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Review

The Evolution of Fabrication Methods in Human Retina Regeneration

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Abstract: Optic nerve and retinal diseases such as age-related macular degeneration and inherited retinal dystrophies (IRDs) often cause permanent sight loss. Currently, a limited number of retinal diseases can be treated. Hence, new strategies are needed. Regenerative medicine and especially tissue engineering have recently emerged as promising alternatives to repair retinal degeneration and recover vision. Here, we provide an overview of retinal anatomy and diseases and a comprehensive review of retinal regeneration approaches. In the first part of the review, we present scaffold-free approaches such as gene therapy and cell sheet technology while in the second part, we focus on fabrication techniques to produce a retinal scaffold with a particular emphasis on recent trends and advances in fabrication techniques. To this end, the use of electrospinning, 3D bioprinting and lithography in retinal regeneration was explored.

Keywords: retina; tissue engineering; retina regeneration; biofabrication; 3D bioprinting; electrospinning; ophthalmology

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1. Background

The retina, lining the inner surface of the eye's posterior segment, is a thin light-sensing tissue responsible for light absorption, conversion to an electrical signal and transmission to the brain through the optic nerve. The retina comprises multiple layers of different cells as shown in Figure 1 [1]. Photoreceptors are specialized neurons that convert visual stimuli into electrical impulses. The signal is then processed and transmitted from photoreceptors to the brain by neural cells including retinal ganglion cells (RGCs). The photoreceptors collaborate closely with the underlying retinal pigment epithelium (RPE), which is essential for visual function [2–4]. Hence, the dysfunction or degeneration of RPE cells results in the death of photoreceptors and vision loss [5]. The RPE cell basal surface faces Bruch's membrane, a thin (2–4 μm) acellular matrix located between the retina and the choroid. This membrane presents a nanofibrous structure composed of collagen I-V, laminin and fibronectin [6]. Bruch's membrane serves as a physical support for RPE cells and as a barrier regulating the diffusion of biomolecules, nutrients, oxygen, fluids and waste between the retina and the choroidal blood supply [7,8].

Pathologies of the retina and optic nerve represent a leading cause of visual impairment and irreversible blindness in high-income countries [9]. These diseases are usually divided into those caused by mutation in only one gene (monogenic diseases) such as inherited retinal diseases (IRDs) and those caused by mutations in multiple genes and environmental factors (polygenic and/or multifactorial diseases) such as age-related macular degeneration (AMD) and glaucoma [10–14]. As the retina is composed of neuronal highly specialized cells with a limited healing potential, regenerative strategies are necessary to reverse vision loss caused by these diseases.

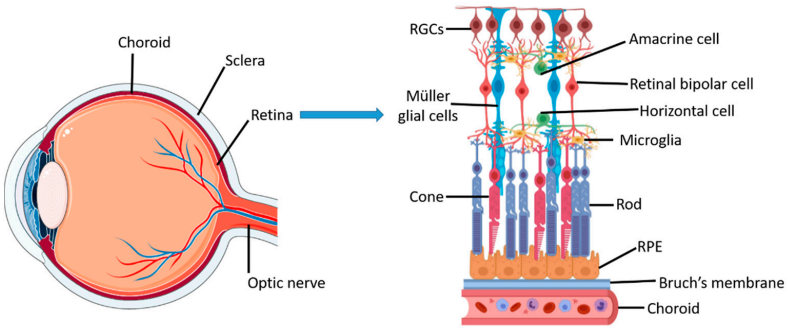

Figure 1. Representation of a cross-section of a human eye and a detail of the multilayer organization of the retina, Bruch's membrane and choroid. The retinal layers include a retinal pigment epithelium (RPE), cone and rod photoreceptors, horizontal cells, bipolar cells, amacrine cells, microglia and Müller glial cells and ganglion cells (RGCs).

This review aims to provide a current account of the developments in retinal regeneration. Retinal regeneration includes tissue engineering approaches and gene- and cell-based therapies, which we refer to as scaffold-free approaches and that are briefly presented in Section 2. Tissue engineering strategies combining cells, a scaffold and chemical cues offer several solutions that are described in Section 3. In particular, we will focus on the regeneration techniques of RGCs, RPE cells along with Bruch's membrane and photoreceptors as these are the cells mainly involved in the majority of pathologies inducing blindness.

