasing significantly the transparency and they obtained a decrease in contraction of the spongy matrix. However, the mechanical properties were really weak with a Young modulus, ranging from 0.94 to 0.366 MPa depending on the time of culture, which is far from native cornea modulus (3 to 13 MPa). Concerning the transparency, they obtained an increase in light transmission of about 50% of that exhibited by a freshly isolated rabbit cornea.

Zhong et al. used electrospinning to develop a nanofibrous collagen-chondroitin sulfate scaffold, supplemented with glycosaminoglycans and crosslinked thanks to glutaraldehyde (Zhong et al., 2007).

Torbet et al. have successfully designed a scaffold with orthogonal lamellae of aligned collagen, oriented mechanically (Torbet et al., 2007).

Tanaka et al. (Tanaka et al., 2010) have designed a stratified artificial cornea mainly composed of collagen type I. The different assembled lamellae were 2 to 5 μm thick and were obtained by oriented flow-casting. Thus, the fibrils forming one layer were perpendicular to the fibrils of the adjacent layer. This system had appropriate mechanical stiffness and transparency thanks to EDC and NHS reticulation and several cycles of vitrification. Calderon-Colon et al. have also used collagen to develop an artificial cornea, with three major steps: a jellification followed by vitrification and rehydration. They obtained a good transparency of 85% and a Young modulus of 12 MPa (Calderon-Colon et al., 2012) while values for native cornea in the literature vary from 3 to 13MPa.

G. Mosser et al. (Mosser et al., 2006) have recently developed a method to control both the organization and the transparency of dense collagen scaffolds thanks to the liquid-crystal properties of collagen. A plywood-like liquid crystal phase was obtained using mixtures of acetic and hydrochloric

acids as solvents. The use of the two acids allows getting a transparent and cornea-like organized fibrillated collagen I scaffold. The development of corneal epithelium as well as the preservation of epithelial stem cells was demonstrated *ex vivo* (Mosser et al., 2006, Tidu et al., 2015; Tidu et al., 2017).

Mi et al. used plastically compressed collagen to create a scaffold (Mi et al., 2011). The keratocytes were embedded in the collagen gel, which was then compressed and laminin-coated. On this system, limbal epithelial cells were able to grow and to differentiate into a stratified epithelium that expressed specific markers such as CK3. This scaffold was comparable to amniotic membrane for limbal epithelial cell expansion.

Shojaati et al. also used compressed collagen to deliver corneal stromal stem cells for corneal therapy (Shojaati et al., 2018). The collagen from rat tail tendons was neutralized and then dehydrated in a laminar flow, producing a plastic compressed gel that adhered well in mouse cornea. The embedded cells maintained viability throughout the rapid embedding and compression steps and were highly effective in corneal regeneration.

Using human adipose tissue derived stem cells (hASCs), Sorkio et al. (Sorkio et al., 2018) have developed a layered stroma-mimicking structure. Functional bioinks were based on recombinant human laminin and human sourced collagen I. They have bioprinted three types of corneal structures and particularly a lamellar corneal stroma using alternating acellular layers of bioink and layers with hASCs, and finally, structures with both stromal and epithelial parts. The printed constructs allowed cell viability and proliferation, and expression of key proteins such as p63 α , CK3, CK15 and collagen type I. Isaacson et al. have employed a similar method and shown that 3D bioprinting can be effective at elaborating human corneal substitutes from low viscosity bioinks (Isaacson et al., 2018).

