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The human cornea as a model tissue for additive biomanufacturing: a review

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Abstract

The recent development and clinical success of molded recombinant collagen corneal substitutes offers a tantalizing prospect for the application of additive biomanufacturing in corneal tissue engineering and regenerative medicine (TERM). The cornea has a number of advantages over other tissues for additive biomanufacturing in that it is relatively homogeneous and completely avascular and has low metabolic demand. Additive biomanufacturing could bring a number of advantages over molding to corneal TERM, including increased visual acuity and mechanical strength. We have developed a method for producing thin collagen films, which are suitable for the culturing of corneal mesenchymal stem cells (MSCs), using additive manufacturing techniques, and we hope to use this technique to produce functional corneal substitutes.

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Keywords: Cornea; collagen; additive manufacturing; corneal substitute;tissue engineering

1. Introduction

The cornea serves as the main refractive component and structural coat of the dioptric system of the eye. It is composed of five layers (Fig. 1): the epithelium, Bowman's Membrane, the stroma, Descemet's Membrane, and the endothelium. The stroma is the largest of these and accounts for over 90% of the corneal thickness [1]. Collagen type I makes up 70% of the corneal stroma's dry weight, giving it a relatively homogenous composition which is advantageous in tissue engineering and regenerative medicine (TERM). Damage to the stroma through trauma or infectious and degenerative diseases can cause corneal scars, which may lead to substantial visual impairment. This visual impairment is a result of the transformation of stromal cells, also known as keratocytes, into (myo)fibroblasts due to inflammation [2]. Left untreated, this wound healing process leads to opacification of the cornea, and hence blindness. While the majority of cases of corneal opacification globally are a symptom of poverty and best treated through preventative actions such as the World Health Organization (WHO)'s SAFE program for trachoma, there are still many cases where surgical intervention is the only treatment [3].

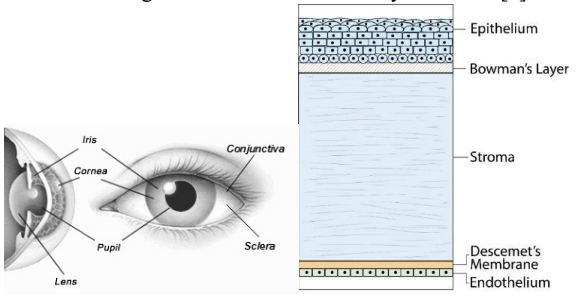

Fig. 1. Cross-section and anterior view of the eye highlighting its main anterior components; source: [60]. A cross-section of the cornea illustrating its 5 layers.

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The WHO estimates that 39 million people are bilaterally blind and 246 million people suffer from impaired vision [3]. More than 5% of these cases are due to corneal opacification and scarring [4]. Treatment options for these patients are limited to the transplantation of a human donor cornea through penetrating keratoplasty (PK) or to the implantation of an artificial cornea. Artificial corneas are ultimately flawed by their failure to integrate with local tissue. The main use of current artificial corneas is for patients that have experienced multiple rejections of human donor corneas or have been given a poor prognosis for PK. Patients who receive artificial corneas are exposed to a significant risk of rejection, retroprosthetic membrane formation, and glaucoma. Human donor corneas have a much higher success rate; however, the global lack of donor corneas has led to only 120 000 corneal transplants being performed each year, while millions of patients remain untreated on waiting lists worldwide [5,6]. Improved surgical methods have increased the number of transplantable corneas. For example, anterior lamellar keratoplasty (ALK) is a partial thickness transplant of the cornea that allows partially damaged, imperfect corneas to be transplanted [7]. However, this increase is small, and the knowledge is localized to the developed world. To truly address the need for donor corneas, a corneal substitute should be developed that has the potential to be mass produced, and this has led to much progress in the field of TERM for cornea.

The relative homogeneity of the corneal stroma's composition and its low metabolic demand make it an ideal candidate for TERM. Oxygen and nutrients are supplied to the cells of the corneal epithelium and stroma mostly through diffusion from the tear film and aqueous humour, which negates the common challenge to achieve vascularization in TERM constructs used for other target tissues. Hence, much research has been done in TERM to develop a corneal substitute, most notably by May Griffith and colleagues, who have gone into clinical trials with a corneal substitute made by molding recombinant collagen type III (RHCIII). However, as the molds used to produce the corneal substitutes of Griffith et al. are not patient specific, additive biomanufacturing could produce customized corneal substitutes to enhance visual acuity and mechanical properties. Here, we present a number of materials and methods that provide inspiration to produce an additively manufactured cornea as well as a method developed ourselves with which we aim at producing RHCIII corneal substitutes.

