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A review of retinal tissue engineering and the possible application of dECM bioink in retinal 3D printing.

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A Lisa e Francesco

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Contents

List of abbreviations

AM:	Amniotic membrane
AMD:	Age related macular degeneration
BrM:	Bruch's membrane
Co-dECM:	Cornea specific decellularized extracellular matrix
dECM:	Decellularized extracellular matrix
decell-retina:	Decellularized retina
DI water:	Deionized water
DIW:	Direct ink printing
DR:	Diabetic retinopathy
ECM:	Extracellular matrix
EL:	Elastin layer
GA:	Geographic atrophy
GAGs:	Glycosaminoglycans
HA:	Hyaluronic acid
hESC:	human Embryonic stem cells
hiPSC:	human induced Pluripotent stem cells
HS:	Heparan Sulphate
ICL:	Inner collagenous layer
LIFT:	Laser induced forward transfer
oBRB:	outer Blood retina barrier
OCL:	Outer collagenous layer
PBS:	Phosphate buffer saline
PCL:	Poly (ϵ-caprolactone)
PGA:	Poly glycolic acid
PLA:	Poly lactic acid
PLGA:	Poly(lactic-co-glycolic acid)
RGC:	Retinal ganglion cell
RP:	Retinitis pigmentosa
RPC:	Retinal progenitor cells
RPE:	Retinal pigment epithelium

SDS: Sodium dodecyl sulphate

TE: Tissue engineering

3D: Three-dimensional

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Abstract

Visual impairments affect on average 2.1% of European population, and this percentage grows with growing age. Among visual impairments, degenerative retinal diseases are a group of diseases that if left untreated can cause irreversible blindness, since they are characterized by progressive irreversible cell death. Nowadays, there are not treatment strategies to reverse the degeneration of these diseases. Thus, in this work recent progresses in retinal regenerative medicine strategies are investigated.

First of all, a brief description of the anatomy of the retina is performed; a particular focus is put on the retinal pigment epithelium (RPE) and Bruch's membrane (BrM) but also on main characteristic of the neural retina. In fact, they are the main target of retinal tissue engineering strategies. Then, some of the most diffused retinal degeneration diseases such as age related macular degeneration (AMD), retinitis pigmentosa (RP), glaucoma and diabetic retinopathy (DR) are described. Finally, the main perspective in retinal tissue regeneration are reported.

In the second chapter, among the most interesting approaches to retinal tissue engineering, both scaffold-free and scaffold-based methods are summarized. In particular, scaffold-free methods comprise cell suspension injection and cell sheet engineering approach. However, generally this approach does not allow obtaining good outcomes in terms of cell attachment to the retina.

On the contrary, scaffold based methods are based on the use of material to lead cell growth, proliferation and differentiation. Of course, scaffold can be fabricated using natural or synthetic polymers, but also using natural membranes such as decellularized extracellular membrane (dECM). dECM are appealing material for tissue engineering because of their ability to mimic the native extracellular environment. They can be obtained through a decellularization process and dECM was already applied for retinal tissue engineering.

In the third chapter, it is analysed the possibility to develop retinal dECM gels to be used as bioinks for 3D bioprinting applications. 3D bioprinting is an evolving biofabrication technique that provide the printing of cell-laden structures. Actually, 3D bioprinting allow the printing of biomaterials containing living cells or cell suspensions that can be deposited in a precise way. This technique is so appealing because it gives the possibility of controlling

scaffold architecture, composition and porosity. In this final chapter, there is a focus on bioinks for 3D bioprinting, on their most important requirements and on the main materials used as bioinks in literature. Finally, the use of dECM gels as bioink in 3D bioprinting is considered. dECM bioink preparation strategies are summarized and some of the most interesting work on the use of dECM gel as bioink are reported.

As a conclusion, a perspective for 3D printing of the retina using a retinal dECM bioink is presented.

Sommario

I difetti visivi colpiscono in media il 2.1% della popolazione europea e questa percentuale cresce all'aumentare dell'età. Fra i difetti visivi, le malattie degenerative della retina sono un gruppo di patologie che se non trattate possono causare cecità irreversibile, poiché essi sono caratterizzati dalla perdita progressiva delle cellule retinali. Quindi, in questo lavoro verranno analizzati i recenti progressi svolti nell'ambito delle strategie di medicina rigenerativa della retina.

Prima di tutto, viene svolta una breve descrizione dell'anatomia della retina con particolare focus sull'epitelio retinico pigmentoso (RPE), sulla membrana di Bruch (BrM) ma anche sulle principali caratteristiche della retina neurale. Infatti, questi sono i principali target delle strategie di ingegnerizzazione della retina. In seguito, vengono descritte alcune delle più diffuse malattie degenerative della retina come la degenerazione maculare senile, la retinitis pigmentosa, il glaucoma e le retinopatie diabetiche. Infine, vengono riportate le principali prospettive dell'ingegneria tissutale della retina.

Nel secondo capitolo, si svolge una riassunto dei più interessanti approcci alla rigenerazione della retina, distinguendo gli approcci basati sull'utilizzo di scaffold (scaffold based) e quelli senza scaffold (scaffold-free). Fra questi ultimi, si citano l'iniezione di sospensioni cellulari e l'ingegneria dei foglietti tissutali. Tuttavia, generalmente queste strategie non consentono di ottenere buoni risultati in termini di adesione delle cellule alla retina in vivo.

Al contrario, i metodi basati sull'utilizzo di scaffold utilizzano dei materiali che possano guidare la crescita, la proliferazione e la differenziazione delle cellule. Ovviamente, anche nel campo della rigenerazione della retina, gli scaffold possono essere fabbricati tramite polimeri naturali o sintetici, ma anche attraverso l'utilizzo di membrane naturali come le membrane extracellulari decellularizzate (dECM). dECM sono materiali molto attrattivi per l'ingegneria tissutale in quanto la loro capacità di mimare il microambiente extracellulare nativo è superiore a quello di qualsiasi altro materiale. Possono essere ottenuti tramite un processo di decellularizzazione e sono già state applicate anche alla rigenerazione della retina.

Nel terzo capitolo, viene infine analizzata la possibilità di sviluppare dECM gel partendo dalla retina che possano essere applicati come *bioink* per l'applicazione del 3D *bioprinting.*

Il 3D *bioprinting* è una tecnologia di *biofabrication* che è in costante evoluzione per permettere lo stampaggio di strutture caricate di cellule. Dovrebbe garantire lo stampaggio di biomateriali contenenti cellule vive o semplicemente sospensioni cellulari, che possano essere depositate su un substrato in modo controllato. Questa tecnologia è così interessante poiché dà la possibilità di poter decidere a priori e quindi controllare l'architettura finale dello scaffold ma anche la sua composizione e la sua porosità. In questo terzo capitolo, si pone attenzione ai *bioink* ad oggi sviluppati, sui requisiti tecnici necessari ma anche su quali materiali siano finora stati applicati nel 3D bioprinting. Infine, viene considerato l'utilizzo di dECM gel come *bioink*, vengono riassunte le principali metodologie per la produzione di un dECM bioink trovate in letteratura e vengono analizzati alcuni dei principali lavori nei quali questi materiali vengono applicati.

In conclusione, viene presentata la prospettiva dell'utilizzo del 3D printing della retina sfruttando un dECM bioink ottenuto dal processamento della retina.

CHAPTER 1

INTRODUCTION: THE RETINA

1.1 The anatomy of the retina

The retina is a thin multilayer sheet of tissue organized in layers, barely half a millimetre thick that lines the inside of the eyeball. The retina is charged with the critical task of receiving, modulating and transmitting visual stimuli from the external world to the optic nerve and, ultimately, the visual cortex of the brain. [1] Adequate conveyance of visual signals depends largely upon the highly specialized anatomy of the retina.

The adult retina is located behind the vitreous that separates the retina from the lens. It is a laminated structure, bordered apically by the retinal pigment epithelium (RPE). Anatomically, the retina can be divided into inner and outer layers. The inner layer comprises neuronal cells, known as photoreceptors, whereas the outer layer comprises the highly specialized pigmented cells of RPE. Outer retina is avascular, receiving nutrients from the choroid, that resides between the Bruch's membrane (BrM) and the sclera. The BrM is a specialized basement membrane to which the retinal pigmented epithelial cell monolayer adheres. The neural retina is composed of nine different neural or glial cells in a highly coordinated manner [2], light must transverse all these layers before initiating signal transduction in the rods and cones photoreceptors.

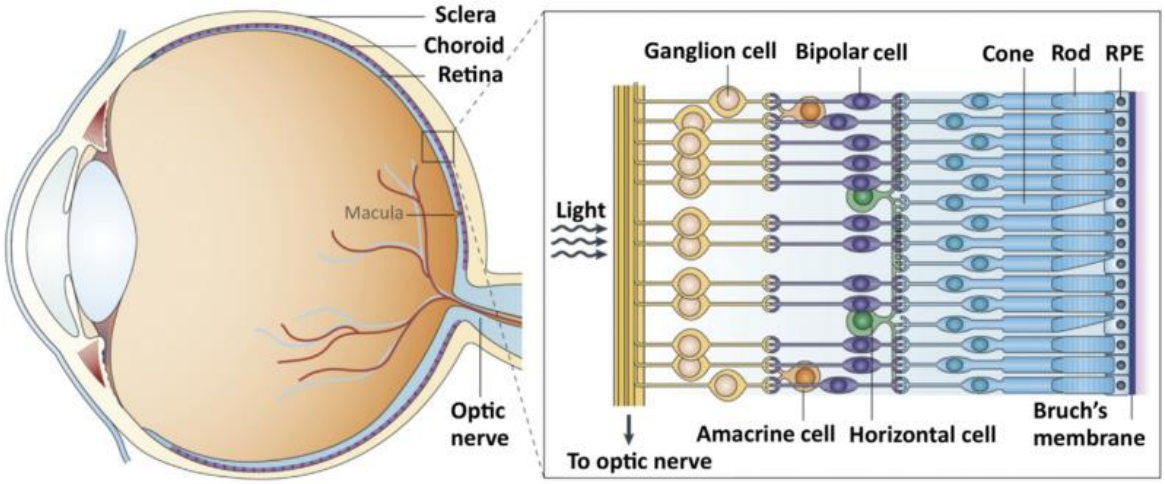

Figure 1.1 Location and architecture of human retina.

Since this work is about tissue engineering of the retina, in the next section there will be a description of the three main components of the retina to which regenerative strategies are addressed: RPE, Bruch's membrane and the neural retina. They are particularly interesting because of the role they play in retinal pathology and in retinal regeneration strategies.