2. Scaffold-Free Approaches

Gene therapy has been proven to restore vision by replacing absent or abnormal genes causing monogenic retinal diseases [15–17]. Viral vectors and non-viral gene nanocarriers have been used to deliver specific genes to target cells [15]. For instance, for RPE65-associated IRDs, nowadays there is an FDA (Food and Drug Administration) and EMA (European Medicine Agency)-approved gene therapy (Luxturna (voretigene neparvovecrzyl), Spark Therapeutics) based on the delivery of a functional copy of the RPE65 gene into RPE cells [17]. Therapies targeting monogenic retinal dystrophies are the most promising. Gene augmentation therapy, however, has its own limitations. Only the recessive forms where the mutations cause a loss of function, also called haploinsufficiency, such that the resulting protein is too little or absent can be currently treated with gene therapy [18].

Another scaffold-free strategy explored to regenerate damaged cells or replace dead cells is cell therapy. Healthy cells can be injected as cell suspension or transplanted as sheets to replace pathological cells, thus preventing further degeneration and improving visual function. For instance, as a possible treatment for glaucoma, the transplantation of RGCs derived from human pluripotent stem cells and from human Müller glia cells was investigated. However, difficulties remain in integrating the transplanted RGCs into the complex neurological network of the host retina [19]. Cell transplantation in animal models has been experimented also to treat AMD. Capela et al. reported that a subretinal injection of human central nervous system stem cells in pigmented dystrophic rats enhanced the proliferation of the host RPE cells, providing a new mechanism for RPE regeneration and thus preserving the viability of photoreceptors [20]. Lu et al. demonstrated that the embryonic stem cell-derived RPE implanted in a pathological mouse model was able to restore visual function in the short term. After 90 days, the visual acuity started decreasing [21]. This reduced efficacy could be due to the lack of interaction between the transplanted cells and Bruch's membrane, thereby not forming a functional monolayer. Phase I/II clinical trials were carried out to determine the primary endpoints of safety and tolerability of a subretinal injection of a cell suspension [22]. No serious adverse effect was encountered; however, concerns about integration efficiency and long term cell survival were raised. A possible approach to overcome this issue is represented by cell sheet engineering [23,24]. Cell sheet engineering is based on harvesting a sheet of cells along with their extracellular matrix (ECM) without the use of enzymes [25]. Harvesting

cells is performed with thermoresponsive coatings that enable reversible cell detachment by switching their surface hydrophobicity. Such an approach may allow the formation of an intact RPE cell monolayer to be transplanted. Furthermore, the presence of an ECM should improve the attachment to the host tissue once implanted [26]. However, the use of cell sheet engineering for the RPE is limited by the insufficient amount of the ECM secreted by these cells [18]. In addition, when multiple structures are involved in the pathology, as in AMD, cell therapy cannot be effective in the long term. For example, Bruch's membrane is also compromised in AMD; therefore, it needs to be replaced along with the RPE monolayer. Alternative strategies are needed to fabricate a mechanically strong tissue that promotes cell attachment and survival while maintaining cell functionality in the long term.

3. Tissue Engineering Approach

Tissue engineering has been commonly employed in biomedical applications [27–30]. This approach relies on the use of scaffolds as support systems for adherent cells. Scaffolds should provide a proper in vivo-like microenvironment for tissue regeneration.

Autologous Bruch's membrane explants or its constituent layers were first used as a scaffold for RPE cells to regenerate the outer retina in patients with AMD. According to early studies, the degree of structural support for cell attachment decreases with a decreasing concentration of proteins contained in the RPE basal lamina such as specific laminins [31–35]. To improve the RPE attachment, Bruch's membrane explants were coated with fibronectin, laminin and vitronectin [36–38]. The cell attachment was also enhanced by seeding the RPE cells onto Bruch's membrane explants coated with an ECM previously secreted by corneal endothelial cells [39]. However, the inter-donor variability and limited availability of a native Bruch's membrane have urged researchers to use artificial scaffolds for clinical applications.

The fabrication of artificial scaffolds offers a valid alternative to native tissues. A variety of three-dimensional (3D) scaffolds have been designed and developed for the skin, the bladder, cartilage, bones and muscles [40–44]. Selecting a scaffold fabrication technique and chemical composition is crucial to reproducing the microarchitecture of a native ECM. Here, we provide an overview of the fabrication methods used to engineer a human retina (Figure 2). First, we present the conventional and common methods for retinal scaffold production. We then lay emphasis on the most recent and promising advances in fabrication techniques used for retina tissue engineering.

