Chapter 1
Introduction

1.1 Cornea

1.1.1 Anatomy, Function, and Composition of Cornea

The cornea is the outermost front component of the eye and it together with the eyelids and sclera serves to protect the interior section of the eye from injury or infection (Sridhar 2018). The structure of the cornea is both transparent and avascular in nature. Together with its transparency, the curved curvature of the cornea adds to its refractive ability in the eye (figure 1.1). Males have an average corneal diameter of 11.04–12.50 mm, while females have an average corneal diameter of 10.7–12.58 mm (Rüfer, Schröder, and Erb 2005). Cornea provides approximately 40–44 D of refractive power and accounts for roughly 70% of total refraction. Cornea has a refractive index of 1.376 (Fares et al. 2012).

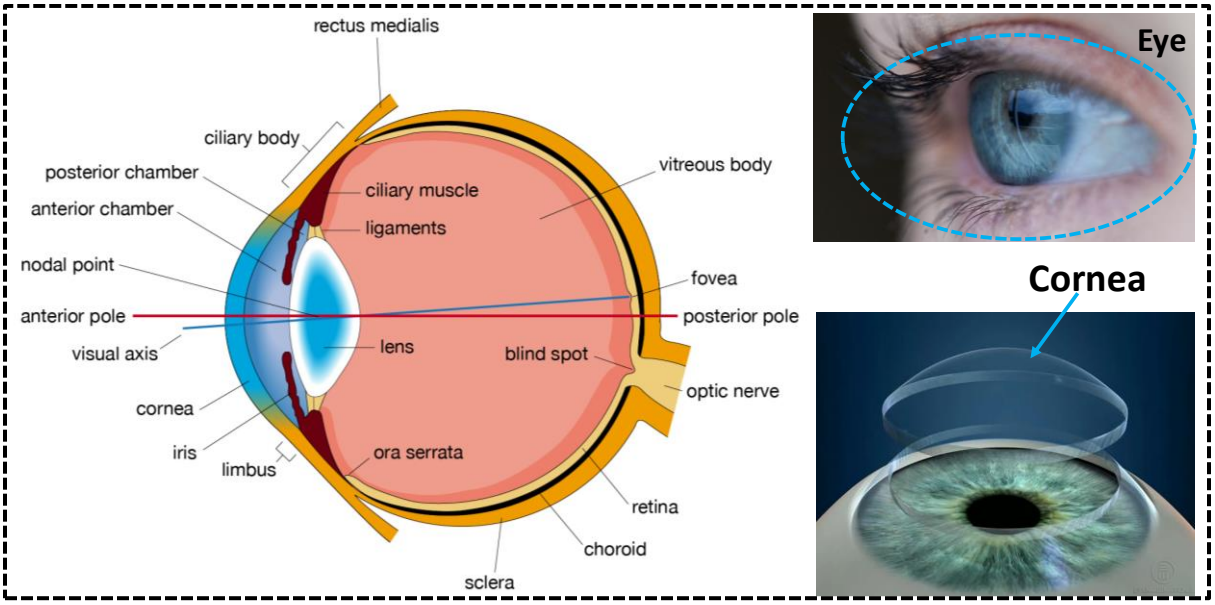

Figure 1.1 Diagram and anatomy of a human eye. (Source: Encyclopedia Britannica, Inc., 2013)

The cornea is composed of up to 78% of water and this precise degree of hydration is another critical aspect of the cornea's ability to operate as an optical element. Because the cornea is an avascular tissue, material movement into and out of it is largely dependent upon diffusion

(Larrea and Büchler 2009; Sridhar 2018; Beebe 2008). The aqueous humour, which is situated just under the cornea, is essential for transporting growth agents and nutrition to the cells. Tears are the second pathway and the cornea's only supply of oxygen (O_2). Diffusion is also used to remove waste products and carbon dioxide (CO_2).

1.1.2 Microstructure of Cornea

The cornea is comprised of five different layers, which are as follows: three of these layers are cellular in nature, while the other two are membranous in composition (figure 1.2). The epithelium, stroma, and endothelium are the three major layers of cornea. Bowman's membrane and Descemet's membrane act as a barrier between these three layers, separating them from each other.