1.1.1 Retinal pigment epithelium (RPE)

RPE is composed of a monolayer of non-regenerative cells that is essential for maintaining vision [3]. RPE is located between retinal photoreceptors and is basally bordered by the Bruch's membrane (BrM), which separates the RPE from the choriocapillaris. RPE with the choroid form the metabolic support system of photoreceptor cells of the inner layer of the retina. This is a support layer, whose cells are an important mean of nourishment for the overlaying neurosensory retina. Cuboidal cells of RPE contains melanosomes, from which cells derive their pigmented colour [1].

The size and shape of RPE cells vary as a function of retinal eccentricity, from a diameter of 14µm at the fovea to 60µm in the peripheral retina. The height of RPE cells is 10-15µm at the fovea and 7,5µm at the periphery. Cell density of RPE is greater in the fovea compared to the equator. Approximately 30-40 photoreceptors overlie each RPE cells. [4]

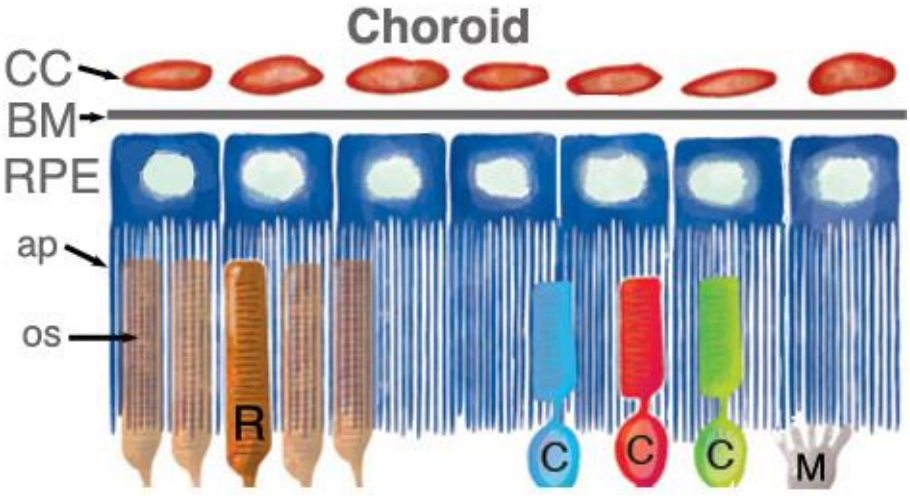

Figure 1.2 Representation of RPE

The apical membrane of RPE faces the photoreceptor outer segment (fig 1.2). Long apical microvilli are projected from RPE and envelop the light-sensitive outer segments establishing a complex of close structural interaction. [5] Generally, the length of outer

segments cloaked by microvilli is greater for rods than cones. This relationship between RPE and photoreceptors is crucial to sight; this is evident from basic and clinical studies demonstrating that primary dysfunctioning of RPE can result in visual cell death. [6]

The lateral membranes of RPE are the sites of cell-cell adhesion and cell communication. The apical junction complexes of RPE play a role in maintaining cell polarity by forming a physical barrier that prevents intermembranous diffusion of components between the apical and basolateral domains.

RPE has numerous function in maintaining vision:

- It is a pigmented epithelium that absorbs light energy focused on the retina by the lens. This is important to both optimize image quality and to protect the retina from oxidative damage caused by reactive free radicals. The pigment melanin is stored and synthetized in melanosomes, its density increases towards the centre of the retina. Melanin absorbs and filters harmful light in the blue wavelength region of the spectrum, which is known to cause photo-oxidation of lipofuscin, a lipid-protein pigment that accumulates with age and from phagocytosis of the photoreceptor outer segments. [5]
- Blood retinal barrier: tight junction between adjacent RPE cells form the outer blood retinal barrier, preventing paracellular transport of large molecules, toxins and water. The resistance to paracellular movement is 10 times higher than the resistance to transcellular movement. [4]
- Phagocytosis of photoreceptors outer segments: photoreceptor membranes undergo renewal process whereby the outer segment tips are shed and subsequently phagocytosed by RPE before new outer segments are constructed at the cilium: this is necessary to maintain photoreceptor excitability [7], to recycle nutrients and to prevent oxidative damage from photo-oxidation of damaged photoreceptor outer segments lipid and protein components.
- Production and secretion of growth factors: RPE produces and secretes several protein mediators, cytokines and growth factors. These factors are essential for maintenance of the structural integrity of retina and choriocapillaris, supporting the survival of photoreceptors and ensuring a structural basis for optimal circulation and supply of nutrients. [5]

- Transport of ion, water and metabolic end-products from the subretinal space to the blood: the close anatomical apposition of the RPE to the photoreceptors is necessary for facilitating the transcellular movement of nutrient, wastes and fluid. The delivery of nutrients such as glucose, ascorbate, fatty acids from the choroidal vasculature to photoreceptors occurs through transporters within the RPE membranes. Constant transport of water away from the subretinal space to the choriocapillaris is achieved through active transport. It maintains close apposition of the RPE to photoreceptors (in fact large volumes of subretinal fluid are generated from metabolic turnover in photoreceptors as well as intraocular fluid flux from the vitreus body to the retina).
- Transepithelial transport and maintenance of subretinal ion levels: this role is strictly connected with the preservation excitability of photoreceptors by careful regulation of subretinal ionic concentration through transepithelial transport of ions. Active transport is necessary to provide the energy gradient, producing high subretinal sodium concentration and high potassium concentration in the RPE cytosol. The elevated subretinal sodium is critical to the dark current. [5]
- Reisomerization of all-trans retinal and transport of retinoids in the visual cycle: the visual cycle is the metabolic process through which the visual pigment (rhodopsin in rods) is regenerated after light activation, in order to maintain light sensitivity of the opsin pigments. [8] This triggering event of the visual cycles the absorption of light by rhodopsin in the photoreceptors, which results in the conversion of the chromophore 11-cis retinal into all-trans retinal. This causes a conformational change in rhodopsin, activating the regulatory protein, transducin, to initiate signal transduction cascades. All-trans retinal must be isomerized back to 11-cis retinal and that occurs in the RPE.
- Immune privilege: the outer blood-retinal barrier formed by tight junction between RPE cells creates a microenvironment, which allows the regulation of the infiltration by immune system components into the retina. Moreover, RPE itself is capable of secreting immunosuppressive factors to downregulate T cell activity [9]. Furthermore, RPE cells, which express mass histocompatibility complex I or II, are able to act as antigen-presenting cells in the eye [10].

1.1.2 Bruch's membrane

Recently, the interest in BrM has increased because of its strategic location between the retina and general circulation and so its crucial role in retinal function, aging and diseases. It is characterized by a pentalaminar structure that forms a single functional unit with the RPE and the choriocapillaries. Its nature is highly dynamic and it is involved in cell-cell communication, cellular differentiation, proliferation, tissue modelling and shaping pathologic processes. [11]

BrM consist of five layers, from the RPE toward the choroid:

- the basement membrane of the RPE that resembles other basement membranes of the body;
- the inner collagenous layer (ICL) that consists of thick striated fibers of collagen type I, III and V organized in a multi-layered grid-like structure;
- the elastin layer (EL) that is composed by several stacked layers of linear elastin fibers of varying shapes and sizes. The fibers form a perforated sheet with interfibrillary spaces of about 1μm. In addition to elastic fibres, the EL contains collagen type VI, fibronectin and other protein-associated substances.
- the outer collagenous layer (OCL) characterized by the same structure of ICL
- the basement membrane of the choriocapillaris is a non-continuous, interrupted BM layer due to the so called intercapillary columns of the choroid. It is predominantly composed of laminin, heparan sulphate (HS) and collagen type IV, V and VI. RPE synthetizes laminins that preferentially adhere BrM to the RPE through interaction with integrins. HS is a common glycosaminoglycan in the BrM, the HS polysaccharide side chains bind to a variety of protein ligands in BrM and regulate a range of biological activities. Collagen type IV in the basement membrane of choriocapillaries may inhibit endothelial cell migration into the BrM. Collagen type V is present in most types of connective tissue, particularly in pericellular spaces and near basement membranes and plays a role in platelet aggregation, epithelial cell migration and binding of interstitial collagen fibrils. Type VI collagen is the major structural component of microfibrils and specific for the choroidal basement membrane. It may be involved in anchoring BrM to the capillary endothelial cells of the choroid. Collagen VI possibly interacts with collagen I, which is abundant in both

the BrM-OCL and the choroidal matrix. A remarkable structural feature of the choriocapillaries adjacent to the choroidal basement membrane is the endothelial fenestrations or pores that are permeable to macromolecules.

BrM is structurally different in the macular area compared with the retinal periphery. BrM is three to six times thinner in the macular area and two to five times more porous than in the retinal periphery. Furthermore, structural proteins are lower in the macular area than in retinal periphery. [11]

The three primary functions of BrM include:

- regulating the diffusion of biomolecules between choroid and RPE: It acts as a semi-permeable filter for the reciprocal exchange of biomolecules between the retina and the choroid (passive transport)
- providing physical support for RPE cell adhesion, migration and perhaps differentiation: BrM from young donors is much more efficient in the attachment of RPE cells than BrM from old donors. Not all layers of BrM show equally strong adhesion properties. RPE-BrM adhesion is mediated by integrin cell surface receptors. Integrins are a group of membrane proteins that is capable of binding to a number of extracellular and BrM matrix components such as laminin isoforms and type IV collagen in anchoring plaques. The RPE continues its development until 6 months after birth. After that, RPE is generally considered to be post-mitotic, however, in case of mechanical or light induced damage, RPE cells can proliferate similarly to other epithelial tissues.
- acting as a division barrier restricting choroidal and retinal cellular migration: the outer blood retina barrier (oBRB) is formed by RPE cells that are connected to each other by tight junctions. oBRB prevents transport of molecules larger than 300kDa into and out the retina. [12] This function is physically supported by BrM that acts as a semi-permeable molecular sieve.

1.1.3 Neural retina

The neural retina is composed of nine different layers and by numerous cell types. Firstly, it comprises photoreceptors, specialized cells that transduce light signal, thanks to the presence of photoreceptive molecules. In human retina, they can be broadly categorized into rods and cones. [13] The rod and cone layer is composed of the outer and inner segments of the rod and cone cells. Rods have exquisite sensitivity to light and can even detect a single photon [14], as a consequence they are responsible for scotopic vision. Cones are 100 times less sensitive than rods, but exhibit much faster response kinetics during phototrasduction. Cones are engaged in bright light, high acuity colour vision. [14] They are concentrated in the fovea where they achieve the highest resolution. There are different cone photoreceptor type, each one sensitive to a specific wavelength of light.