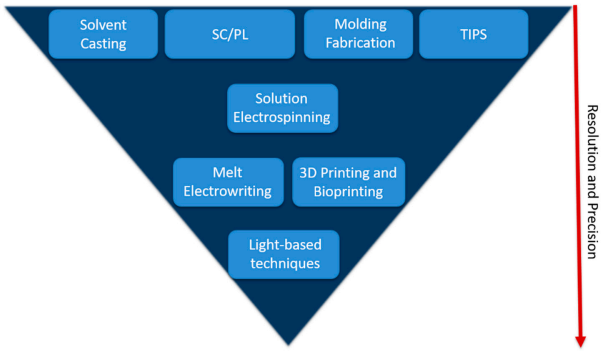

Figure 2. Overview of fabrication methods used for retinal tissue engineering. SC/PL stands for solvent casting-particulate leaching and TIPS for thermally induced phase separation. The red arrow indicates the increase in precision and resolution of the techniques.

3.1. Conventional Fabrication Techniques

Solvent casting techniques have been highly utilized in ocular tissue engineering [45]. They consist of dissolving a polymer in an appropriate solvent followed by casting and solvent evaporation [46]. The resulting scaffold is a uniform and non-porous film. In retinal tissue engineering, these films were seeded with RPE cells to regenerate the RPE layer and replace Bruch's membrane for AMD treatment, thus preventing the loss of photorecep-

tors [47–51]. The prosthetic Bruch's membrane manufactured by solvent casting displayed a similar thickness to the human Bruch's membrane and promoted correct implantation and orientation of the cellular graft in the subretinal space. Moreover, as the degradation of the polymer proceeds, transplanted RPE cells should re-establish interactions with the native Bruch's membrane [47]. To enhance the survival of a functional RPE monolayer in the long term, the film surface was modified [52,53]. Singh et al. showed that silk fibroin scaffolds coated with type I collagen promoted the in vitro development and survival of a functional RPE monolayer for 90 days [52]. Hasirci et al. used oxygen plasma treatment to render a film surface hydrophilic, thus enhancing cell attachment and spreading [53]. However, the film's non-porous structure did not match the open fibrillar structure of the native Bruch's membrane and could prevent nutrient diffusion from the choroid in vivo. To generate a porous scaffold, a solvent casting/particle leaching (SC/PL) method was adopted in biomedical applications [54]. In the SC/PL technique, the solvent, where the polymer is dissolved, contains leachable particles known as porogen [55]. This method allows the production of scaffolds with high porosity (up to 93%) in a simple, inexpensive way [54]. Prosthetic Bruch's membranes for RPE transplantation were successfully prepared using the SC/PL method [56–58]. Moreover, to develop an optimized cell therapy strategy, the influence of the scaffold pore size on the cell differentiation into the retinal precursor has been investigated [59]. However, the low reproducibility limits the use of the SC/PL technique for retinal tissue engineering applications [60].

Over the last decade, hydrogels have received considerable attention as leading candidates for engineering soft tissues such as the retina due to their unique compositional and structural similarities to the ECM [61]. As such, they are highly hydrophilic and biocompatible, thus promoting cell attachment, proliferation and differentiation. Various methods have been used to produce hydrogels depending on the desired structure and application [61]. The most common method of hydrogel preparation involves the crosslinking of the polymer solution poured into a mold of a specific shape. Crosslinking may occur by physical or chemical methods; the type and degree of crosslinking strongly affects the properties of hydrogels such as the elastic modulus, nutrient transport and swelling capacity [62]. In retina tissue engineering, hydrogels have been employed as scaffolds for RPE, photoreceptors and the regeneration of RGCs [63–70]. For instance, implantable hydrogels with encapsulated cells were proposed as promising candidates to treat those blinding diseases caused by the degeneration of the RPE. Hydrogels acted as a cell delivery vehicle providing an appropriate environment for RPE regeneration [63–65]. Marmorstein et al. seeded an induced pluripotent stem cell (iPSC)-derived RPE onto the surface of thin fibrin hydrogels as a support material for RPE transplantation [66]. The findings of this study indicated that fibrin hydrogels could be easily manipulated with surgical tools and degraded rapidly into products recognized and metabolized by the body. IPSC-RPE cells on a fibrin hydrogel were viable and organized into a functional monolayer that could be preserved during and after implantation thanks to the hydrogel support [66]. However, despite their high biocompatibility, hydrogels have low mechanical properties and, hence, they are not suitable as a Bruch's membrane substitute. In fact, hydrogel stiffness ranges from a few Pa to few kPa whereas the elastic modulus of Bruch's membrane is estimated to be around few MPa [5]. Hydrogels were also employed as 3D scaffolds for neural retina tissue engineering to regenerate damaged photoreceptors and RGCs [69,70]. Conjunctiva stem cells encapsulated in fibrin gels differentiated into photoreceptor-like cells, thus providing a new potential therapy for diseases involving photoreceptor injuries [70]. Similarly, retinal ganglion-like cells were differentiated from dental pulp stem cells suspended in fibrin gels [69]. This construct could be transplanted into patients with glaucoma to repair axonal damage and prevent further RGC death. The 3D networks resembled the physiological 3D microenvironment of the neural retina, thus promoting cell viability, differentiation and function when compared with 2D cultures [68–70]. However, the molding fabrication method has a limited microarchitecture and cell distribution controllability.