2. Cornea structure

The understanding of the nature of collagen in the cornea has evolved over the years, and it has become clear that the structure of the cornea is key to its transparency. Collagen molecules in the cornea are arranged into microfibrils, which in turn are arranged into fibrils, which are then arranged in parallel into lamellae, typically up to 200 µm broad and 2 µm thick [8]. These lamellae make up the collagenous layers within the corneal stroma, and the number of these layers from the center of the cornea to the limbus (border of the cornea and sclera) varies from ~300 to ~500, respectively [9] (see also Fig. 1). Orientation of the lamellae within these layers also varies, with orthogonally orientated layers more dominant in the center of the cornea and annular layers towards the limbus [8]. The transparency of these lamellae relies upon the regularity of the arrangement of its component collagen fibrils, and this arrangement is believed to be controlled by the electrostatic repulsions generated by the ionic balance [10]. Disruption to this arrangement can lead to corneal opacification, therefore it may be advantageous for a corneal substitute to emulate this arrangement.

The cornea also comprises three main cell types: corneal epithelial cells, keratocytes, and corneal endothelial cells. The epithelium consists of five to seven layers of epithelial cells that form the outermost barrier of the cornea [11,12]. These cells are constantly being replenished from a population of stem cells residing at the limbus [13] because the oldest epithelial cells are shed off by friction with the eyelid. The stroma is sparsely populated with keratocytes, cells that are mitotically quiescent under normal physiological conditions [14]. Extended cellular processes connect the keratocytes with each other throughout the stroma, as this cell type only accounts for approximately 5% of the corneal tissue [15,16]. Upon injury of the corneal stroma, keratocytes are activated, transform into a fibroblast-like phenotype, and start migrating towards the injured area. Within the wound another activated cell type is found: the myofibroblast. These cells express alpha smooth muscle actin (αSMA) and are responsible for wound contraction during corneal repair [17–19]. *In vitro* corneal stromal cells show high proliferative capacity and meet all criteria required for mesenchymal stem cells (MSCs, Fig. 2a) when cultured in serum-supplemented media [20]. This

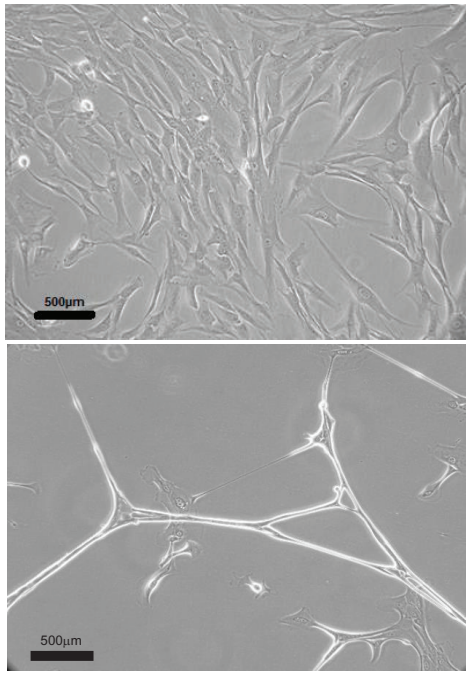

Fig. 2. (a) *in vitro* expansion of corneal stromal cells in the presence of serum leads to dedifferentiation into MSCs with a characteristic fibroblast-like phenotype; (b) corneal stromal cells retain the typical keratocyte phenotype when cultured in the absence of serum-supplemented media.

dedifferentiation into a more stem cell-like phenotype is reversible by serum deprivation, making these cells ideal candidates for TERM approaches focused on the cornea.

3. TERM of the corneal substitute

The most basic needs of a corneal substitute are adequate mass transport properties to support keratocytes and prevent angiogenesis from the limbal region to the cornea, suitable transparency and mechanical properties, and low immunogenicity, and it should also support nerve in-growth to allow for restoration of the blink reflex. Many of these needs have been met or are likely to be met using collagen hydrogels, silk fibroin-based scaffolds, or decellularized corneas. However, to achieve the appropriate mechanical performance most biopolymers require some form of crosslinking to enhance the mechanical properties of the construct, and this may be done through chemical or physical means.