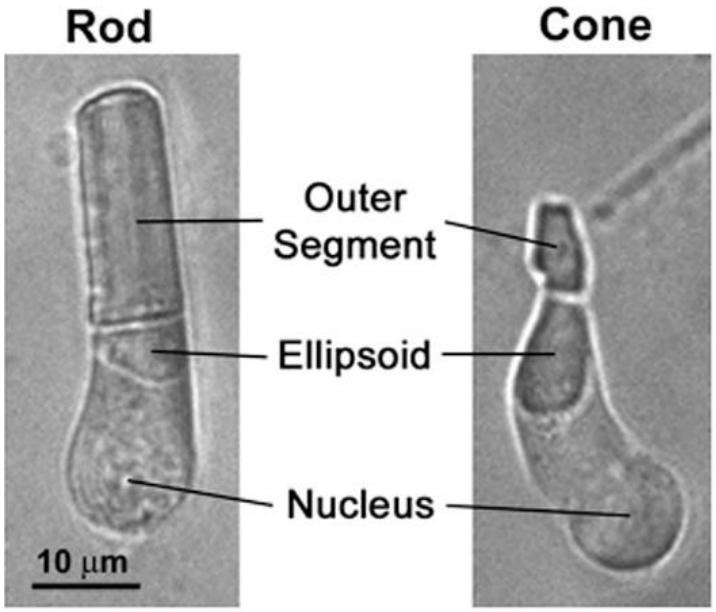

Figure 1.3: Brightfield images of living rod and cone photoreceptors

The outer segment contains stacks of membrane disks, which enclose virtual pigment molecules and are constantly renewed. This part is specialized for phototrasduction. New disks are added to the base of the outer segment at the cilium. At the same time, old disks are replaced outwards. Anatomically, the outer segment constricts at the cilium beginning the inner segment, the part where cell's nucleus and organelles reside. The external limiting membrane separating the inner and outer segment from the photoreceptor nuclei is not a true

membrane but is rather firmed by the terminal bar attachment of the cell bodies of rods cones and Müller cells.

The other cells comprised in the neural retina are fundamental to transmit the signal generated by photoreceptors towards the optic nerve and thus to the brain.

The main cell types that compose the inner neural retina are the bipolar cells that connect photoreceptors ultimately with the dendrites of the ganglion cells in the outer plexiform layer. The horizontal cells are GABAergic neurons that connect to either rod or cone cells and synapse with bipolar cells, where on and off responses are generated, to regulate signal transduction. Amacrine cells receive excitatory input from bipolar cells and inhibitory input from other amacrine cells mediated by GABAc receptors. They can synapse back onto bipolar cells, ganglion cells or other amacrine cells. [1]

Müller cells act as specialized glial cells to form retinal scaffolding, support the inner segments of the photoreceptors and create the acellular fibrous internal limiting membrane. [1] This is composed by laterally contacting Müller cells and it represents the inner surface of the retina bordering the vitreous humor that forms a diffusion barrier between the two compartments.

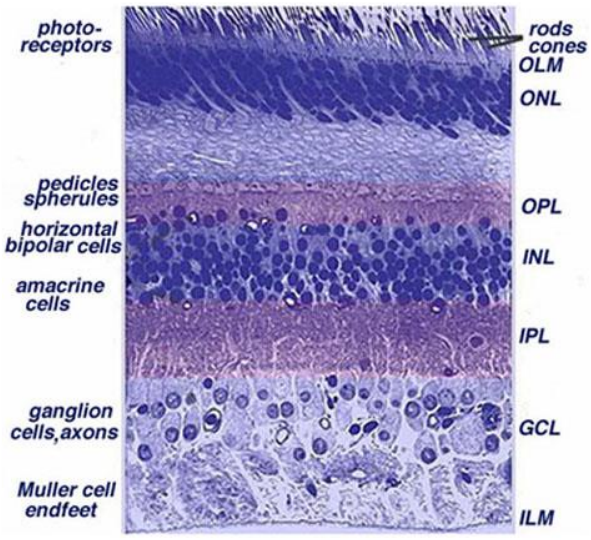

Figure 1.4 Hystology of the central retina

1.2 Diseases of the retina

The human retina is a delicate organization of neurons, glia and nourishing blood vessels. In some eye diseases, retina becomes damaged or compromised, and degenerative changes set

in that eventually lead to serious damage to the nerve cells that carry the viral messages about the visual image to the brane. [15]

Globally, 285 million people are estimated to be blind or visually impaired. 90% of them live in low income countries. In higher income countries, retinal diseases account for a large portion of those blind, with macular disease, mostly age related macular degeneration (AMD), as well as vitreo macular interface abnormalities such as macular hole. In Italy, AMD is estimated to affect 1 million people, 200000-300000 of which in an advanced form. According to the ISTAT report "*Condizioni di salute e ricorso ai servizi sanitari in italia e EU",* visual impairments affect on average the 2.1% of European population over 15 years old, whilst over 65 the percentage grows to 5,6% and to 8,7% in over 75 population. In Italy more than one third of old people (4,5 million people) are affected by visual impairment. [16] Other highly prevalent blinding retinal diseases account for a 25%, including glaucoma, diabetic retinopathy (DR), hereditary retinal conditions (6%) and retinal vascular occlusions. AMD, glaucoma and DR account for 14% of worldwide visual impairment and all increase in prevalence with age. With the ageing population demographic, more people are therefore becoming at risk of visual impairment. [17]

Degenerative retinal diseases are a large group of condition that if left untreated can result in irreversible blindness. [18] Damage to any type of retinal neuron results in irreversible changes and therapeutic modalities that can reverse these degenerative processes are not available. Despite different etiologies, these diseases are characterized by progressive photoreceptor cell death. [18] Given that the retina is unable to regenerate damaged cells, vision loss is irreversible. [19]

1.2.1 Age related macular degeneration

AMD is a complex multifactorial disease that is the leading cause of blindness in individuals over 60. [2] It is manifested fundoscopically in the early an intermediate stages by the appearance of yellowish cellular debris called drusen, accumulating between the RPE and its basement Bruch's membrane in the macular retina [17]. It can be divided into two categories:

- neovascular (wet AMD): it is characterized by aberrant choroidal blood vessel growth through RPE affecting the function of the overlaying neurosensory retina by vascular leak, haemorrhage and fibrosis with subsequent outer retinal degeneration.
- atrophic (dry AMD): it is characterized by degeneration of RPE and subsequently the overlaying photoreceptors [17]. It is the most common form of AMD (50-80%).

Advanced stages of AMD (both dry and wet) are called geographic atrophy (GA). They manifests as the loss of choroid and RPE in the macular region of the retina, causing gradual central vision because of dysfunctional macular photoreceptors.

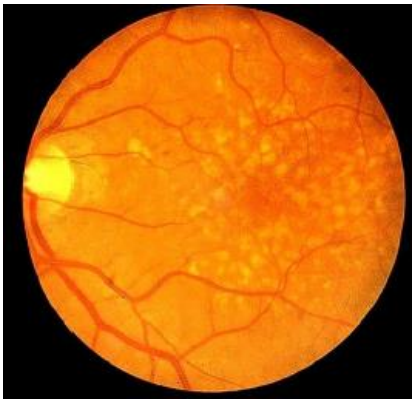

Figure 1.5 A view of the retina of a patient affected by AMD presenting drusen

1.2.2 Retinitis pigmentosa

RP is a group of inherited disorders associated with progressive photoreceptors degeneration and blindness. It is a highly variable disorder, caused by a large number of genetic mutations (most of those which have been discovered concern the rod photoreceptors); some patients develop symptomatic visual loss in childhood whereas others remain asymptomatic until mid-adulthood. As the disease advances, they lose fare peripheral vision, eventually develop tunnel vision and finally lose central vision by the age of 60 years. [20] Visual symptoms indicate the gradual loss of the two photoreceptor types (tipically, rods die first followed by cones death). The outer nuclear layer of the retina that consists of rod and cone nuclei is severely attenuated in patients with RP. The inner nuclear layer, composed of amacrine, bipolar and horizontal cells, and the ganglion cell layer are fairly well preserved, but many of these cells degenerate later in the disease. RP can be associated with extraocular findings

such as deafness (Usher syndrome), obesity (Bardet-Biedl syndrome) or cardiac conduction defects (Kerns-Sayre syndrome). These patients most commonly present with visual impairment in the first quarter of life, although there is significant variability from individual to individual.

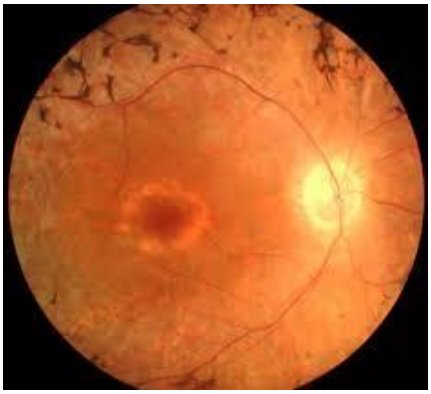

Figure 1.6 Fundus oculi of a patient affected by retinitis pigmentosa

1.2.3 Glaucoma

Glaucoma is the name given to a group of eye diseases, which result in ganglion cell loss with a typical damage to the optic nerve seen fundoscopically as optic disc cupping and slow progressive peripheral field loss finally affecting central field. [17] The most common form is usually associated with raised intraocular pressure related to reduced aqueous humor outflow at the trabecular meshwork within the eye becomes elevated. The pressure rises because the anterior chamber of the eye cannot exchange fluid properly by the normal aqueous outflow methods. The pressure within the vitreous chamber rises and compromises the blood vessels of the optic nerve head and eventually the axons of the ganglion cells so that these vital cells die. The traditional treatment is the use of medical therapies (beta-

Figure 1.7 A view of fundus oculi of a patient with an advanced form of glaucoma

blockers, alpha-agonists, prostaglandin analogues…) to reduce intraocular pressure. As an alternative, laser trabeculoplasty and surgical treatments are applied too. [21]

1.2.4 Diabetic retinopathy

Diabetes mellitus affects over 120 million people worldwide and the prevalence of diabetic retinopathy (DR) correlates with the duration of diabetes mellitus and is 75% after 10 years and 95% after 20 years. [22] DR occurs secondary to hyperglycaemia with the main pathological events occurring primarily in the pericytes and vascular endothelial cells of the retinal blood vessels, which transverse the inner retina, from their entry into the eye at the optic nerve head, although there is increasing evidence that retinal neuronal and glial cells are also affected. DR is therefore characterized by vasodegenerative changes that lead to areas of retinal ischemia, retinal edema and aberrant retinal blood vessel growth. Visual loss is caused by macular ischemia, edema and the tractional and haemorrhagic consequences of new vessel growth. [17]

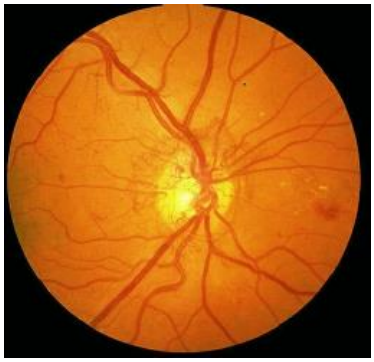

Figure 1.8 A view of the fundus of the eye of a patient affected by an advanced form of diabetic retinopathy

Broadly speaking, AMD and hereditary retinal diseases affect the outer retina, glaucoma the inner retina and DR all retinal layers.