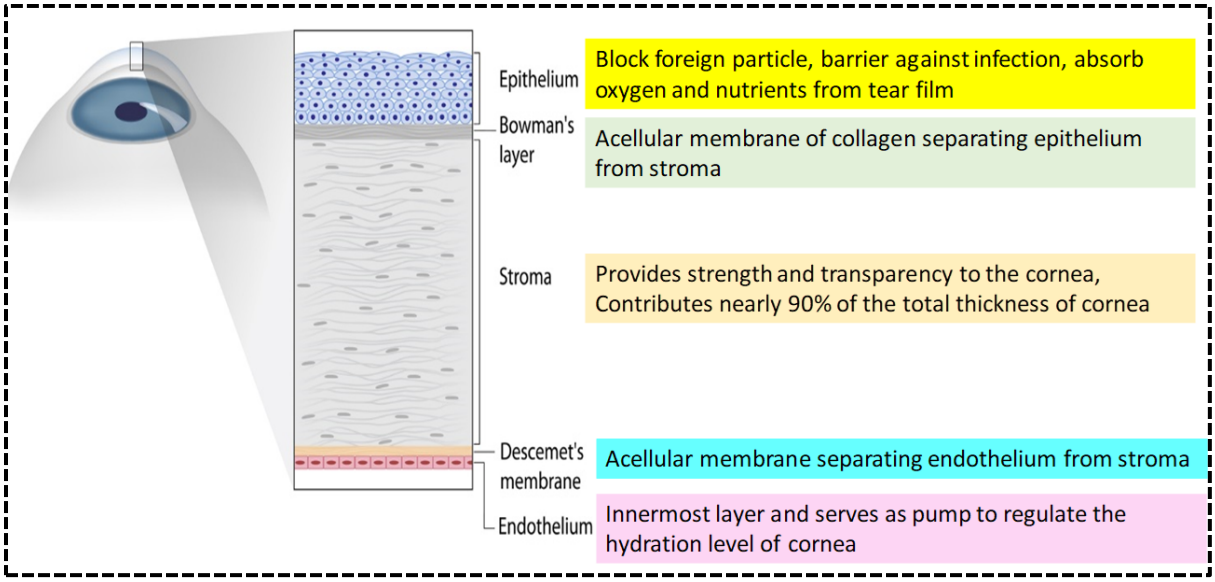

Figure 1.2 Diagram showing the microstructure of cornea and its function (Ahearne and Lynch 2015)

1.1.3 Functions of corneal layers

1.1.3.1 Corneal Epithelium

This layer is the outermost and makes up around 10% of the overall corneal thickness. The corneal epithelium is made of 5–7 layers of cells that are very equally distributed across the cornea (Sridhar 2018). It acts as a barrier against and prevents foreign particles entering into eyes. It absorbs nutrients and oxygen from tears and transports them to the rest of the cornea. The epithelial cells have unattached nerve endings. The presence of large quantities of the intracytoplasmic enzyme crystalline, as found in lens epithelial cells, may be critical for maintenance of optical transparency.

1.1.3.2 Bowman’s Layer

Bowman's membrane is a collagen and proteoglycan condensate. It is a structure composed of collagen types I and V as well as proteoglycans. Bowman's membrane lies just anterior to the stroma and is not a true membrane and incapable of regenerative ability. This smooth layer helps the cornea in maintaining its shape.

1.1.3.3 Stroma Layer

The corneal stroma constitutes the majority of the cornea's structural framework and accounts for roughly 90% of its thickness. Stroma is the transparent layer, made up of collagen fibers that are regularly spaced, with interconnected keratocyte cells that are responsible for common repair and maintenance. Collagens, proteoglycans and cells are the three major non-aqueous elements of the corneal stroma. Additionally, it includes specialized glycoproteins and ions that are critical for organizing the collagen fibrils and ensuring their transparency (Espana and Birk 2020). Numerous properties of corneal collagen and its structural organization have been previously reported. Collagen types I, V, VI and XII, as well as glycosaminoglycans decorin, keratocan, lumican, and mimecan are the primary components of the ECM (Meek and Boote

2004). The Stroma layer is important not just for the cornea's transparency, but also for its mechanical strength.

1.1.3.4 Descemet's Membrane

Descemet's membrane is an acellular sheet of collagen IV and laminin protein that acts as a basement membrane for endothelial cells (Sridhar 2018). It is a 5-20 μm thick membrane continuously secreted by endothelial cells (Dua et al. 2013).

1.1.3.5 Endothelium Layer

It is a single layer of polygonal flat cells known as the corneal endothelium situated at the posterior end of the corneal surface. Corneal endothelial cells are around 5 μm thick and have a distinctive hexagonal structure (Hertsenberg and Funderburgh 2015). Endothelial layer governs the movement of fluid and solutes between the aqueous and corneal stromal compartments by regulating sodium/potassium and ATPase pumps (Eghrari, Riazuddin, and Gottsch 2015).