Table 2 : Different processes involving collagen to create an artificial cornea

Study	Collagen type / cells	Production process	Produced molecule	Molecule assembly	Domains	Plywood organization	Transparency
Tidu et al., 2018	Rat type I / Human corneal epithelial cells	Liquid crystal process, sol-gel transition	Triple helix, with telopeptides	Fibrils, 67-nm periodicity	yes (200-400 μm wide)	yes	85% (500 μm)
Majumdar et al., 2018	Bovine type I	Vitrification	Triple helix, no telopeptides	Fibrils shape not mentioned	yes	yes	85% (400 μm)
Hoffman et al., 2002	Bovine type I	Cross-linkers glutaraldehyde	Triple helix, no telopeptides	Aggregate of triple helix	no	no	Transparency not measured
Fagerholm et al., 2010	Human recombinant type III	Cross-linkers EDC/NHS	Triple helix, telopeptides	Fibrils, periodic striation	yes	no	95% (493 \pm 27 μm)
Liu et al., 2008	Human recombinant types I and III	Recombinant, cross-linkers, EDC/NHS	Triple helix, telopeptides	Periodic striation	no	no	80-90% (500 μm)
Torbet et al., 2007	Rat tail type I	Magnetically oriented, fibril formation by heating	Triple helix, telopeptides	Fibrils, periodic striation (30 $^{\circ}\text{C}$)	yes	yes	Scaffold transparent, but not evaluated
Tanaka et al., 2011	Bovine type I, pepsin treated	Flow-casting, collagen laminates, cross-linkers EDC/NHS	Triple helix, no telopeptides	Fibril-like, no periodic striation	yes	Lamellar structures	75% at 550 nm (100 μm)
Calderon-Colon et al., 2012	Bovine type I	Gelation, vitrification, rehydration	Triple helix, no telopeptides	Fibril-like, no periodic striation	yes	Layers of collagen fibrils	85% (500 μm)
Mi et al., 2011	Rat tail type I	Plastically compressed, photochemical cross-linking	Triple helix, telopeptides	Fibril-like, periodic striation not analyzed	no	no	80% (80 μm)
Liu et al., 2009	Bovine type I, pepsin treated, recombinant type III	Hydrogel with cross-linkers (EDC/NHS) interpenetrating network with Phosphorylcholine (photopolymerization)	Triple helix, no telopeptides	Fibril-like, periodic striation not analyzed	no	no	80-90% (500 μm)

One limitation of these biomimetic materials is the absence of keratocytes within the collagen fibril scaffold. Scaffold colonization by recipient keratocytes can occur, at least to a certain extent, after transplantation. However, normal keratocyte density with the appropriate anterior-posterior gradient of density after transplantation has not yet been demonstrated. In addition, in case of genetic disease involving keratocytes, colonization by recipient keratocytes is not expected, as it could be associated with recurrence of the primary corneal disorder. These scaffolds also differ from the native tissue by collagen domain size, distribution and 3D-organization (i.e. differences between anterior, mid and

posterior stroma, presence of stromal striae). The viscoelastic behavior of these scaffolds has not been fully investigated except for a few materials, which have been evaluated for Young modulus.

5.2.2 Other materials

The approaches using materials other than collagens tend to produce a transparent and biocompatible scaffold stiff enough to be transplanted. These materials however cannot be considered as fully biomimetic.

A first example of these materials is gelatin. Mimura et al. have used gelatin, which is a denaturalized form of collagen with no triple helix organization, to create hydrogels and used them as lamellar grafts in a rabbit model (Mimura et al., 2008). The hydrogels were cross-linked with glutaraldehyde and seeded with rabbit fibroblast precursors. The authors did not observe any opacity after 4 weeks. Another group has added chondroitin sulfate to gelatin hydrogels using NHS/EDC as cross-linkers (Mimura et al., 2011), improving the biocompatibility. The gelatin was also used to improve the mechanical properties of alginate hydrogels (Lai, 2013).

Other corneal substitutes were based on silk derivatives. Bray et al. (Bray et al., 2012) used silk fibroin to generate a corneal tissue. The fibroin, extracted from silkworm cocoon, allowed to creating a tissue substitute that consisted of a 3D network of degummed fibroin fibers with stromal stem cells and an overlying stratified epithelium.

Lawrence et al. also developed an equivalent of the corneal stromal tissue using silk films. Upon silk films stacked to mimic the corneal stroma, human and rabbit corneal fibroblasts adhered, proliferated, and produced native matrix (Lawrence et al., 2009). In addition, the patterned silk films seemed to induce an alignment of cells and actin filaments. However, the authors provided no values for mechanical properties and transparency.