1.3 Regeneration of the retina

Unlike lower vertebrates, which can regenerate retina, adult mammalian retina does not possess the ability to self repair. Nowadays, although there are lots of studying going on, we

lack a clinical relevant medicine approach to treat retinal diseases, such as those descripted previously. Current treatment strategies including gene therapy, anti-angiogenic therapy and growth factor treatment, focus mainly on slowing down the degeneration process. [23] However, recent progress in regenerative medicine has provided emerging hope for visual restoration.

Transplantation approaches such as autograft, xenograft and tissue transplantation have major drawbacks included the need for tissue donors, tissue banks, heavy surgical intervention and immune suppression therapy to prevent any rejection response. Tissue engineering offers an attractive alternative to regenerate defective tissues. It is an interdisciplinary that relies on the development and use of bioactive support structures, scaffolds, which act as a template to which cells are able to adhere and proliferate to regenerate a particular tissue.

Tissue engineering has emerged in the last years as a potential and promising alternative to treat blindness by replacing diseased or damaged tissue with healthy tissue. Advances in this field has been rapid, particularly for outer retinal diseases. In this case, the site of interest is surgically easily accessible specially if compared to other central nervous system locations and retinal imaging and functional assessment of the site is at an advances level with cellular level imaging possible. [17]

Each of the degenerative diseases previously descripted has some specificity regarding the type of cells affected but also the progression of the disease itself. Thus, there are many research and clinical trials going on with different approaches. For example, in AMD or RP, it is clear that the areas of RPE atrophy are associated with the degeneration of the adjacent photoreceptors. Two major strategies have emerged to regenerate the degenerating retina. The first one is to restore the photoreceptors themselves, while the second attempt is to replace directly RPE. [18] The first approach, as a consequence is targeting the neural retina whilst the second one is targeting the RPE and BrM.

However, there are some specific issues related to retinal tissue engineering:

- The human retina is a highly complex vascularized tissue that contains at least 60 functionally different cell types. These retinal cells need to act in concert with each other to successfully relay visual information from the eye to the brain. This means

that simple injection of cells of a given type requires that the added cells connect to each other and to other cells in a correct way, which is not efficient in adults. [24]

- Findings in mice, the most common mammalian disease model, cannot be directly translated to humans. Although the overall cellular architecture is similar, the two species exhibit certain differences that can complicate translation. There are differences in retinal cell-type compositions, in cell-type-specific gene expression and in the organization of the retina at a macroscopic level.
- The presence of the inner limiting membrane between the retina and the vitreous limits the diffusion of molecules.
- Cell sources are also a complex issue. In fact, autologous transplant has been investigated for example for the replacement of RPE, however the amount of tissue available is limited and there are additional risks associated with the creation of a second surgical site. Several human RPE cell lines have been investigated including ARPE 19, which is interesting for in vitro studies but unsuitable for transplantation. Allogenic fetal tissue is considered both for RPE and for neural retinal repair but it is limited in availability and ethically problematic. The alternative is to derive retinal tissue from hESC, which have been shown to be differentiated into mature RPE and neuroretinal cells. [17]

CHAPTER 2

STRATEGIES FOR RETINAL REGENERATION

Having written about the reason why retina needs to be regenerated, in this chapter the main approaches applied in the tissue engineering of the retina are considered. Two main strategies can be studied in order to regenerate defective retina: scaffold-free and scaffold-based approach. Figure 2.1 represents an overview on them. The following description comprises an analysis of the main works, material and cells.

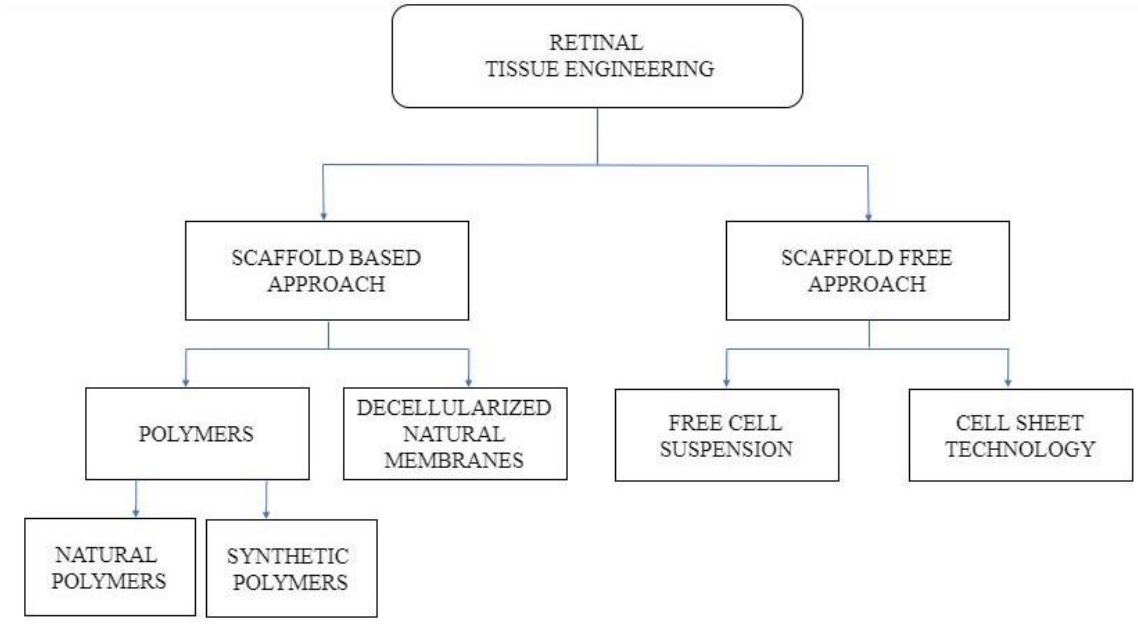

Figure 2.1 Overview of the main strategies applied in TE of the

2.1 Scaffold-free approaches

These approaches are based on the ability of cell to fuse into larger cohesive constructs and to produce a functional tissue without the need for an external support system. These approaches can be classified into direct cell suspension injection and cell sheet engineering. Both of them have been investigated in clinical trials. These approaches are however limited in terms of clinical outcome. In fact, transplanted retinal tissue and photoreceptors from fetal or embryonic donors, or derived in laboratory, without a scaffold have been shown to result in rosette formation, where the photoreceptors exhibit abnormal orientations with the outer segment facing inward rather than being associated with the RPE. Similarly, RPE cells

transplanted without scaffold often fail to adhere to BrM and aggregates without forming a normal monolayer. [17]

2.1.1 Injection of free cell suspension

This is the first approach developed, it is based on direct bolus injection of free cell suspension. First of all, the injection can be done directly into the vitreous humor, this is a very simple way to dispense cells. However, injected cells might not survive or diffuse through the viscous vitreous humor to reach the target site. Subretinal injection of cells is more plausible because this route of administration leads directly to the retina. This methodology minimizes surgery time and damage to adjacent tissues. [25] This had been used to transplant homologous RPE cells, but no visual benefits were reported. By contrast, the transplantation of autologous RPE via the subretinal injection route showed a significant improvement in vision. The main drawback is the need to isolate healthy cells from the patients.

Concerning RPE cell transplantation, in 2004, Haruta et al showed that primate ESC derived RPE cells were capable of mature protein production and phagocytic activity in vitro [26].

Regarding the regeneration of the neural retina, many studies have been performed using neural and retinal progenitor cells. A number of authors have demonstrated that retinal progenitor and retinal precursor cells into the subretinal space are able to integrate within the host retinas, differentiate and express photoreceptor specific proteins. [27]

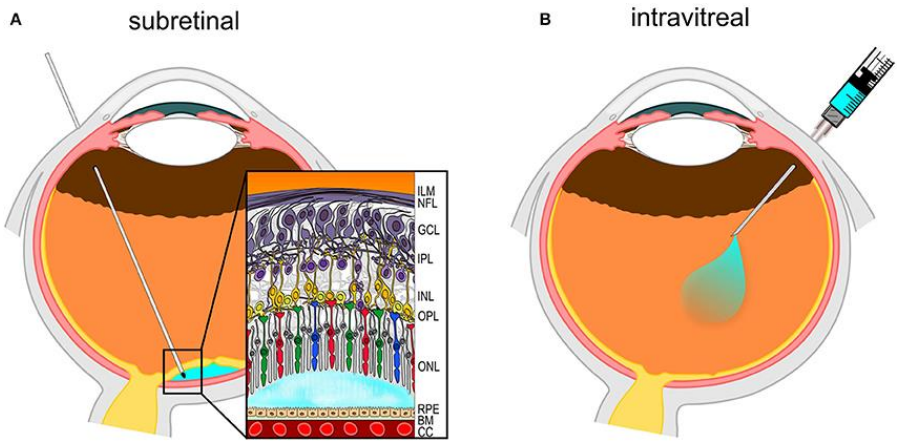

Figure 2.2: The difference between subretinal (A) and intravitreal injection (B)

These studies demonstrated the potential of cell-based therapies to regenerate the degenerating retina, but they also shows that the bolus injection of stem and progenitor cells to the subretinal space resulted in disorganized and poorly localized grafts. The outcomes are inherently related to the transplantation procedure and result in low rates of cell survival due to poor donor cell integration and injection reflux [28].