1.2 Problems and needs

1.2.1 Corneal diseases and dystrophies

The cornea, being the most exposed portion of the eye, is susceptible to a variety of illnesses such as keratoconus, corneal dystrophy, corneal scarring, and bullous keratopathy, all of which can result in significant vision impairment or corneal blindness (Kadar et al. 2009; Mamalis et al. 1992; Moffatt, Cartwright, and Stumpf 2005). Cornea issues can also be caused by physical damage such as etching by strong acids or bases, excessive exposure to ultraviolet radiation, hereditary disorders, or fungal or bacterial infections. Although the cornea is a tough tissue with substantial regeneration capacity, sometimes there are rare instances where significant

damage cannot be healed using the conventional methods (Sitalakshmi et al. 2009; Puangsricharern and Tseng 1995). Genetically inherited dystrophies can also have a detrimental effect on a patient's later years (Puangsricharern and Tseng 1995; Du et al. 2009). Although corneal infections are uncommon, they can occur as a consequence of contaminated contact lenses and penetration of fungus or bacteria into the cornea; causing significant damage as well as resulting in loss of ocular transparency and perhaps even scarring (Huang and Hazlett 2003). Some significant cause of vision loss due to corneal impairment is exposure to chemical agents such as strong acids and bases, whose fast penetration capabilities can cause serious damage to the cornea, rendering it incapable of recovery.

1.2.2 Burden of corneal diseases

According to the World Health Organization (WHO), corneal blindness is the fourth most common cause of worldwide blindness in the world (Pineda 2020). Around 6.8 million individuals in India are believed to have vision less than 6/60 in at least one eye due to corneal disorders; around a million of these have bilateral involvement. By 2020, India's population of individuals with unilateral corneal blindness was expected to achieve 10.6 million (Gupta et al. 2013).

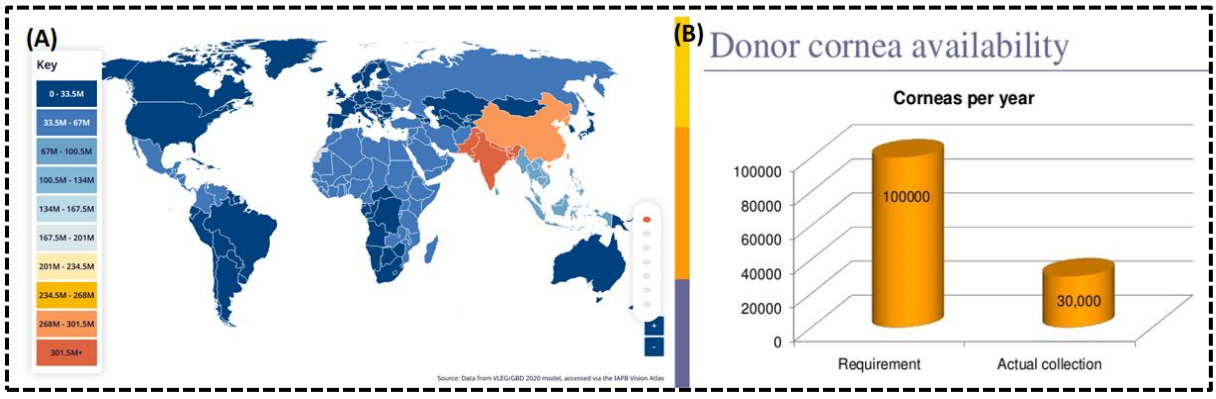

Figure 1.3 (A) Global map of the age-standardized prevalence of blindness, 1990-2020. Source: Vision Atlas (https://theophthalmologist.com/subspecialties/a-way-out-of-the-dark) (B) represents the requirement and availability of healthy donor cornea.

Vision Loss Expert Group (VLEG) in collaboration with the International Agency for the Prevention of Blindness (IAPB) released database information through Vision Atlas. According to the Vision Atlas released in 2020, emerging nations such as India, Pakistan, Afghanistan and Nepal shared the highest number of people affected by vision loss by 2020 (figure 1.3a).

1.3 Solutions for Health Issues Associated With the Cornea

While penetrating keratoplasty (PKP) continues to be the gold standard for corneal transplantation, the prognosis in these patients may be compromised by immunological rejection, chronic endothelial cell loss and higher intraocular pressures; all of which can result in graft failure (Armitage, Dick, and Bourne 2003). Consequently, the development of lamellar keratoplasty (LK) has resulted in a significant shift in the favoured practice pattern of the majority of ophthalmologists around the world. When the cornea is irreversibly injured, a procedure such as penetrating keratoplasty or corneal transplantation is recommended. A donor cornea is used to replace the deteriorated cornea in this procedure, which takes about an hour. In order to enable this procedure to be effective, the donated tissue must be compatible with the recipient's own tissue type. Due to the fact that the cornea is a non-vascularized tissue, there is a better likelihood of finding an appropriate substitute (Claesson et al. 2002). However, the corneal availability is only 30% of the total requirement of cornea (figure 1.3b). To overcome such scenario artificial corneas may be utilized instead of transplanting donor tissue. Prostho-keratoplasty is the term used to refer to the technique of replacing the cornea with an artificial substitute, and the artificial corneas are termed as keratoprostheses. Keratoprostheses (when using artificial cornea) also have certain drawbacks, such as extrusion, inflammation, infection,

epithelial down growth or glaucoma. This is where tissue engineering comes into play since it enables the fabrication of bioartificial corneas with high acceptance rates.