Another technique that has been explored in retinal tissue engineering is thermally induced phase separation (TIPS). TIPS induces the destabilization of a homogenous solution composed of a polymer dissolved in a solvent [71]. After dissolution, a polymer can be thermodynamically unstable at low temperatures, thus causing a spontaneous separation of the solution into a polymer-rich phase and a solvent-rich phase. Lowering the temperature in a controlled manner causes the solidification of the solvent and subsequently the separation of the polymer from the solvent. The solvent crystals are then removed through extraction, evaporation and sublimation resulting in porous structures [72]. The TIPS method has been largely employed for tissue engineering applications in general, for example, bones and cartilage, due to its ability to produce highly porous scaffolds with interconnected pores [73–75]. Porous membranes, fabricated by a separation phase, were investigated as a scaffold for RPE transplantation [76]. TIPS was also used to fabricate scaffolds for retinal progenitor cells isolated from mice eyes [77]. Seeded scaffolds were tested in vitro and then implanted into models of retinal degeneration. The outcomes of this study indicated that the scaffolds promoted cell differentiation in vitro and cell survival in vivo [77]. The guidance of cell differentiation by the substrate could be exploited to form functional photoreceptors to replace the cells lost due to degenerative diseases. However, the use of the TIPS technique is limited by the low control of pore size and geometry [73].

The retinal scaffolds produced by conventional techniques are listed in Table 1. Due to their own limits, the conventional fabrication methods have been mostly replaced by innovative techniques that hold great promise as potential treatments for retinal degenerative diseases.

Table 1. A summary of scaffolds produced by conventional methods and used for retinal tissue engineering. This table highlights the fabrication technique, the structure of the scaffold and the cell type used. SC/PL: solvent casting-particle leaching; TIPS: thermally induced phase separation; RPE: retinal pigment epithelium; ESC: embryonic stem cells; iPSC: induced pluripotent stem cells; RGC: retinal ganglion cells.

Fabrication Technique	Scaffold Structure	Cell Type	Research Stage	Reference
Solvent casting	Non-porous film	Human fetal RPE cells	In vitro	[47]
Solvent casting	Non-porous film	Human cell line ARPE-19	In vitro and in vivo (rabbit)	[48]
Solvent casting	Non-porous film	Human cell line ARPE-19	In vitro and in vivo (rabbit)	[49]
Solvent casting	Non-porous film	Human cell line ARPE-19	In vitro	[50]
Solvent casting	Non-porous film	Human RPE cells	In vitro	[51]
Solvent casting	Non-porous film	Human iPSC-RPE cells	In vitro	[52]
Solvent casting	Non-porous film	Human cell line D407	In vitro	[53]
SC/PL	Porous membrane	Human cell line ARPE-19	In vitro	[56]
SC/PL	Porous membrane	Human fetal RPE cells	In vitro	[57]
SC/PL	Porous membrane	Human ESC-RPE cells	In vitro	[58]
SC/PL	Porous membrane	Human or pig RPE	In vitro	[59]
Silicon Mold	3D hydrogel with cells encapsulated	Rabbit RPE cells	In vitro	[63]
Petri dish Mold	3D hydrogel with cells encapsulated	Human cell line ARPE-19	In vitro	[64]
Mold	3D hydrogel with cells encapsulated	Human iPSC- and ESC-derived embryoid bodies	In vitro	[65]
Custom or well plates mold	Thin hydrogel	Human iPSC-RPE cells	In vitro	[66]
Custom mold	Thin gel film	Human cell line ARPE-19	In vitro	[67]
Mold	3D hydrogel	Rat RGCs and amacrine cells	In vitro	[68]
Well plate mold	3D hydrogel with cells encapsulated	Rat dental pulp stem cells	In vitro	[69]
Well plate mold	3D hydrogel with cells encapsulated	Human conjunctiva mesenchymal stem cells	In vitro	[70]
TIPS	Porous membrane	Human cell line ARPE-19	In vitro	[76]
TIPS	Porous membrane	Rat retinal progenitor cells	In vitro and in vivo (rats)	[77]