2.1.2 Cell sheet engineering

Cell sheet engineering is a promising approach that relies on the ability of cells to secret their own ECM on reaching confluency. A thermosensitive surface is used to form a dense cell sheet that can be detached when temperature decreases. The detached cell sheet can be stacked on top of one another according to the thickness of cell sheet for the specific tissue regeneration application. [29] To harvest cells, a thermo-responsive culture dish is used enabling reversible cell adhesion to and detachment from the dish by switching the hydrophobicity of its surface. That approach ensures the non-invasive harvest of cultured cells such that an intact monolayer cell sheet along with the deposited ECM is generated. The presence of ECM allows faster adhesion and attachment to the host tissue without pre-treatment.

As far as retinal tissue engineering is concerned, the susceptibility of retinal RPE cells render the harvesting of an intact cell sheet from cell culture medium impossible. As a consequence, in a work of Tano et al. many culture conditions were analysed to produce transplantable RPE cell sheet. It was shown that it is necessary to optimize the RPE culture medium through the addition of supplements, such as TGF-β2, to the growth medium to harvest the sheet without defects and limited contraction due to cytoskeletal reorganization. [26] However, construction of an artificial BrM was not successful because the amount of ECM secreted by the cells was insufficient.

In a recent work of Matsumotu et al., RPE cell sheet derived from human induced pluripotent stem cells (hi PSC) were fabricated via an automated cell culture system. [30]

2.2 Scaffold-based approaches

Scaffold-based approaches rely on the use of a template support system that serves as a skeleton to be filled with cells and subsequently form 3D tissues. [19] The scaffold should provide the correct microenvironment for the development of living cells, both *in vitro* and *in vivo*. [31] The seeded cells should populate the scaffold pores and secrete their own ECM. To obtain a functional tissue it is fundamental to proper design the scaffold so that it mimic the ECM structure of the original tissue. This is essential to support cell interactions, to provide cellular attachment and to encourage cell proliferation within the scaffold.

For retinal tissue engineering, the development of the scaffold should mimic BrM in terms of its permeability and flexibility because this is the natural support provided by BrM to RPE cells. This is essential to avoid damage of the surrounding tissue. Finally, the scaffold must be mechanically stable to withstand surgical manipulations.

There are some fundamental properties of the scaffold that have to be considered to correctly analyse scaffold based approaches:

- *Biocompatibility* it is an intrinsic property of materials. Biocompatible materials are expected to provide favourable environment conducive to cellular attachment and viability. Furthermore, they should not trigger any inflammatory, adverse or immunological responses that might result in scaffold rejection by the body. [32]
  Among the large variety of biomaterials applied in retinal tissue engineering, polymers are the most interesting and among them, we can classify natural and synthetic polymers. Natural polymers are more appealing for their similarities to ECM and for their biocompatibility. Synthetic polymers are interesting due to the possibility to be produce them in large quantities and for their longer shelf life. Moreover, they are also more uniform then natural polymers, since they are produced under controlled conditions they are characterized by high purity and their biological and physiochemical properties can be tailored. Often to combine the properties of natural and synthetic materials hybrid scaffold are developed. Other biomaterials that have been applied in retinal tissue engineering are natural membrane such as amniotic membrane or BrM itself, since they are native tissue they are considered biocompatible.

- *Biodegradability*: since artificial scaffold are generally considered as non-permanent implants so they must biodegrade and their degradation products must be non-toxic, meaning that degradation products need to be metabolized and eliminated by the body without adverse effects. However, biodegradation has to be tailored so that it matches the regeneration rate of the specific tissue. The ideal degradation rate of retinal scaffold has yet to be identified. [33]

  Scaffold degradability is dependent on several factors. Firstly, the chemical structure and composition of the material, in case of polymers, their molecular weight and polymerization degrees. Moreover, the microenvironment conditions to which the scaffold is subjected, such as pH, play an important role. Enhancing the hydrophobic properties of the scaffolding system is expected to reduce water uptake and consequently compromise the degradation process. By contrast, designing scaffold with higher surface area to volume ratio could accelerate polymeric matrix degradation. [34]
- *Mechanical properties and stability*: scaffold should have a Young's modulus similar to the delicate sensory retina, yet it has to be robust enough to support surgical manipulation. [18]
- *Architecture*: scaffold architecture characteristics, such as the surface chemistry and morphology, can affect the response of cells and subsequently tissue formation. [35] The geometrical design of the scaffold is crucial to support cells and to guide their distribution. In retinal applications, the scaffold must be thin enough to accommodate the subretinal space: the optimal width is 5-90μm and thickness is 3-5μm [33]. It should be mechanically robust to cope with in vivo introduction procedures but at the same time, it must be well tolerated by ocular environment. It has to display the correct permeability and flexibility in order to support RPE cell growth. Finally, scaffolds must display an open interconnected architecture with a high degree of porosity to provide a large surface area to allow cell ingrowth, diffusion of nutrients to cells and a uniform cell distribution. [36]
- *Micro*/*nanostructure*: micro and nanoscale topological cues are known to influence the morphology and differentiation of multiple cell types. The topological cues interact with cell surface receptor, which activate internal signalling pathways. On a substrate with microscale topological cues, neural progenitor cells acquired more

neuronal morphology than on a planar structure. Thus, some microfabrication techniques, such as micro contact printing, can be applied to pattern the surface of materials for example to simulate the natural retinal tissue patterns. [37]

In the next part, there will be a description of most interesting biomaterials applied in retinal tissue engineering, among them natural and synthetic polymer but also natural platform will be analysed.

2.2.1 Natural polymers

Natural polymer are appealing materials due to their similarities to ECM as well as their chemical versatility and biocompatibility. Naturally occurring polymers such as collagen, alginate and fibrin have been used as hydrogels and films in almost tissue engineering fields. [37] While these natural materials have the benefits of biocompatibility and biomedical cues present in the natural extracellular environment, serious drawbacks such as issues with product purity, disease transmissions, immune response and difficulty in functionalization or modification do arise. [33] These are some of the most interesting natural polymers applied in retinal tissue engineering.

- Collagen: it has been widely used to fabricate scaffold in TE, it exists naturally in most soft and hard mammalian tissues, including cornea, sclera and vitreous humor of the eye. [36] Since the Bruch's membrane consists of various types of collagens, collagen is the most studies polymer for BM scaffolds. Collagens have the ability to interact with each other and with other ECM molecules to create a variety of structures. [36] In an interesting work, Lu and coworkers used thin collagen films for the culture of RPE cells. This scaffold had a thickness of 2.4±0.2 μm was able to maintain cell viability and morphology. [38] Warnke et al. compared thin films to electrospun nanofiber collagen scaffolds and on these nanofiber scaffolds demonstrated better morphology of RPE cells, including more defined apical microvilli, a strong indicator of the health of RPE cells. [39]
- Alginate: it is an hydrophilic polysaccharide derived from brown seaweed that exhibit excellent biocompatibility, biodegradability and chelating ability because of

its unique structure. [19] It is a very versatile material that can be modified to produce hydrogels, sponges, microspheres and fibers. In an interesting work, Hunt et al, demonstrated the potential of RGD-alginate scaffolds for the derivation, transport and transplantation of neural retina and RPE. They succeeded in generating a laminated neural retina and RPE using human embryonic stem cells in a RGD - alginate hydrogel. [40] Purified alginate films have also been manufactured and used as a scaffold for RPE cells, indicating that these films allowed high cell proliferative rate and phenotypic expression. [41].

- Hyaluronic Acid (HA) is a non sulphated polysaccharide, shown to be a major component of the retinal ECM, including the interphotoreceptor matrix, which surrounds the outer segments of photoreceptors. Shoichet and coworkers developed HA-based gels for both RPE and neural retina tissue engineering. In particular, they work on an injectable hydrogel containing cells that can form a scaffold once injected without large incisions and so with minimal surgical invasiveness. [42]

  Another interesting work analysed a complex scaffold composed of HA, gelatin and silk fibroin that was studied as a BrM substitute. The membrane was seeded with both human umbilical vein endothelial cells and RPE cells to develop a construct capable of engineering the choroid together with BrM and RPE. [43]
- Silk fibroin: this material is obtained from a variety of insect sources such as *Bombyx mori* and *Antheraea pernyi* [33] and it is very promising as a substrate for ocular tissue engineering. Silk sutures are already FDA approved and commonly employe in ocular surgery. Silk is itself is cell adhesive, and its adhesion properties can be increased by coatings with ECMs or functionalization with RGD peptides. Silk has a slower degradation rate than collagen or fibrin due to the formation of β-sheets that are exceptionally stable. Shadford et al. developed a porous silk fibroin membrane for culturing ARPE 19 cells demonstrating that cells maintained their characteristic morphology. Same results are obtained using primary RPE cells. [44]

  Silk fibroin combined with a blend of PCL and PLLA has been assessed as a scaffold for engineering neural retina. This scaffold of 60-100 μm thickness was evaluated with neonatal mouse retinal progenitor cells (RPC) demonstrating to support the viability of these cells.

- Cellulose: this is a versatile material derivable from plant material or bacteria and even if it is not yet approved from FDA and EMA for in vivo use in humans, it has been extensively researched for the replacement of spinal cord and retina. In fact, cellulose seems to be well tolerated intraocularly without causing adverse immunological response. Since humans do not synthesize appropriate enzymes to mediate cellulose degradation, but through chemical modification host mediated degradation is possible. [45] Cellulose as a BrM substitute has been investigated by the Rodriguez group: they studied whether surface modification of bacterial cellulose using acetylation or coating with polysaccharides, such as chitosan, could promote hRPE cell adhesion or proliferation. In further studies, the acetylated cellulose scaffold was coated by decellularized ECM from porcine bladder showing better properties in terms of cell viability and adhesion. However, limitations are related to the elastic modulus of these material, that exceeds that of native BrM but also the fact that the mature RPE phenotype was not maintained [46].

2.2.2 Synthetic polymers

Several synthetic polymers are approved for clinical use in the field of tissue engineering and regenerative medicine. They can be better controlled in terms of properties such as degradation, biocompatibility, melting point, transitional temperature and mechanical strength. [37] Scaffold based on synthetic biomaterial have high purity and can be designed in a reproducible manner. Among them poly(lactic acid) (PLA), poly (glycolic acid) (PGA) and their copolymers have been investigated since their degradation occurs in vivo hydrolytically through de-esterification.