Wang et al. (Wang S et al., 2017) developed a 3D silk-based co-culture system (for human corneal stromal stem cells and human epithelial cells) including the corneal stroma, the corneal epithelium and dorsal root ganglion neurons. The scaffold was made to be biomimetic with patterned silk films coupled with RGD peptide for corneal epithelial and stromal cell growth. It was surrounded by a silk

sponge containing the dorsal root ganglion neurons, which simulated the limbus. The scaffolds appeared to be transparent after 38 days of co-culture but no quantification of transparency was given. Guan et al. used silk fibroin with chitosan to create a stroma equivalent for implantation in rabbits. If the implants were not synthesized with a high transparency, they however became more transparent after transplantation. The transparency was approximately 62.4 % while the mechanical tensile strength was 1.3 MPa (compared to 3.8 MPa for the human cornea). Also, it contained more than 90% of water (Guan et al., 2013).

Van Essen et al. have designed an artificial cornea based on Tilapia scale, mainly composed of collagen type I (Van Essen et al., 2016). The scales were first decellularized, decalcified, and irradiated. The matrices obtained showed good properties for cell adhesion and growth. Holan et al. used copolymer polyamide 6/12 nanofibers for the growth of mesenchymal stem cells and limbal stem cells. They showed by mesenchymal stem cells penetrated into the structure to form pseudopodia (Holan and Javorkova, 2013) while Hunt et al. have encapsulated corneal cells within alginate hydrogels (Hunt et al., 2009).

The use of collagen-like peptides can also be promising. They have similar physiochemical and biological properties as collagens. Indeed, collagen-like peptides can self-assemble to create a hydrogel, as shown by O'Leary et al. (O'Leary et al., 2011). These authors presented a 36 amino acid peptide, (Pro-Lys-Gly)₄(Pro-Hyp-Gly)₄(Asp-Hyp-Gly)₄ that mimicked collagen fibrils, but was only capable of forming a hydrogel with insufficient robustness for *in vivo* applications.

Islam et al. thus added polyethylene glycol maleimide to improve mechanical properties (Islam et al., 2016). They also modified the amino acid composition by adding a glycine and a cysteine. These changes allowed covalent attachment of the polyethylene glycol maleimide previously mentioned. An EDC cross-link was also performed. Their modified scaffold thus provided a transparency of 92.4 %, which is higher than the value they indicated for human cornea (87.1%). However, as the hydrogels contained approximately 90% of water (compared to 78% for human cornea), they exhibited poor mechanical properties. The modulus was 0.18 MPa compared to 3-13 MPa for human corneas. The hydrogels were tested on rats and mini pigs, and resulted after a year innervation, in cellular integration and in deposition of types I and V collagens.

6 Conclusion

Thus, it appears that there are a lot of promising methods and materials to develop artificial corneas, but none of them allows, today, to fit with all the requirements for native cornea-like function. None of the current bioengineering processes reproduces the biological development of cornea. The bioengineered stromal equivalents that are the closest to the native tissue feature the presence of oriented collagen I fibrils close to the native fibrils, and a change in fibril orientation between two adjacent domains, high transparency and acceptable Young modulus. None of the processes used at present to synthesize these scaffolds allows reproducing anterior to posterior differentiation of the corneal stroma regarding cell density and collagen lamellae organization. The viscoelastic behavior of these scaffolds has not yet been demonstrated. Further bioengineering advances thus are needed to better mimic the actual development of the cornea *in vivo*. Importantly, there are still several steps of stroma formation that remain unclear and which need to be investigated further before they can be reproduced *ex vivo*. In this regard, it should be useful to determine the exact roles of several genes and the temporality of numerous events like the emergence of stroma structure and the cellular and molecular factors that drive keratocyte penetration in the stroma. Whereas corneal transparency and development of an appropriate epithelium appear largely solved issues, the colonization of the stroma by keratocytes associated with adequate mechanical properties and a cornea-like structure, need to be further developed.

7 Disclosure / Conflict of interest

This research did not receive any specific grant from funding agencies in the public, commercial or not-to-profit sectors

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