1.4 Tissue Engineering

1.4.1 Definition of Tissue Engineering

Tissue engineering is a multidisciplinary discipline that incorporates fundamental sciences and engineering concepts intending to enhance, repairing, or replacing damaged, dysfunctional, or diseased tissue with a healthy, artificial equivalent (Vacanti and Vacanti 2007; Thein-Han and Misra 2009). The goal of tissue engineering is to create tissues with radically distinct features by imitating the unique biological, physical, and chemical qualities of their natural counterparts (Ikada 2006). A tissue-engineered product is composed of two primary components: cells and a carrier. Tissue engineering requires the development of an appropriate carrier for the target tissue, as well as the acquisition, expansion, and manipulation of the cells to be employed (Figure 1.4).

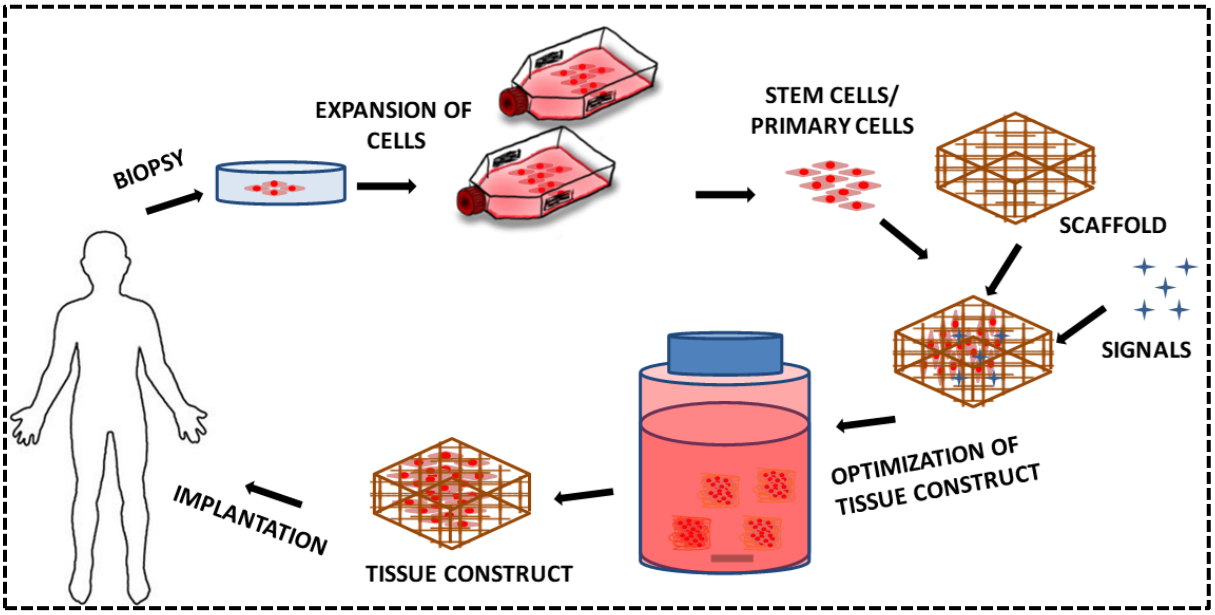

Figure 1.4 General scheme of the tissue engineering process.

Tissue engineering enables various approaches to fabricate corneal stromal constructs and/or equivalents to meet the specifications similar to the natural cornea (Ghezzi, Rnjak-Kovacina, and Kaplan 2015; Lin and Jin 2018; Sharif et al. 2018). Corneal tissue engineering applications have gathered countless interest across several fields of science with few foremost limitations such as graft rejection, scar formation and infection (Griffith and Naughton 2002; Karamichos 2015).