- PCL: poly (ϵ-caprolactone) is extensively applied for the fabrication of artificial scaffold, it is an easily processable material that can be fabricated in fibers, scaffolds and membranes. After biodegradation, it produces pH neutral and non inflammatory by-products. Tan et al. worked on an ultrathin PCL porous membrane scaffold to replicate BrM for tissue engineered retinal epithelium transplantation. They developed a new phase separation technique to synthetize a 9μm thickness membrane. The membrane was assessed with human retinal pigment epithelial cell

line (ARPE-19) that form tight junctions indicating the formation of a well developed barrier, similar to that characterizing the blood retinal barrier. [47]

In a recent work, the response of primary fetal retinal progenitor cells (hRPC) to various PCL film scaffold has been assessed. PCL film were coated with vitronectin-mimiking peptide, since vitronectin is known to support the culture of hRPCs. [48]

- PLGA: this is a biodegradable copolymer, composed of PLA and PGA, approved by US Food and Drug Administration (FDA). It offers tailored degradation, according to the ratio of the two components. It has been researched for engineering tissues including bone, skin cartilage and retina. It has been recently demonstrated that its degradation in the subretinal space is slow with a scaffold of 60µm thickness still remaining after 3 month in vivo in monkey. After implantation in the subretinal space, no adverse effects nor inflammation or fibrosis were observed [17]. Electrospun PLGA fibers and PLGA films were also investigated as a BrM substitute by Warnke et al. They seeded these substrate with RPE cells and assessed the phagocytic ability of RPE but also the microstructure and the tensile strength of the scaffold. Although the value obtained were significantly higher than those of the native tissue, electrospun scaffolds allowed the formation of microvilli. [39]
- PLA is a synthetic polyester extensively applied in regenerative medicine applications. Its degradation occurs hydrolytically via de-esterification, and the by products can be eliminated through the tricaboxylic acid cycle [17]. It is used for the production of biomedical devices, including resorbable sutures and membranes. In 2016, Calejo et al [49] developed a honeycomb PLA films as a BrM substitute. This substrate were composed of 96/4 ratio of L-lactide/D-lactide. They obtained an easily handled scaffold, thicker than native BrM. They also perform a coating with collagen IV and seeded pigmented hESC-RPE, demonstrating good cell proliferation on the scaffold and good levels of pigmentation.

  Concerning the regeneration of neural retina, PLA scaffolds were produced by electrospinning to tissue engineer the retinal ganglion cell (RGC) nerve fiber layer. [50] The PLA electrospun fibers were coated with laminin and seeded with mouse RGCs, and the substrate obtained has shown to support cell viability and alignment.

2.2.3 Native membranes

Native tissues such as amniotic membranes or Descemet membranes have been extensively analysed for retinal tissue engineering applications. They are obviously biocompatible and so they offer better cell adhesion properties than natural or synthetic polymers. They are able to activate a range of cell integrins leading to appropriate downstream cell signalling. They can easily bind growth factor and ECM molecules. Finally, since they present the correct extracellular matrix (ECM) composition they present the same mechanical properties of native tissues, correctly mimicking the in vivo microenvironment. Main limitations regard the limited availability of these materials, but also the batch-to-batch variability in terms of composition, degradation rates and mechanical properties. [17]

- Amniotic membrane (AM) is the inner layer of the fetal membranes, composed by epithelium, basement membrane and avascular stroma. When used as a substrate for tissue engineering purposes, the amniotic membrane is often denuded (epithelial cells are removed). AM is a well-known inhibitor of neovascularization on the ocular surface and anti-inflammatory and angiogenic proteins are present in its stroma: these properties are particularly appealing. Its limitations concern the variability and the potential risk of disease transmission. [17] One of the first applications of amniotic membranes as BrM substitute was conducted in 2006, used as support for RPE ingrowth in two pigs with surgically induced choroidal neovascularization. The migration of RPE cells in the presence of the AM was assessed through the analysis of hypo or hyperpigmentated areas. They proved that AM transplanted in subretinal space stimulates the host RPE cells to cover the membrane substrate. [51] In further studies they demonstrate that the isolated amniotic membrane can modify the formation of choroidal neovascularization in a porcine model of subretinal wound healing.[52]
- Resurfaced BrM: even if its availability from young healthy donors is quite limited, BrM itself is the obvious choice as a scaffold for RPE transplantation [17]. Many researches investigate the possibility to resurfing BrM to enhance RPE attachment. Priore and co-workers demonstrated that by exposing the inner collagen layer (ICL) of aged BrM and coating it with a mixture of laminin, fibronectin and vicronectin significantly improved cell adhesion and proliferation of hRPE. They also showed how following this procedure there was an increase in hRPE phagocytosis ability of

photoreceptor outer segments. This activity was not equivalent to that of the native BrM, in fact other ECM components such as heparin sulphate, that drastically decrease with age, might be necessary. [53]

- Retinal decellularized ECM (dECM): the use of decellularized membrane as a support scaffold for tissue engineering is nowadays acquiring more and more popularity. This approach is based on the assumption that native ECM provide the best microenvironment for cells to adhere and develop. In a work of 2015, Kundo et al. reported the first production of dECM of the neural retina.

Among all the material previously described, dECM used as tissue engineering substrates result as the most interesting. Since they perfectly mimic native ECM, they provide to cells the best microenvironment in terms of physical, chemical and mechanical properties. Nowadays the use of dECM as a biomaterial is growing and many studies have been performed on different tissues, even in ophthalmic applications. Moreover, this material can fit many biofrabrication techniques such as 3D bioprinting.

In the following chapter, I will analyse in detail this particular approach i.e. the use of decellularized extracellular matrices (dECM) as bioinks in bioprinting technology with the particular target for retinal tissue engineering.

CHAPTER 3

dECM AS BIOINK FOR 3D PRINTING OF THE RETINA

3.1 Three dimensional (3D) bioprinting

Three dimensional (3D) bioprinting is a technology to fabricate construct from living cells with or without a carrier material in a layer-by-layer manner. [54] It has quickly become an attractive method to rapidly fabricate complex architecture in a top down approach. [55] Whilst conventional techniques, such as porogen leaching, injection molding and electrospinning, are limited in terms of control over scaffold architecture, composition, pore shape, size and distribution, 3D bioprinting enables the fabrication of scaffolds, devices and tissue models with high complexity. [56] This is possible because 3D printing allows construction of tissues from commonly used medical images using computer aided design. The ability of producing a patient specific design with high structural complexity but also low costs and high efficiency are some of the major advantages that makes 3D printing so attractive for medicine. [57]

The material that is printed is referred to as *bioink*. The bioink is generally composed of a prepolymer solution containing living cells or cell aggregates. It is deposited on a substrate, sometimes called *biopaper*. Many biomaterials widely used in 3D printing applications cannot be used as bioink since they can be printed only at elevated temperature or need to be dissolved in organic solvents and thus they are not suitable for live cell printing.

3.1.1 Bioprinting technologies

3D bioprinting process should be mild and cell friendly, as it is required to allow cell printing. This requirement limits the number of printing techniques that are suitable for 3D bioprinting. [56]

There are three main 3D printing technologies suitable to 3D bioprinting: direct ink writing (DIW) which is an extrusion based method, inkjet printing a drop based method and finally, among photolithography techniques, laser induced forward transfer (LIFT).

Extrusion based methods print cell laden biomaterials using mechanical force via screws, pistons or pneumatics. The extruder continuously deposits the bioink as the extruder or the stage is moved through computer control. DIW allows the use of high cell densities with gentle processing but also reduced speed. They are compatible with more polymers than drop based methods. The main limitation can be nozzle clothing and the risk of poor structural and mechanical stability of the bioprinted construct. Moreover, the resolution that can be achieved is lower than other methods (50-400μm). DIW has been applied for the extrusion of high viscosity solution, hydrogels and colloidal suspension, it is suitable for printing cell suspension and cell aggregates with or without a carrier. [56]

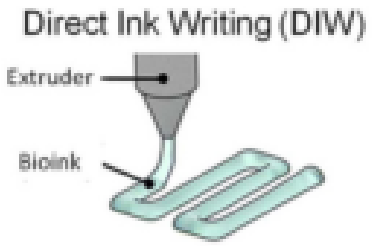

Figure 3.1 Representation of a DIW mechanism

Drop based methods are based on a mechanical print head that deposits the bioink in droplets that can coalesce and gel to form the structure. This method, also known as inkjet printing, has one limitation related to gelation time of the bioink. Gelation time needs to be faster or at least similar to the drop deposition time otherwise it results impossible to obtain a 3D construct with a controllable structure. This limits the choice of the bioink material. Another drawback of this methodology is the risk of droplet spreading effect. The resolution of the printed pattern using inkjet printing is high, i.e. about 20-100μm and the costs are limited. It can be used for printing of colloidal and cell suspensions with relatively low viscosities (<10cP) and relatively high shear rates (10^5-10^6 s^{-1}). [56]

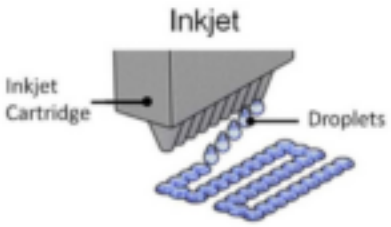

Figure 3.2 Representation of Inkjet printing

Laser-induced forward transfer (LIFT) technology is commonly composed of a pulsed laser source, a target or ribbon coated with a bioink to be transferred and a receiving substrate. The ribbon is a silicate slide with a metallic absorptive coating. The bioink is loaded on top of the ribbon and oriented face-down above the receiving substrate (figure 3.3). High speed laser that is focused onto the absorptive layer creates local pressure to eject the ink layer to the receiving substrate. This method is capable of precisely printing of bioinks in relatively small 3D patterns while maintaining cell viability. It is suitable for a wide range of bioink viscosities but the process requires rapid gelation mechanism of the bioink to reach high resolution of the printed cell pattern.

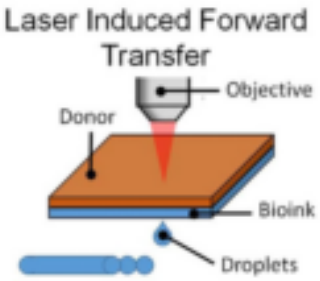

Figure 3.3 Representation of LIFT

Each one of the descripted methods have different resolution but also limitations in terms of printing speed but mostly in in terms of suitable material and cell densities.