3.2. Electrospinning

The electrospinning process has been suggested as a promising technique to fabricate a prosthetic Bruch's membrane as it is able to recapitulate the nanofibrous structure of a native Bruch's membrane [78]. Indeed, the electrospinning technique is able to produce 3D thin nanofibrous membranes by using natural and synthetic polymers [79]. These fibrillar networks are highly permeable for solutes, thus facilitating cell adhesion and proliferation [79]. The electrospinning setup involves a high-voltage supply, a capillary tube/syringe with a needle, a syringe pump and a collector. The high-voltage supply applies a positive charge to a polymeric solution (solution electrospinning) or melt (melt electrospinning) that is extruded from the needle forming a jet. The jet becomes unstable and thin and forms fibers while the solvent evaporates. The fibers are deposited onto a collector [80]. The morphological properties of electrospun membranes such as thickness, fiber size and orientation can be tuned simply by changing the specific parameters of the electrospinning process such as the polymer concentration, solution flow rate and collector distance [81]. Many studies have reported the fabrication of electrospun scaffolds for vascular, cardiac and neural tissue engineering [82–84].

Solution electrospinning has been utilized to produce a prosthetic Bruch's membrane to engineer an RPE layer. The hypothesis is that the RPE monolayer engineered on thin electrospun membranes could become an effective therapy to cure blindness and the deficiencies associated with RPE and Bruch's membrane degeneration. Synthetic and natural polymers including poly(lactic acid) (PLA), poly(ε-caprolactone) (PCL) and poly(lactic-co-glycolic acid) (PLGA), silk fibroin and silk fibroin-PCL-gelatin were used to fabricate electrospun Bruch's membrane-like scaffolds [85–100]. Typically, a blend of natural and synthetic materials is electrospun as synthetic polymers exhibit good mechanical properties and a controlled degradation rate while pure naturally-derived polymers promote cell adhesion and proliferation. Warnke et al. successfully produced ultrathin nanofibrous scaffolds based on collagen and PLGA that closely imitated the structure of a native Bruch's membrane [85]. Human RPE cells seeded onto these scaffolds formed a functional monolayer with a typical cobblestone morphology and abundant sheet-like microvilli on their apical surfaces; however, no results of the scaffold mechanics and permeability were reported [85]. In our previous paper, we fabricated a Bruch's membrane-like membrane composed of a blend of *Bombyx mori* silk fibroin and PCL to study the pathological mechanisms of AMD in a 3D in vitro model [100]. The resulting scaffolds showed similarities with a human Bruch's membrane with regard to the architecture, permeability and mechanical properties [100]. These in vitro studies indicated the feasibility of using electrospun membranes as a prosthetic Bruch's membrane on which a functional RPE monolayer is formed. As such, RPE patches, composed of electrospun scaffolds previously seeded with RPE cells, have been investigated in vivo. The RPE patches were proven to be biocompatible when implanted in animal models showing no adverse reactions [86,89,92,95,98]. Sharma et al. also evaluated the functionality of clinical-grade iPSC-RPE patches in rats and in a porcine laser-injured model. According to the findings, the use of the scaffold improved the patch integration and efficacy over the cell suspension [95]. In fact, an increased photoreceptor preservation was encountered in animals transplanted with the patch in respect to those injected with the cell suspension. This study has led to a phase I/II clinical trial (NCT04339764) based on patch subretinal transplantation in patients suffering from advanced stages of dry AMD.