1.4.2 Cell sources in Tissue Engineering.

Cell sources for tissue engineering can be classified as embryonic stem cells, adult stem cells, or fully developed cells, depending on their differentiation state. Obtaining fully differentiated cells from a biopsy of any tissue is possible following cell isolation from the sample. It is then necessary to expand the number of these cells in order to achieve the desired number for the construction of a fully functional tissue. The cell type and its proliferation capacity mostly determine the choice between primary and stem cells. Keratinocytes, osteoblasts, and chondrocytes may be separated and grown very rapidly from a small biopsy. This is not true for all cell types though. A variety of cell types can be utilized to fabricate a corneal construct with a complete or divided thickness, consisting of one or two layers of tissue. The structure is mostly made up of cells separated from the various corneal layers. The epithelial layer is reconstructed using isolated human corneal epithelial cells and limbal corneal epithelial cells (Liu et al. 2012; Alaminos et al. 2006). The stroma was constructed by seeding several cell types onto the scaffolds, including isolated primary stromal cells, precursor cells or cell lines, and dermal fibroblasts (Mimura et al. 2008; Carrier et al. 2009).

1.4.3 Biomaterials in Tissue Engineering.

The use of polymeric biomaterials is a supporting component of tissue engineering. Numerous materials have been utilized in the delivery of drugs, micropatterning, microfluidics, and other technologies (Kohane and Langer 2008). Three-dimensional matrixes have been created and rendered deliverable using minimally invasive methods. An ideal biomaterial should have mechanical strength, controllable biodegradability, high porosity and proper pore size (Karageorgiou and Kaplan 2005). Additionally, these materials and their degradation products must be biocompatible, which means they must be non-toxic, non-immunogenic, and non-carcinogenic. There are several materials that meet these criteria. Multifunctional scaffolds made of natural and synthetic materials have been designed to imitate the extracellular matrix (ECM) characteristics of an in vivo milieu in order to stimulate tissue development. In the synthetic category, the most notable members are polyglycolic acid (PGA), polylactic acid (PLA), and copolymers of poly(lactic acid-co-glycolic acid (PLGA) (Alaribe, Manoto, and Motaung 2016). Synthetic biomaterials have several benefits, including the fact that their chemical compositions are well specified, which allows for greater repeatability. Moreover, they have the potential to regulate mechanical characteristics, controlled degradation rate and benefit of being able to form independently of one another. Chemically synthesized polymers are extremely valuable in the biomedical area because their characteristics may be customized to meet the needs of certain applications. Synthetic polymers are less expensive than biologic scaffolds, and they may be consistently manufactured in huge quantities with a long shelf life. However, many of the synthetic materials lack cell adhesion moieties and require chemical alteration in order to activate these moieties (Alaribe, Manoto, and Motaung 2016). In total, the biocompatibility of the material and its appropriateness for in vivo transplantation are also

examined, including the likelihood of an immunological reaction to the substance and its by-products. Natural biomaterials exhibit various advantages, including their abundance from renewable agricultural food supplies, biodegradability, and biocompatibility. Natural biomaterials are also well-known for their ecological safety and their capacity to generate a large number of enzymatically modified derivatives for a variety of purposes or applications (Watanabe et al. 2007).

1.4.4 Tissue Engineering Scaffolds

A scaffold for tissue engineering can be characterized as a two-dimensional (2D) or three-dimensional (3D) framework constructed of a biodegradable substance, either synthetic or natural, that provides a hospitable milieu for cells to grow, differentiate, and perform their normal metabolic functions (Doillon et al. 2003). Cells grown on 2D surfaces are compelled to develop in a monolayer and spread freely in a horizontal plane due to the force apical-basal polarity. When grown in a three-dimensional matrix, the cells adopt a stellate shape. Additionally, because the polarization is just from front to back, they can spread vertically as well (Mseka, Bamburg, and Cramer 2007). Moreover, Cells may distinguish between 2D and 3D geometries of the ECM based on the formation of integrin-mediated adhesions on one side (2D) or in all directions (3D) of the ECM (3D). 3D settings provide persistent temporal gradients ranging from hours to days, which is necessary for investigating morphogenetic processes. 3D settings enable the study of morphogenetic processes to occur over an extended period, which is necessary while developing scaffolds based on corneal tissue engineering (Baker and Chen 2012) (figure 1.5). Some of the relevant techniques that could be exploited for the accomplishment of the 3D tissue constructs are hydrogels (Mantha et al. 2019), electrospinning (Bhattarai et al. 2017; Kong and Mi 2016; Tonsomboon and Oyen 2013), three-

dimensional (3D) bioprinting (Isaacson, Swioklo, and Connon 2018; Jang et al. 2016), Topographic library (Miyoshi and Adachi 2014), decellularization (Badylak, Taylor, and Uygun 2011; Jang et al. 2016), salt leaching (Boote et al. 2006) and freeze-drying (Liu, Ren, and Wang 2014) (Figure 1.6).