3.1.2 The bioink

One fundamental component of bioprinting technology is the bioink. The ideal bioink should satisfy material and biological requirements. Material properties are printability, mechanics, degradation and functionalizability whilst biological requirements mainly include biocompatibility, cytocompatibility and bioactivity. [56]

Printability is probably the most important parameter to consider, it comprises both the processability of the bioink formulation, and the print fidelity associated with the mechanical strength of the printed structure to self-sustain a 3D structure post printing. Of course, printability is dependent on the printing technique; it could involve viscosity, surface tension and crosslinking properties. Viscosity is a crucial parameter for bioink formulation as it both affect the printing fidelity and cell encapsulation efficiency. In a study by Tirella et al, a diagram showing the interplay between bioink viscosity, print velocity and applied pressure

to obtain high print fidelity. [58] The bioink formulation should have tunable viscosity in order to be compatible with different bioprinters.

The overall mechanics, i.e. the achievable stiffness is important both to create self-supporting constructs and to direct cell behaviour.

Degradation is important for functional integration of the 3D construct in vivo, degradation products should be biocompatible and avoid inflammatory host responses.

Functionalizability is required to incorporate biochemical cues, such as RGD sequences, suitable to direct cell behaviour such as adhesion and differentiation.

Two main types of bioinks materials have been used in bioprinting of 3D tissue. The first and most common is scaffold-based bioink where cells are loaded in hydrogels or similar exogenous materials. In the second type of bioink cells are bioprinted without the use of an exogenous biomaterial, in a scaffold free process, mimicking the embryonic development. In this case, neo tissues are obtained and finally deposited in specific patterns. [59] Scaffold free bioinks comprise tissue spheroids (cells organized spherically into 200-400μm diameter cell aggregates), cell pellet (cells concentrated through centrifugal or gravitational forces), and tissue strands (cylindrical neo-tissues that are engineered for bioprinting of scale-up tissue).

Scaffold-based bioinks include hydrogels and decellularized extracellular matrices (dECM). Hydrogels are a class of polymeric substances capable of absorbing and retaining large quantities of water and they are generally classified in naturally derived and synthetically derived. They most common bioinks because they can be easily formulated for DIW, inkjet and LIFT technologies. Both natural and synthetic hydrogels are able to mimic the native tissue environment, possessing several essential features of the native ECM components. Thanks to their highly hydrated 3D environment, they are suitable materials for cell encapsulation. Hydrogels can absorb up to 1000 times their original weight in aqueous medium without dissolving and this high hydration level makes them high biocompatible material. Moreover, they are highly permeable to oxygen and nutrients and to other water-soluble compounds and embedded cells are able to migrate and communicate with each other through a porous flexible network. Although not hydrogels are bioprintable, their bioprintability is superior to that of other bioinks, in fact the crosslinking mechanism is

incorporated into the layer-by-layer fabrication scheme. The main challenge related to this material is the fact that they do not contain specific ECM protein for particular cell types, thus they unable to provide a native environment. Moreover, it is difficult to achieve a high cell density as in native tissue through bioprinting. The need for improved mechanical properties leads to an increased concentration of hydrogel that on the other hands limits biological activities, lowering cell mobility resulting in lower cell proliferation and deposition of ECM proteins.

To better recapitulate the natural tissue environment, bioinks based on decellularized ECM has been developed. dECM bioinks require cell removal through chemical, physical or enzymatic processes without damaging the ECM. The advantages of dECM are based on the nature stimulating biocomposition, such as mechanical properties and appropriate micro-environment for cells, for their proliferation and differentiation activities and porous microstructure retaining bioactive agents in the scaffold. They are excellent allogeneic or xenogeneic biomaterials. Even after decellularization, risks of immunoreaction by the recipient remains. Moreover, the volume of the final product compared to the size of the initial organ is extremely limited (low yields). Toxic residues from decellularization process can remain. The major weakness of this material is related to weak mechanical properties, often a 3D printing support frame is essential (figure 3.4).

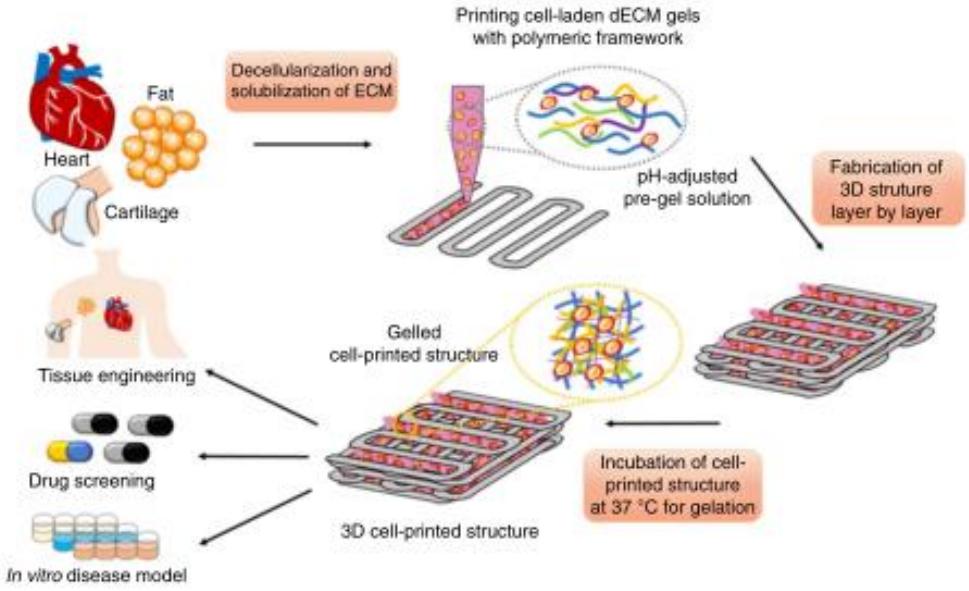

Figure 3.4 Main steps to be taken for the development of dECM bioprinting

3.2 3D bioprinting of the retina

3D printing has also been applied to the ophthalmology field for ocular prosthesis, intraocular devices; in 2018 the first 3D printed human cornea was developed and research is still going on for retinal applications. 3D printing of the retina could not only be interesting for retinal tissue engineering but also to obtain retinal models that may provide additional information for both the physician and patients, with a better grasp of the anatomy and pathology of lesions and surrounding tissues. [60]

3D bioprinting techniques that allow for precise deposition of living cells, biomaterial and growth factors are very interesting considering retinal tissue engineering. Its major advantages such as precision, high throughput and reproducibility could play a major role in BrM scaffold fabrication. Printed film of natural material could potentially serve as a BrM substitute for retinal cell attachment. [37] Moreover, many efforts have been done to develop retinal cell printing methods.

Before describing the most promising works towards the regeneration of a functional retina, it is important to analyse the challenges of retinal 3D printing.

- The primary challenge includes printing cells accurately at high densities to achieve the high cell numbers needed to make up a functioning retina. [61] This is most crucial in printing photoreceptor cells, to achieve the close packed arrangement of the rod cone receptor mosaic a biomimicry or self-assembly approach, in addition to accurate printing at the single cell level, will need to be employed. Similar approaches will be required to print other retinal cell types to allow them to synaptically connect together correctly and establish horizontal and vertical connection between cells in different layers to ensure proper physiological function and transmission of visual information in the printed retina. Retinal ganglion cells that transmit visual information to the brain need to retain the regeneration promoting properties to successfully extend their nerve processes through the optic nerve toward the brain. [61]
- It is difficult to maintain cell viability during the printing process without a reliable high-throughput printing method. For example, in inkjet printing cell sedimentation can lead to a significant cell loss, so modifications to reduce this phenomenon are needed. With the continuous improvement to other printing methods, such as laser-

assisted printing, may turn out to be better suited to achieve the goal of printing a functional retina. [61]

- To ensure long term survival of retinal cells it is necessary to provide nutritional support and oxygen, ways need to be found in order to construct vascularized retinal tissues.
- As any other 3D printed tissue, 3D printed retinas will undergo a rigorous approval process by regulatory government body before human transplantations. The implantable 3D printed retina will most likely be defined as a Class III medical device and therefore, it requires more stringent approval review processes. [61]

Despite these limitations, there is a lot of research about this topic and some interesting works allow advantages in the construction of 3D structures potentially relevant to the creation of a 3D printed retina.

One of the most interesting work was performed by Shi et al., who developed a hybrid 3D bioprinted retinal equivalent. This construct was composed of a PCL ultrathin membrane that used as a substrate onto which human retinal pigmented epithelial cell line (ARPE 19) and human retinoblastoma cell line (Y79) were bioprinted. The printed construct is interesting as a meaningful retina model for the investigation of RPE and Y79 interactions and tissue regenerative strategies. An ARPE-19 cell monolayer, representing RPE, is bioprinted on the ultrathin PCL membrane, representative of BrM, in order to finally obtain a cell monolayer. Subsequently, an Y79 cell-laden alginate/pluronic bioink was bioprinted onto the ARPE-19 cell monolayer (fig 3.4). The Y79 cell viability was not compromised into the bioink. Thus, they obtained a meaningful retinal model to mimic the relationships between RPE and photoreceptor cells, the retinal equivalent had acceptable cytocompatibility with advanced structure aiming to simulate native retina. [62]

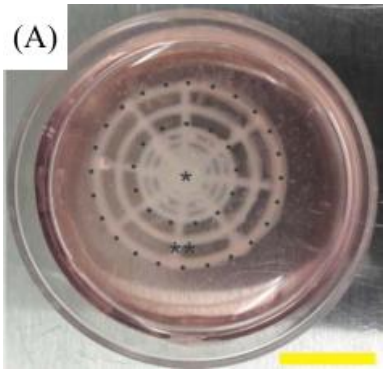

Figure 3.5 The bioprinted retinal equivalent with high cell density at the center

As far as retinal cell printing is concerned, studies on rat retinal ganglion cells had been printed using piezo inkjet technology. This printing technique utilizes the rapid movements of a piezoelectric element to eject liquid drops from a nozzle. Lorber et al. showed that the printing process does not affect the viability of different retinal cell types, although a reduction of cell number caused by sedimentation within the print head did occur. In particular, they printed retinal ganglion cells and retinal glial cells and they evaluated survival and regeneration- promoting properties of these cells. Printed cells exhibited similar survival rates and regeneration properties in culture compared with their non-printed counterparts, suggesting that cells are not adversely affected by the printing process. [63]

3.3 dECM as bioink

Decellularized ECM are a promising tool for the production of scaffold for regenerative medicine.