3.1. Collagen

Collagen type I is the most abundant type of collagen found in the human cornea, and therefore it is the most used type in corneal TERM. Collagen type III is also well-studied due to its superior mechanical characteristics compared to collagen type I [21,22]. Glutaraldehyde, carbodiimides, and polypropyleneimine dendrimers have all been used to increase the mechanical strength of collagen scaffolds for corneal TERM [6,23,24].

The clinical trial of May Griffith et al. mentioned earlier was a 4-year phase I clinical trial. Ten patients were treated by removing damaged corneal tissue and replacing it with an acellular anterior lamellar graft and compared to 9 similar patients who had received a full thickness human corneal transplant via PK. The corneal substitutes were fabricated by mixing a concentrated RHCIII solution with a carbodiimide crosslinking agent (EDC) and dispensing it into a polypropylene contact lens mold (500 µm thick, 12 mm in diameter). Within 1 to 3 months, nine patients showed a full coverage of regenerated epithelium, whereas one patient achieved this only after five months facilitated by an amniotic membrane patch. Cellularization in the body of the scaffold by ingrowth of stromal cells from the periphery and regeneration of nerves were reported seven months after transplantation as well as stable regeneration of the tear film [25,26]. During the four years, no severe infections or rejection episodes occurred in patients with RHCIII corneal substitutes. However, one of the nine patients who received a human donor cornea experienced rejection [27], and all experienced high density of inflammatory dendritic cells in the area of the implant, although it should be noted that different surgical methods were used for the biosynthetic implants and the human donor corneas. The RHCIII corneal substitutes were more prone to changes in thickness, likely due to collagen compression and remodeling by keratocytes. Central corneal thickness in the patients who received the RHCIII corneal substitutes was reported to be 358 ± 101 µm, compared to 578 ± 50 µm for patients treated with human donor corneas, and 534 ± 30 µm for untreated healthy human corneas. Much of this thinning of the RHCIII corneal substitute was associated with suture placement. The mechanical properties of the corneal substitutes were insufficient for the use of running peripheral sutures commonly used in corneal transplants, therefore mattress sutures had to be used. Mattress sutures overlay the graft piercing only the periphery of the native cornea and are known to lead to an uneven topography, (depicted in Fig. 3). As a result, patients who received RHCIII corneal substitutes had to be fitted with corrective lenses to regularize the uneven surface of the resultant cornea whereas patients with human donor corneas could use spectacles. Distance-corrected visual acuity achieved at the end of the 4-year study was significantly lower for patients with RHCIII corneal substitutes (20/54) relative to those with human donor corneas (20/36). Despite some of these drawbacks, the relative success of these corneal substitutes is an inspiration to the field to generate improved corneal substitutes through the incorporation of differentiated keratocytes and new fabrication methods, such as additive manufacturing (AM), for improved mechanical properties.

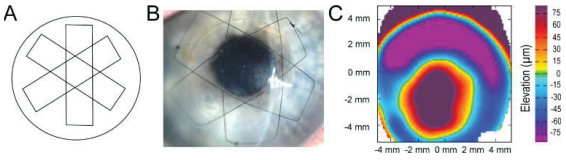

Fig. 3. Hexagonal pattern of the mattress sutures imprinted on the anterior corneal surface of the BSS implant (patient #4). (A) Diagram of the mattress sutures placement. (B) Slit-lamp photograph of the cornea showing the mattress sutures over the BSS implant. (C) Corneal topography. Source: [61] © ARVO Journals 2016. Reprinted with permission.