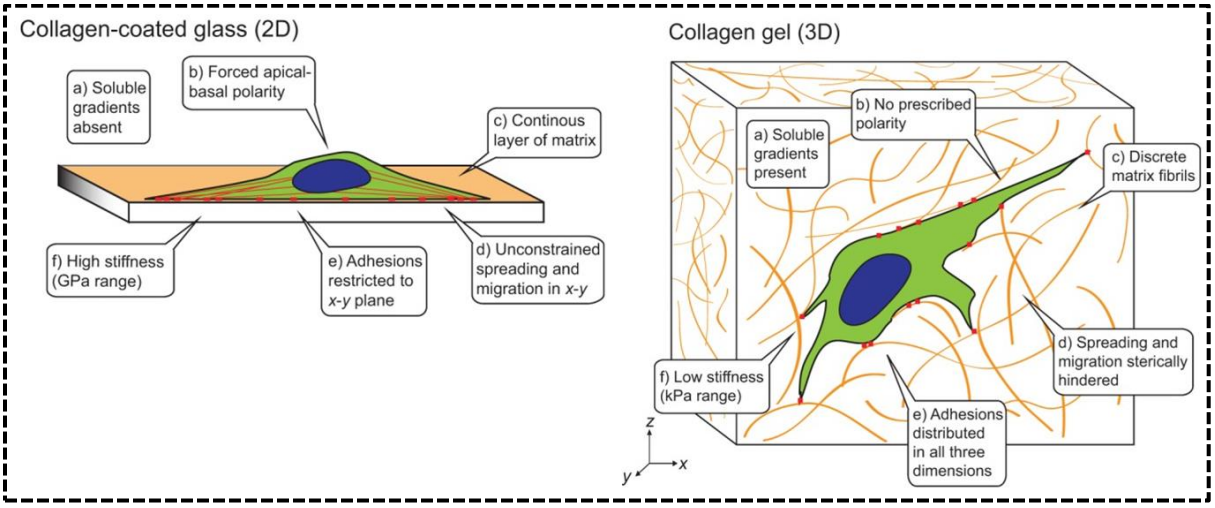

Figure 1.5 A diagrammatic representation of the difference between a two-dimensional and three-dimensional cell culture environment. (Baker and Chen 2012).

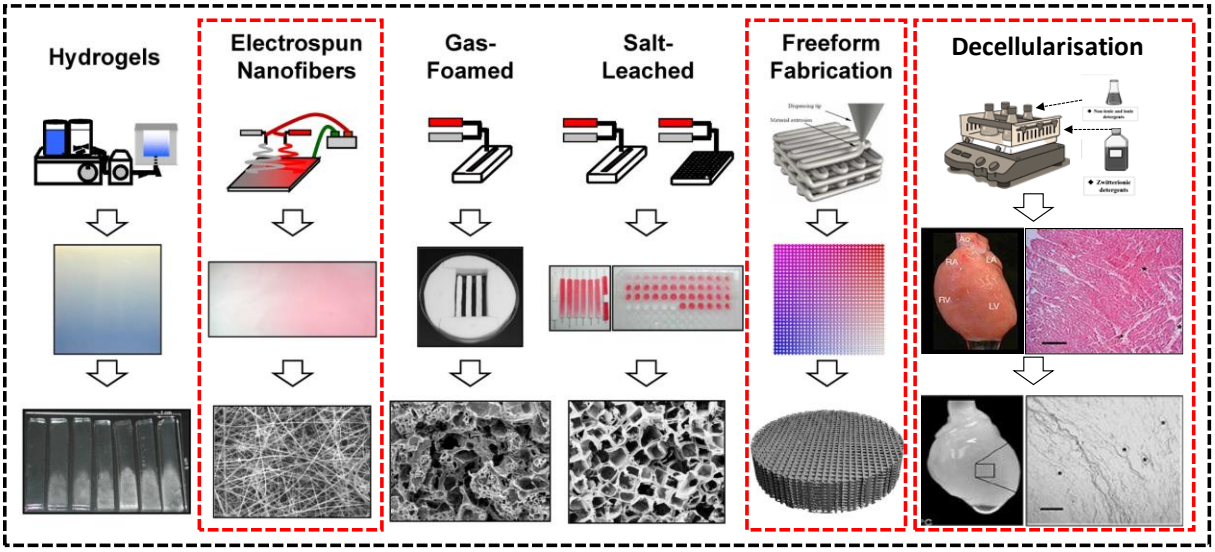

Figure 1.6 Approaches for developing a wide range of 3D scaffolds (Tutak et al. 2013).