Tissue and organs contain cells and ECM components, such as collagen, fibronectin, laminin and glycosaminoglycans (GAGs). The composition of each tissue is specific with cells and ECM mutually interacting with each other. While cells produce the ECM, the ECM in reverse interacts and influence cells behaviour. This is made through cell receptors such as integrins; cell-ECM interaction activate signalling pathways essential for cellular functions. [64] Proteoglycans, with attached GAGs, are large molecules and occupy more space than ECM fibers. The presence of charged groups on GAGs allow the movement of molecules and metabolites in tissues. ECM traps several growth factors, enzymes and other molecules, influencing cell function and fate. [64] Collagens and fibronectin, instead, provide physical strength needed by cells to maintain form and for migration. Other fibrous proteins, such as elastin and laminin provide elasticity. The specific ECM of each tissue contains different percentage of each component and is important to allow cells to perform specific functions. Each tissue specific ECM is needed to support specific cell growth and function. Thus, the use of mixture of ECM proteins can hardly recapitulate the native ECM properties. [65] This is the reason why to recapitulate the native tissue properties the use of dECM as a biomaterial is so appealing.

dECM have been already used as biological sheets or for coatings and become an appealing material for 3D bioprinting process because it can easily form soft gels, which is required for the printing process. The presence of both physical and biological cues allows the dECM to induce restoration of normal tissue or organ homeostasis and cellular processes. [65] What dECM lacks as a bioink is mechanical strength and in order to be printed properly, there might be a need to modify the dECMs before or during printing.

3.3.1 dECM bioink preparation

The first study on dECM bioink was performed by Pati et al. in 2014. They produce dECM bioink of cardiac, adipose and cartilage tissues [66]. Based on their procedure, other researchers obtained dECM bioinks derived from skeletal muscle, liver, skin tissue, kidney, etc. Despite some specificity related to the use of specific chemical agents suitable for each tissue type, the preparation of dECM bioinks can be summarized in some general steps:

1. Decellularization step: the native tissue is decellularized to remove cellular components and involves cellular lysis followed by separation of cells from ECM. This is a crucial step since cell removal has to be performed without modifying the composition of the tissue. The amount of residual DNA after decellularization process must be below 50ng dsDNA/mg weight. [65] The choice of decellularization method plays a key role in determining the final properties of the dECM bioink obtained. Decellularization is achieved through a combination of the following methods:

   a. Chemical method through chemical agents such as ammonium hydroxide, sodium dodecyl sulfate (SDS) and Triton X-100. They can cause some damage to ECM proteins or a reduction of growth factors.

   b. Biological method through enzymes such as nucleases (DNAse and RNAse) and proteases (trypsin, dispase) or chelating agents. Exposure of the tissue to trypsin for too long can result in damage to the structure. Nucleases are known to decrease the amount of collagen and it may be difficult to remove them from the tissue.

   c. Physical method though freezing, osmosis agitation or direct pressure. They cause less disruption of the ECM, but they can result in incomplete removal of cellular debris.

Since dECM bioinks only require maintenance of ECM composition tissues and organs are generally cut and sliced into small pieces and exposed to chemical and enzymatic agents. Slicing into small pieces the initial tissue is useful in order to increase the surface area and thus to reduce exposure times to the chemical agents and decellularization time as well.

2. Sterilization or disinfection step: generally, ethanol and peracetic acid are used as sterilization agents. Sometimes gamma irradiation can also be applied to the material even if they can cause changes in strength of the dECM.
3. Lyophilisation step: after a washing step in distilled water and phosphate buffer saline (PBS) to remove detergents, enzymes and solvents, dECM is lyophilized and pulverized.
4. Solubilization and pH adjustment step: the lyophilized dECM is solubilized in an acidic solution and digested with pepsin. finally, pH is neutralized to 7.4 using NaOH. The digestion with pepsin is necessary to obtain the pre-gel solution, in fact pepsin digestion cleaves the telopeptide regions of the collagen, which allows solubilisation of the dECM in dilute acids. [66]
5. Storage of dECM pre-gel solution below 10°C to avoid gelation that naturally occurs at 37°C. Cells are encapsulated in the pH adjusted pre-gel before the loading in the sterilized syringe for cell printing.

Of course, the characterization of the obtained dECM bioink is needed. In the previously cited work of Pati et al, the characterization was mainly related to the analysis of rheological properties of the dECM bioink before and after gelation (figure 3.6). This analysis is important to understand if the material is printable. The dECM pre-gels showed shear thinning behaviour required for extrusion based printing. The storage and loss moduli of the pre-gel were measured at 15°C the bioinks that exhibited more like a liquid mater. Whilst at 37°C they behaved like a crosslinked gel with greater storage modulus than loss modulus, demonstrating that the dECM gel can retain shape and form after gelation, without needing chemical crosslinking. Moreover, they demonstrated that cells survive shear stress during the printing process of these constructs. This is possible because of the shear thinning behaviour of dECM gel. They were able to print a precisely defined structure with homogeneous distribution of cells, without modifying in cell survival and proliferation. [66]

Figure 3.6 Cartilage dECM pregel solution (on the left) and dECM gel (on the right)

Similarly, in 2019 a work published by Kim et al [67] the development and characterization of a cornea specific-dECM bioink (Co-dECM) was reported. This bioink, which is capable of 3D printing with encapsulated cells, is optically transparent, biochemically similar to the native cornea and compatible in vivo. The Co-dECM was prepared according to the procedure previously described. Cornea was decellularized, sterilized, lyophilized and pulverized. Then, it was digested with pepsin in a solution of acetic acid for 3 days and after filtration, pH was adjusted at 7.4. Finally Co-dECM pre-gel was stored at 4°C.

Figure 3.7 Schematic representation of Co-dECM gel preparation and its validation

Both the physical and chemical properties of the gel were investigated, the obtained gel showed high transparency in visible wavelength range. Co-dECM gel contained various growth factors, such as fibroblast growth factor and insulin-like growth factor that are observed also in the native cornea. Moreover, the gel showed shear-thinning behaviour resulting suitable for extrusion bioprinting techniques and rheological analysis demonstrated that physical crosslinking occur at 37°C. The gel was also tested with human turbinate mesenchymal stem cells that were induced to differentiate into keratocyte-like cells expressing corneal specific markers. Thus, they obtained bioink that can be applied to 3D bioprinting to provide cornea-mimicking microenvironment.

3.4 dECM for 3D bioprinting of the retina

3D Bioprinting using dECM bioink is an emerging field and as previously described it was applied also in the ophthalmic field, especially in corneal tissue engineering. On the contrary, in retinal tissue engineering works on the development of retinal dECM bioinks are still lacking.

However, the idea of using retinal dECM as a substrate for retinal regeneration was considered by Kundo et al. in a work of 2015. In this work, decellularized retinal based substrate were obtained and their impact on human retinal progenitor cells (hRPC) was observed in terms of cell attachment, viability, morphology, proliferation and gene expression. [68]

This approach is still based on the decellularization of the retina but instead of obtaining a retina dECM gel, they fabricated decellularized retina (decell-retina) films onto which they seeded and cultured hRPCs for 7 days. They demonstrated that the obtained substrates were capable of supporting cell attachment, survival, growth and differentiation, in fact the films promote the maturation of retinal progenitor cells and photoreceptor marker expression. In detail, the process can be described as follows:

1. Retinas were isolated from bovine eyes and floated with phosphate buffer saline (PBS) and transferred into deionized water.
2. Decellularization was performed in 1% SDS at room temperature on an orbital shaker for 3 hours. Finally, retinas were centrifuged at 25°C at 10000rpm for 15 minutes. After resuspension in DI water, retinas were purified through dialysis to eliminate residual SDS. A decell-retina suspension was obtained.
3. Decell-retina suspension was frozen at -80°C overnight and then subjected to lyophilisation through a freeze drier for 48-72h. Finally, they were pulverized using a mortar and pestle to obtain lyophilized powder.
4. Digestion was performed using pepsin-HCl for 48h under constant stirring. Finally, the partially digested decell-retina was diluted with PBS and NaOH was added.
5. Fabrication of decellularized retina films was performed through solvent casting: the decell-retina solution that was cast onto tissue culture plate surface at room temperature and left overnight for drying. Finally, the obtained decell-retina film were sterilized using UV light and ethanol.

They demonstrated that their decellularization protocol was suitable since it allow the general maintenance of bulk composition of the material (mostly in terms of collagen, GAGs and hyaluronic acid) but also preserves growth factors within the retinal ECM.

The main limitation of this work is related to the fact that films obtained by solvent casting while presenting biological cues of the native tissue, do not preserve the 3D environment provided by the native ECM. Thus, authors themselves suggest that starting from this approach, future efforts will lead to the development of retina ECM gels preserving more of the 3D stimuli of native tissue.

Starting from the analysis of this work, it is also clear that few modifications are needed to define a methodology for the production of retina dECM gel. If this is suitable to obtain a dECM gel, it is easy to understand that this process can be used to produce dECM pregel solution, suitable for the development of a retinal dECM bioink.

Since the decell-retina films provide good retinal progenitor cells viability, proliferation and adhesion properties, the bioink obtained through a similar methodology should result as biocompatible and cytocompatible and in particular should allow cell growth and proliferation.

Of course, the development of this dECM bioink should include analysis of the mechanical properties, in particular rheological one. In fact, the obtained dECM gel should have a shear thinning behaviour in order to be printable through extrusion-based methods. However, it should also be demonstrated that digestion with pepsin is enough in order to perform physical crosslinking of the material. This is suggested by numerous work on dECM bioink, but eventually some chemical modifications could be done to the decellularized solution in order to obtain chemical crosslinking. For example, chemical crosslinking was performed on a kidney dECM derived bioink. After dECM digestion with pepsin the material was methacrylated and as a consequence gelation of the material can be obtained through photo-crosslinking [69]

The combination of the attractive method of 3D bioprinting, that allow the fabrication of customized product, and the use of dECM bioink that ensure to obtain a perfect microenvironment for cells growth and proliferation is perhaps one of the most appealing technique for retinal tissue engineering.

The use of a retinal dECM bioink should be used to bioprint layer-by-layer structure that can be tailored for example in terms of cell types. Using a multi head printer method, containing different bioinks, it might be possible for example to develop a retinal equivalent composed of a bioprinted BrM substitute, RPE cells, which can be cell printed though a scaffold free printing, and neural retinal cells, which can be encapsulated in a retinal dECM gel.

Although several research needs to be done to finally develop a retinal dECM bioink, the application of this highly biocompatible material that mimic the native ECM, both in terms of mechanical, physical and mechanical properties is very attractive for biomedical applications. Moreover, the possibility to transfer recent findings on dECM bioinks 3D bioprinting could be useful to be applied in retinal regeneration. As a conclusion, the use of dECM bioink in retinal 3D bioprinting could be the next step towards the development of functional 3D bioprinted retina.

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