3.2. Silk fibroin

Silk fibroin has become an attractive biopolymer for a number of applications in TERM due to its high mechanical strength, extensibility, tunable degradation rate, optical properties, and low-immunogenicity [28]. It is a structural protein obtained from the cocoons of the silk worm Bombyx mori [29]. The mechanical strength of silk fibroin can be further enhanced by crosslinking with sonication by low frequency ultrasound or photo-initiated crosslinking with riboflavin, and it can also be chemically crosslinked. Silk sutures have been in use for thousands of years and are still in use, particularly in ocular surgery, due to their excellent handling and tying capabilities [30]. Silk fibroin films have successfully been used as a substrate for the culture of corneal endothelial cells [31] and epithelial cell-equivalents [32], and more recently a study performed on enucleated porcine eyes showed that riboflavin-crosslinked silk fibroin films could adhere to a de-epithelialized cornea [33]. In most of these studies, silk fibroin is presented simply as a substrate for epithelial or endothelial cells, yet there is potential for it to be used as a bulk material for a corneal substitute.

3.3. Decellularized corneas

Removing all cells and cellular components from an existing cornea is an approach used specifically in TERM for ophthalmology. A scaffold is obtained, composed of native

extracellular matrix (ECM), which can be repopulated with the patients' own cells. It is hypothesized that this approach will significantly reduce rejection rates, but some issues with regards to limitations in available donor tissue are not resolved. Li et al. have investigated the use of human decellularized cornea in a clinical trial [34]. Human donor corneas were decellularized using glycerol cryopreservation, and 24 months post transplantation, the rejection rate was significantly reduced. Other methods of corneal decellularization include the use of detergents with or without the application of high hydrostatic pressure [35,36], liquid nitrogen, hypertonic NaCl [37,38], polymers such as poly(ethylene glycol) [38], and sodium dodecyl sulphate (SDS) [37,39].

Recellularization of decellularized corneas can be achieved through superficial seeding, injection, or the addition of cells in between multiple thin layers of decellularized corneal stroma as described by Ma et al. in 2015 [40]. Corneal mesenchymal stem cells (MSCs) can be isolated from the stroma and have the potential to differentiate into functional keratocytes, which makes them ideal candidates to populate corneal stromal replacements [14,41]. It has been shown that cells align along the axis of a stress applied to a substratum and that the ECM is deposited in an aligned way [42]. The use of bioreactors has been investigated to provide a mechanically stimulated environment for the development of a tissue-engineered cornea [43–47]. By decellularizing a donor cornea and repopulating it with the recipient's cells, it is hoped that there will be fewer cases of rejection and that it will eliminate the time-dependent aspect of transplant surgery because, after a cornea has been decellularized, it can be stored longer than a donor cornea.

4. Additive manufacturing: potential in ophthalmology

Current use of AM in ophthalmology is limited to optical frames and surgical guides with many possible applications in conjunction with TERM yet to be realized [48]. For example, with the resolution achievable with some AM techniques, it is possible that biopolymers could be printed in a way that reflects the layered structure of the cornea. It may therefore be possible to produce orthogonally oriented scaffolds with aligned fibers using AM to emulate the corneal stroma using collagen or other biopolymers. If lamellar fiber alignment can be achieved in AM corneal substitutes, it could improve their mechanical properties relative to their molded counterpartslimbus. These improved mechanical properties may allow peripheral sutures to be used. Peripheral sutures would be preferred to mattress sutures given the issues of corneal thinning and suboptimal visual acuity seen from their use in the RHCIII corneal substitutes of Griffith and colleagues [27]. A number of AM techniques have been developed to produce TERM constructs, most notably electrospinning and various forms of 3D printing (inkjet, extrusion, and laser-based printing). However, inkjet printing and laser-induced forward transfer are mostly used for cell patterning. Much work has been published on the advantages and disadvantages of each platform, most recently Ozbolat et al. [49].

Extrusion printers are the simplest and most adaptable printers, able to print a wide range of viscosities. They use either a mechanical or pneumatic-driven system to extrude a material (hydrogels, molten polymers, cell-laden inks) through a small nozzle, which can be 5 µm or smaller where shear in the nozzle is not of concern or as wide as 1 mm when low shear within the nozzle is desired to protect cells or the printed material. Multiple nozzle systems have also been developed to allow for simultaneous deposition of different materials. Although the movement of a print platform can be highly accurate with a resolution below 1 µm, much of the accuracy of printing relies on the gelation or drying of the material being printed. Biopolymers tend to swell after extrusion leading to a lower resolution relative to inkjet printing, and gelation of the deposited layer is required before a proceeding layer can be printed on top. Without adequate gelation, the added material can lose its deposited shape.