1.4.4.1 Hydrogel Based Scaffolds

Hydrogels have emerged as one of the most famous and diverse groups of materials utilized in tissue engineering (Hunt et al. 2014). Hydrogels are three-dimensional networks of cross-linked, hydrophilic polymers that can retain a significant quantity of water in a swelled scaffold (El-Sherbiny and Yacoub 2013). The formation of hydrogels results in extremely porous networks, which allow for the diffusion of nutrients, oxygen, and biomolecules, while also permitting for the transfer of byproducts and toxins away from cells. The porosity of the material is often sufficient to allow for cell penetration and interconnectivity, resulting in a good growing medium for tissue (Khan, Tanaka, and Ahmad 2015).

1.4.4.2 Electrospinning Nanofibrous Scaffolds

Electrospinning is a manufacturing technique that uses an electrostatically driven process to produce electrospun fibers, which resembles nanofibrous yarn. The thickness of these fibers is generally found in the range of a few nanometers to a few micrometers (Xue et al. 2019). When a significantly high voltage is administered to a liquid droplet, the liquid's body becomes charged, electrostatic repulsion overcomes surface tension, and the droplet is expanded to form a Taylor cone, a key point of liquid stream ejection (figure 1.7). The jet begins in a straight path and soon whips violently due to bending instabilities. As the jet is extended to smaller dimensions, it rapidly solidifies, depositing solid nanofibers on the grounded collector. Because of its versatility and ease of manufacture of continuous nanofibers from a wide range of materials, electrospinning is often regarded as the most successful way of producing nanofibers (Kong and Mi 2016). The choice of collector influences the fiber orientation and the structure of a construct.

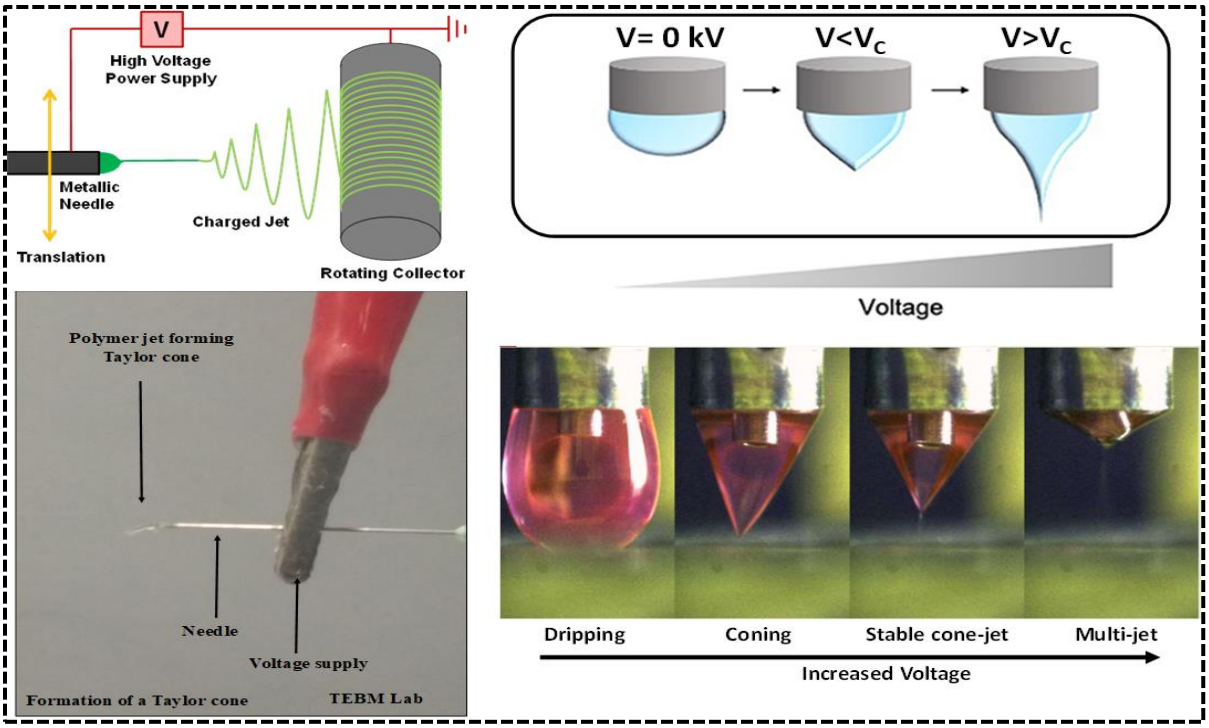

Figure 1.7 Diagrammatic representation of electrospinning unit setup and Taylor cone formation.