Electrospun scaffolds have been employed also in neural retina engineering to guide the growth of RGC axons through the control of fiber orientation [101–103]. This approach could benefit patients suffering from glaucoma and optic nerve diseases. The transplantation of functional RGCs onto a suitable support combined with treatments that promote dendritic integration may regenerate axons and help recreate a healthy axonal transport. Radially oriented scaffolds have been investigated for the transplantation of RGCs as they mimic the radial axon pattern [101]. It was observed that radially oriented scaffolds increased the survival of RGCs while preserving the cellular electrophysiological function. Moreover, these scaffolds promoted RGC axonal integration with the host retinal nerve fibers in retinal rat explants whereas RGCs transplanted directly onto explants grew axons in a random

pattern [101]. Soleimani et al. compared radially and randomly oriented scaffolds to regenerate photoreceptors. They found that the expression of rod photoreceptor-specific genes increased when stem cells were differentiated on randomly oriented nanofibers [104].

Electrospun scaffolds for retina applications are summarized in Table 2.

Table 2. A summary of retinal scaffolds fabricated by electrospinning. This table highlights the fabrication technique, the structure of the scaffold and the cell type used. RPE: retinal pigment epithelium; ESC: embryonic stem cells; iPSC: induced pluripotent stem cells; RGC: retinal ganglion cells.

Fabrication Technique	Scaffold Structure	Cell Type	Research Stage	Reference
Electrospinning	Ultrathin random nanofibrous membrane	Human RPE cells	In vitro	[85]
Electrospinning	Ultrathin random nanofibrous membrane	Human RPE cells	In vitro and in vivo (rabbit)	[86]
Electrospinning	Random nanofibrous membrane	Rat retinal progenitor cells	In vitro	[87]
Electrospinning	Random nanofibrous membrane	Porcine RPE cells	In vitro and ex vivo (pig)	[88]
Electrospinning	Random nanofibrous membrane	Human RPE cells	In vitro and in vivo (rat)	[89]
Electrospinning	Random nanofibrous membrane	Human fetal and adult RPE cells	In vitro	[90]
Electrospinning	Random nanofibrous membrane	Human RPE cells	In vitro	[91]
Electrospinning	Random nanofibrous membrane	Human fetal RPE cells	In vitro and in vivo (rabbit)	[92]
Electrospinning	Random nanofibrous membrane	Human RPE cells	In vitro	[93]
Electrospinning	Random nanofibrous membrane	Human ESC-RPE cells	In vitro	[94]
Electrospinning	Random nanofibrous membrane	Human iPSC-RPE cells	In vitro and in vivo (rat/porcine)	[95]
Electrospinning	Nanofibrous membrane	Human ESC-RPE/bovine RPE cells	In vitro	[96]
Electrospinning	Random nanofibrous membrane	Human RPE cells	In vitro	[97]
Electrospinning	Random nanofibrous membrane	Human cell line ARPE-19/MIO-M1	In vitro and in vivo (rat)	[98]
Electrospinning	Random nanofibrous membrane	Rat retinal progenitor cells	In vitro	[99]
Electrospinning	Random nanofibrous membrane	Human cell line ARPE-19	In vitro	[100]
Electrospinning	Aligned nanofibrous membrane	Rat RGCs	In vitro and ex vivo (rat)	[101]
Electrospinning	Aligned nanofibrous membrane	Rat RGCs	In vitro	[102]
Electrospinning	Random nanofibrous membrane	Human iPSC-RGCs	In vitro and in vivo (rabbit/monkey)	[103]
Electrospinning	Random + aligned nanofibrous membranes	Human conjunctiva stem cells	In vitro	[104]

Despite the advantages, the electrospinning technique has a limited microarchitecture controllability. Therefore, to overcome this drawback, melt electrowriting (MEW) has been recently developed [105]. MEW allows the controlled deposition of a polymer melt fiber starting from a digital model, thus combining melt electrospinning and additive manufacturing principles. To the best of our knowledge, this innovative method has been used in vascular, bone and skin tissue engineering but no studies on a retinal application are yet present [106–108].