Electrospinning is a technique for producing nanofibers of a non-conductive material whereby a DC excitation voltage of one to several tens of kilovolts is applied to a needle, or spinneret, as a non-conductive material is extruded through it. Fibers are induced to form from a bead at the tip of spinneret by the electric field generated between the spinneret and ground. The distance of separation between the spinneret and ground, usually 10-40 mm, and the excitation voltage define the size of the fibers generated.

4.1. Silk fibroin

As mentioned earlier, silk fibroin is being researched in TERM for the cornea due to its myriad of advantageous properties. It has been used with a number of AM techniques including electrospinning, inkjet printing and extrusion printing. Hu Tao and colleagues at Tufts University, USA, published an extensive study on the printability of silk fibroin, its customizable rheological characteristics, and formulations of different doped and biologically active silk inks [50]. A number of methods have been reported to induce a change in silk fibroin from its fluid amorphous state to its β-sheet crystalline conformation, with direct printing into a methanol bath seeming to be the most effective. Ghosh et al. overcame the swelling associated with the extrusion printing of hydrogels by using a methanol bath method [51]. While extruding a 28% w/v solution of silk fibroin through a 5 µm nozzle, filaments retained their extruded diameter of 5 µm, and scaffolds were subsequently proven to support adhesion and growth of MSCs. Thinner filament diameters of 1-10 µm can be achieved using electrohydrodynamic printing, which is a method similar to electrospinning but with lower excitation voltage (10-20 kV) and shorter spinneret-ground separation (1-5 mm). Haashimdeen et al. reported controlled deposition of silk fibroin fibers of 1-6 µm in dimeter when using an electrohydrodynamic printer with an accuracy of 1 µm, which was developed by themselves [52]. Inkjet printing has also been used to print silk fibroin [53], although it was used to encapsulate cells rather than produce a construct for TERM. Low concentrations of 0.1% and 0.05% w/v silk fibroin were printed, likely due to the need for low viscosity in inkjet printing, and dried, which would be a concern when considering the mechanical properties and accuracy of the printed materials. The accuracy achieved for AM of silk fibroin is much higher than that reported for other biopolymers;

however, there have not been any significant publications on the printing of silk fibroin in relation to corneal substitutes.

4.2. Collagen

AM of collagen-based corneal substitutes could improve their mechanical properties relative to the molded RCHIII corneal substitutes of Griffith et al [27]. Although collagen has been used in AM, purified collagen is rarely used as a structural component in resultant constructs. There are just a few publications where the gelation necessary to deposit consecutive overlying layers has been achieved by pH- and temperature-induced fibrillogenesis [9] or pH- and shear-induced fibrillogenesis [10]. Murphy et al. performed an extensive evaluation of different hydrogels for bioprinting, by evaluating gelation time and printability using an extrusion printer developed by themselves, and found that collagen took the longest time to gelate at 40 min, which would vastly increase print-time if multiple layers are desired[54].

Collagen has been used more often in composite inks with alginate or gelatin where its purpose is to interact with cells or as a cell carrier ink in a multiple nozzle printing system. Materials like gelatin and alginate have been used often in AM due to their rapid gelation and crosslinking characteristics, respectively; however, they are limited in TERM due to their bio-inertness. Methods to create a collagen-only scaffold have been developed whereby collagen is printed with alginate, which is crosslinked during or immediately after extrusion. The collagen is then crosslinked after printing, and the alginate is removed by addition of monovalent cations or a chelating agent. Wu et al. [55] recently reported on the printing of human corneal epithelial cells (HCECs) for the first time, with a viability of 94.6 ± 2.5% post-print. This was achieved using a hybrid ink of collagen/gelatin/alginate/HCEC, which was extruded through a syringe heated to 37 °C, deposited in a refrigerated environment, and subsequently crosslinked with $CaCl_2$. The constructs had a mean thread diameter of 445.6 ± 8.0 µm and mean layer thickness of 150 µm (Fig. 4). These were then incubated with different amounts of sodium citrate (SC) to control the degradation of the crosslinked alginate with different SC-alginate molar ratios able to vary the degradation time from a number of hours to a number of days; however, gelatin remained. Although the printed constructs had neither the size nor the shape of the cornea, the printing of HCECs with high cell viability offers a tantalizing prospect for the future of printed corneas. HCECs could be printed on top of other constructs to achieve full epithelial coverage prior to implantation. Printing of keratocytes has yet to be reported.

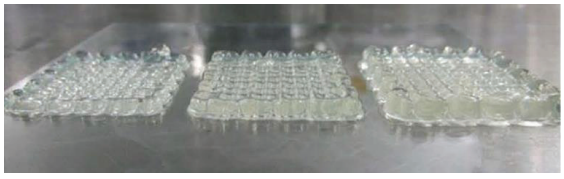

Fig. 4. Collagen/gelatin/alginate/HCEC constructs printed by Wu et al. with 10, 15, and 20 layers respectively from left to right. Source: [55] © Nature Publishing Group 2016. Reprinted with permission.

4.3. Decellularized ECM

While collagen is a major component of the ECM, the use of purified collagen does not reflect the complex composition of native ECM and how its components (collagens, glycosaminoglycans, proteoglycans) interact with surrounding cells and define the function of the tissue. An alternative to using single purified components of ECM is decellularized ECM (dECM), which has come to attention with the improvement of decellularization methods [56]. To prepare dECM, a target tissue is explanted from an animal and put through processes to lyse and remove all cellular contents, similar to those used to create decellularized corneas and aiming to leave just the ECM components in solution with minimal damage. This solution is then usually freeze-dried and printed from solution at a later stage. There are no consensus quantitative metrics to establish what can be considered as decellularized; however, <50 ng double–stranded DNA and lack of visible nuclear material in hematoxylin and eosin (H&E) staining have been reported as appropriate standard metrics [57]. While corneas have been decellularized with some success, they have been kept in their form of a cornea, and a decellularized corneal ECM ink for printing has not been reported.

dECM inks are mostly composed of collagen and gelate in the same way, via pH adjustment and incubation at 37 °C, which may lead to the same complications as with collagen printing. Since the dECM inks are usually quite viscous, their use has been limited to extrusion printing. Many advancements have been made by the group of Dong-Woo Cho at Pohang University, Korea, who have printed dECM inks derived from adipose, cardiac, and cartilage tissues, often in the form of a cell-laden bioink, using an extrusion printer they developed themselves [58,59]. Most of their dECM inks were deposited into the pores of a polycaprolactone (PCL) framework with pore sizes ranging from 50 µm to 600 µm, which would be undesirable in corneal printing, and only cardiac dECM was printed without a PCL framework [58]. If a cornea-derived dECM ink could be achieved, it could be printed using inkjet or extrusion printers to fabricate a cornea scaffold, or further still, it could be mixed with HCECs or keratocytes to create a bioprinted cornea.

5. Research into a bioartificial cornea

In a collaborative project between KU Leuven and the University of Antwerp, funded by the Research Foundation Flanders (FWO), we are investigating AM of a bioartificial corneal substitute. We have developed a method to produce thin collagen films for corneal TERM. Using this method, we can produce layers 2 µm thick with a resolution as low as 20 µm, which is of the same size scale as the collagen layers of the corneal stroma. RHCIII is our selected material due to its enhanced mechanical and optical properties and its current use in corneal substitutes [22].

RHCIII scaffolds were formed by nebulizing a 3.4 mg/ml RHCIII solution in 10 mM HCl and accelerating it onto a substrate using nitrogen gas (see also Fig.5). Layers were formed with a calculated thickness of approximately 2-3 µm

and repeated 2, 10, or 29 times to achieve thicknesses of up to 90 µm. The scaffolds were then crosslinked using EDC [1-ethyl-3-(3-diaminopropyl)carbodiimide] and NHS [N-hydroxy-succinimide] and washed in phosphate buffered saline (PBS). Corneal MSCs were isolated through collagenase digestion (4 hours) of donor corneal stromal tissue. Cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) + 10% fetal bovine serum (FBS). First passage MSCs were seeded onto the collagen scaffold samples consisting of 2 layers (n=4), 10 layers (n=4), and 29 layers (n=4) of RHCIII at a density of 100 cells/sample. For controls, cells were seeded onto glass (n=3) and plastic (n=3). At day 23, the scaffolds were processed for immunocytochemistry (n=9) and inspected via scanning electron microscopy (SEM) (n=3).