3.3. Lithography

Lithography is a technique that allows the formation of precise and complex 2D and 3D microarchitectures and nanoarchitectures. In the field of biomedicine, photolithography has offered a promising alternative to a conventional fabrication method [109]. Photolithography is a photon-based technique that exploits light to project a mask into a photosensitive emulsion (photoresist) coated onto a substrate. The master fabricated by photolithography can be used to create a polymer negative mold typically of polydimethylsiloxane (PDMS) for a polymeric scaffold fabrication [109]. Retinal progenitor cells were successfully seeded onto structures fabricated through photolithography [110–112]. The microfabricated topography enhanced the attachment, organization and differentiation of the progenitor cells into photoreceptor-like cells [111]. Hence, these structures could be used for photoreceptor replacement in the treatment of photoreceptor degeneration. Photolithography might be followed by wet and ion etching. For instance, Lu et al. developed and tested in vitro a parylene-C membrane artificial Bruch's membrane for an RPE cell culture as a potential treatment for dry AMD [8,113]. In vivo studies demonstrated the safety and potential of a parylene membrane as an RPE scaffold [8]. In particular, the implantation of the membrane previously seeded with cells was compared with the injection of a cell suspension. The results showed that cell survival was greater in animals that were transplanted with the cell membrane patch than those that received the cell suspension. Moreover, when injected, cells were observed as clumps whereas an RPE monolayer was visible in rats transplanted with the patch. These findings suggest that this approach may improve visual function at least in the short term in a few patients suffering from advanced stages of dry AMD. Currently, there is an ongoing clinical trial at phase I/IIa (NCT02590692) to study the safety of subretinal implantation of human embryonic stem cells seeded onto a parylene membrane [114].

The retinal scaffolds fabricated via photolithography are listed in Table 3.

Table 3. A summary of retinal scaffolds fabricated by lithography techniques. This table highlights the fabrication technique, the structure of scaffold and the cell type used. RPE: retinal pigment epithelium; ESC: embryonic stem cells.

Fabrication Technique	Scaffold Structure	Cell Type	Research Stage	Reference
Photolithography	Porous scaffold	Mouse retinal progenitor cells	In vitro	[110]
Photolithography	Thin film scaffold	Mouse retinal progenitor cells	In vitro	[111]
Photolithography	Porous scaffold	Mouse retinal progenitor cells	In vitro and in vivo (mouse)	[112]
Photolithography + wet and ion etching	Mesh-supported submicron membrane	Human cell line ARPE-19/H9-RPE	In vitro	[113]
Photolithography + wet and ion etching	Mesh-supported submicron membrane	Human ESC-RPE cells	In vitro and in vivo (rat)	[8]

3.4. 3D Bioprinting

To overcome the lack of a fine control of structural aspects, additive manufacturing (AM) techniques have been introduced into the tissue engineering area. AM is a process by which a digital model for a 3D object is assembled in a layer-by-layer manner [115]. AM advantages include the ability to fabricate complex geometries with multimaterial parts [115]. Among these techniques, 3D printing plays a key role in developing personalized treatments, surgical planning and the testing deployment of devices in realistic pathways due to its potential to fabricate patient-specific 3D structures in a cost-, time- and waste-effective manner [116]. In the medical field, the latest promising evolution of 3D printing is 3D bioprinting.

3D bioprinting is an innovative biofabrication strategy, which allows the precise positioning of non-living materials, as in 3D printing, and living materials in a prescribed 3D hierarchical organization [117]. During the bioprinting process, the bioinks, composed of cells

embedded in biocompatible materials, are dispensed to form the functional desired structures. 3D bioprinting aims to create 3D bioengineered structures serving in regenerative medicine, pharmacokinetics and basic cell biology studies [117]. Such 3D bioprinted constructs can be then cultured in bioreactor systems to obtain mature functional tissues and organs [118]. A few examples of 3D bioprinted scaffolds are represented by synthetic skin to be transplanted onto patients with burn injuries, heart valve replication and bionic ears [119–121]. Currently, there are mainly three types of bioprinting systems (laser-based, inkjet-based and extrusion-based) characterized by a high deposition accuracy, stability and cell viability [117].

Laser-based technology exploits a pulsed laser source and an optical path to focus a laser on a target from which the bioink is printed and deposited onto a substrate. The target is composed of a glass slide, a metal slide and bioink. The laser is focused through the glass slide onto the metal slide inducing a vaporization of the metal-absorbing layer, thus resulting in the production of a jet of bioink. Many researchers have demonstrated that cells were highly viable after laser-based 3D bioprinting [122]. However, cell placement accuracy can be challenging and, in addition, this technology is expensive and relatively slow.

On the contrary, inkjet-based bioprinting is an inexpensive and simple to use technology while offering a relatively high resolution and cell viability [122]. In inkjet bioprinting, small droplets of bioink are ejected from a nozzle through microheater or piezoelectric systems and then dispensed onto a substrate [122]. Inkjet bioprinting was investigated to seed RGCs and glial cells. The technology did not affect the survival and the growth of rat RGCs and glial cells compared with cells seeded onto tissue culture plates [123]. This result opens the way for developing a printed construct to be used in retinal regeneration. Recently, a 3D in vitro retina model comprising of RPE cells and photoreceptors was fabricated using inkjet-based technology to study the interaction between the layers in AMD disease [124]. According to the authors, after bioprinting both cell types were correctly positioned in a layered structure and expressed specific proteins such as tight junction-associated protein ZO-1 in the RPE layer and light-sensitive proteins in the photoreceptor layer [124]. The use of inkjet-printed structures for tissue engineering is limited by their low mechanical properties and their long term durability. Moreover, due to the presence of the nozzle, clogging issues are common with viscous bioink [122].

The extrusion-based technology relies on the extrusion of continuous filaments of bioink through a nozzle using a driving force, i.e., a piston, a screwing system or pneumatic pressure. It is the most versatile bioprinting process as it enables the printing of the broadest range of bioink viscosities [122]. Additionally, it allows clinically relevant constructs to be obtained in terms of size and shape. However, the rheological requirements of the bioink are stringent [125]. Extrusion-based bioprinting was investigated to produce a 3D in vitro model of the RPE and photoreceptor layers [126]. The RPE cell line was bioprinted with a precise pattern and allowed to form a monolayer in 14 days followed by the bioprinting of the photoreceptor cell line [126]. Such a bioprinted construct could be meaningful for biomedical applications such as disease research and high-throughput screening.

So far, the bioprinted retinal construct has been used only as in vitro models that could be used in the future for basic research and drug screening (Table 4). However, this technology is very promising also for retinal tissue engineering due to the possibility of generating layer-by-layer a 3D complex multicellular stratified structure, which mimics the retina architecture and could be transplanted as potential therapies for retinal diseases involving the degeneration or dysfunction of multiple retinal layers.

Table 4. A summary of 3D bioprinting techniques used in retinal applications. This table highlights the fabrication technique, the structure of the scaffold and the cell type used. RGC: retinal ganglion cells.

Fabrication Technique	Scaffold Structure	Cell Type	Research Stage	Reference
Inkjet bioprinting	Not applicable	Rat RGCs and retinal glia	In vitro	[123]
Inkjet bioprinting	3D bilayer retina model	Human cell line ARPE-19 and pig photoreceptors	In vitro	[124]
Extrusion bioprinting	3D bilayer retina model	Human cell line ARPE-19 and Y79	In vitro	[126]

3.5. Hybrid Approach

Recently, researchers have focused on combining two or more fabrication approaches for maximal treatment efficacy (Table 5). Tan et al. combined SC/PL and TIPS to develop ultrathin polymer membranes as a prosthetic Bruch's membrane for an RPE replacement [127]. Solvent casting and photolithography were exploited to produce a polyester-based micropatterned film for RPE cells [128]. Shi et al. developed a hybrid approach to fabricate a 3D in vitro model that comprised an artificial Bruch's membrane, a bioprinted ARPE-19 cell monolayer and a Y79 cell-laden alginate/pluronic bioink [129]. This model could be used in the future to study the pathological mechanisms underlying AMD. Another hybrid approach based on electrospinning and 3D bioprinting has been established for the replacement of RGCs as a promising therapy for glaucoma [130]. RGCs were precisely seeded onto an electrospun scaffold via a thermal inkjet 3D cell printing technique. The electrospun scaffold guided the growth of the RGCs [130].

Table 5. A summary of hybrid approaches used in retinal applications. This table highlights the fabrication technique, the structure of the scaffold and the cell type used. RGC: retinal ganglion cells.

Fabrication Technique	Scaffold Structure	Cell Type	Research Stage	Reference
SC/PL + TIPS	Ultrathin, free-standing, porous membrane	Human cell line ARPE-19	In vitro	[127]
SC + photolithography	Micropatterned film	Human cell line D407	In vitro	[128]
SC + 3D bioprinting	3D construct: ultrathin membrane + bilayer model	Human cell line ARPE-19 and Y79	In vitro	[129]
Electrospinning + 3D bioprinting	Nanofibrous membrane + bioprinted cells	Rat RGCs	In vitro	[130]

4. Conclusions

Regenerative medicine research and optimization carries enormous hope as means to restore visual function compromised by retinal diseases. In this review, we reported both the conventional and recently developed methods to produce scaffolds for retinal tissue engineering. We believe that innovative tissue engineering techniques open the way for new therapies based on retinal grafts that could be implanted and reverse cell loss. Moreover, new tissue engineering strategies in conjunction with gene therapy holds great promise for the development of innovative clinical treatments to cure retinal diseases.

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