Optical coherence tomography (OCT) thickness measurements showed a mean thickness of 28 ± 9 µm for 10-layer samples and 87 ± 23 µm for 29-layer samples. Light microscopy showed that corneal MSCs proliferated on all printed RHCIII scaffolds, regardless of the number of layers. Optical microscopy (Fig.6) and SEM showed the circular print pattern on the surface of the RHCIII film. With immunocytochemistry, we observed collagen type III, and MSCs were observed throughout the samples, indicating penetration into the RHCIII along with superficial growth, which was also observed. The cytoskeleton of the MSCs stained positive for phalloidin (Fig.7), and we observed alignment of the superficial cells following the direction of the collagen in the SEM images. Overall, these results demonstrate that AM can be used to produce RHC III scaffolds that are suitable as a substrate for cultivating corneal MSCs. Presto Blue assay (Fig.8) showed that corneal MSCs proliferate significantly better on RHC III scaffolds than corneal keratocytes, for both printed and non-printed scaffolds (ANOVA. F=26.86, p<0.001) at most of the measured time points (N=12, F=14.20, p<0.001).

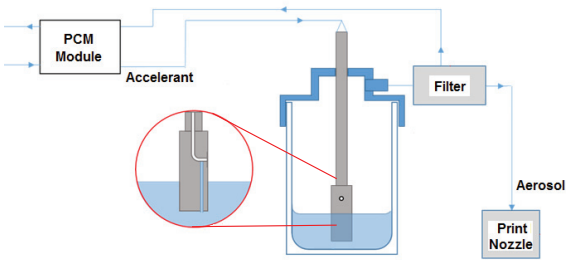

Fig.5: Schematic view of the printing process. Similar to a concentric nebulizer, the material ink is brought through a capillary tube into a low-pressure region and invested by a high-speed gas. Hence, the ink then breaks up into a fine mist at exit of the small orifice. The aerosol is then filtered and accelerated towards the print nozzle.

6. Conclusions

Although TERM has already made some impact in the treatment of corneal visual impairment, the application of AM could enhance the success of tissue-engineered corneal substitutes. Using AM techniques we developed a method to produce RHCIII scaffolds with a layer thickness similar to that of the lamellae in the corneal stroma and which are suitable for the culturing of corneal MSCs.

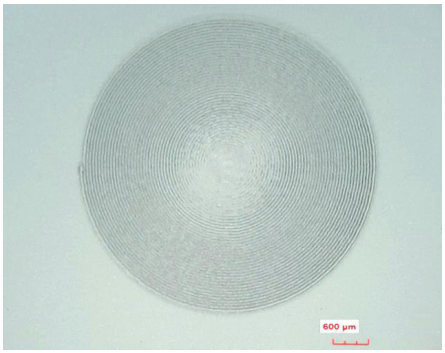

Fig. 6. A RHCIII film seen under an optical microscope with the spiral print pattern clearly visible.

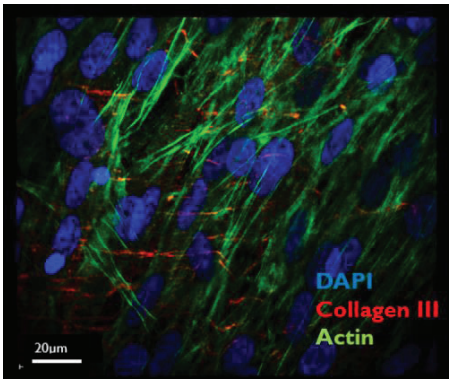

Fig.7. IHC of MSCs grown on 3D printed RHCIII sample. Red: collagen type III; green: actin filaments of the cytoskeleton, phalloidin; blue: nuclei, DAPI

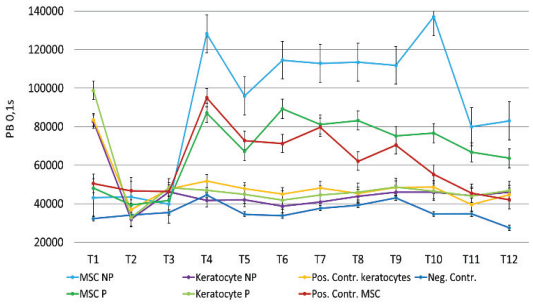

Fig. 8. Presto Blue assay of MSCs and keratocytes grown on 3D printed (P) and non-printed (NP) RHC III scaffolds.

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