Additionally, the diameter and architecture of the fibers are dependent on several parameters, including the distance between the collector and needle tip, the rate of polymer ejection, applied voltage, nozzle diameter of needle, type of solvent used and the polymer concentration (Mulholland 2020; Xue et al. 2017). Electrospun materials have generated great attention in a variety of fields due to their exceptional characteristics, which include high porosity, small diameter, excellent pore interconnectivity and a high surface-to-volume ratio (Park 2011; Aman Mohammadi, Hosseini, and Yousefi 2020).

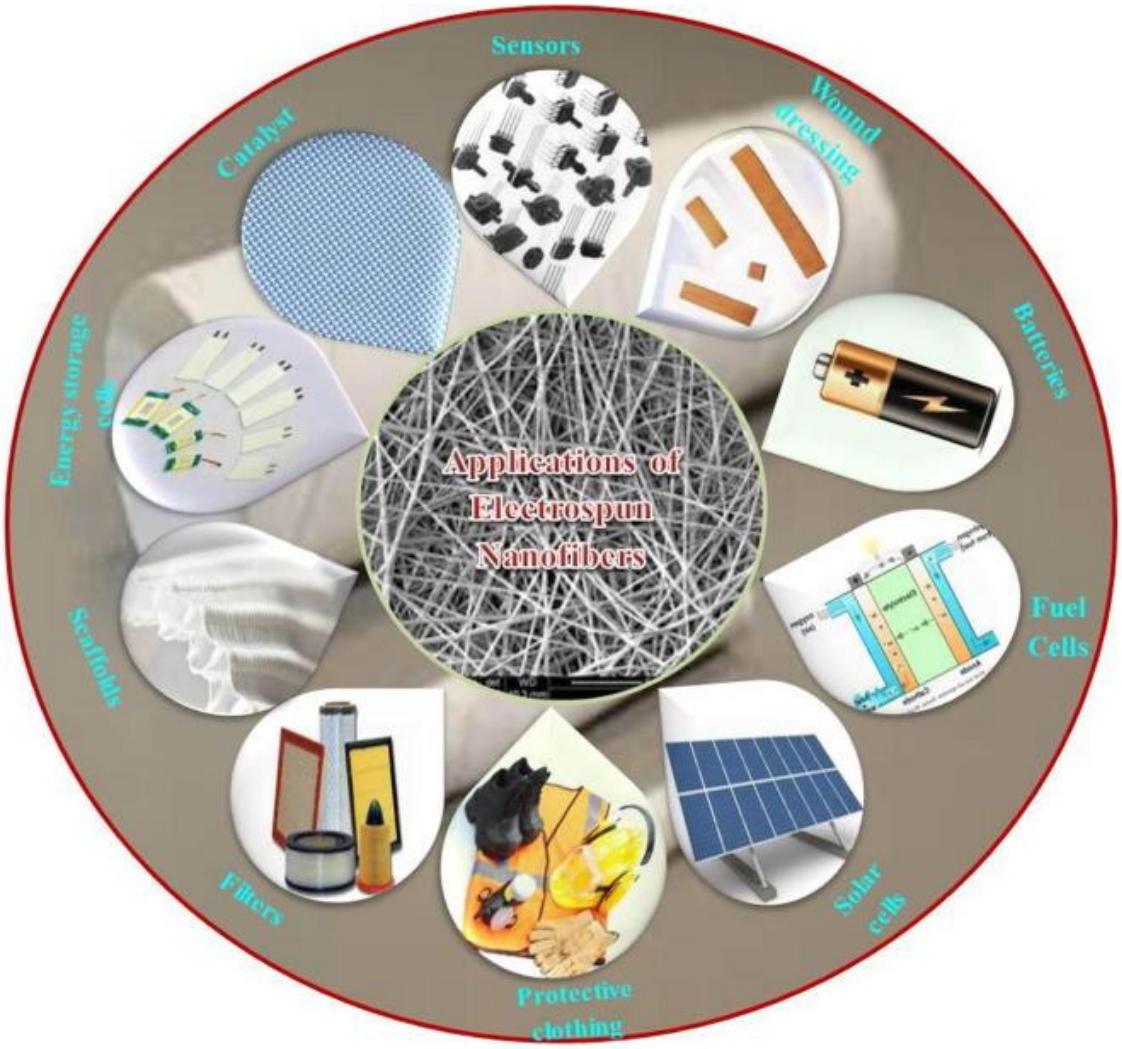

Figure 1.8 Depicts various applications of electrospun nanofibers (Thenmozhi et al. 2017).

Electrospun products have a variety of applications in the field of food packaging, preservatives (Mohammadi, Hosseini, and Yousefi 2020), sensors, filters, solar cells, fuel cells, batteries (Thenmozhi et al. 2017), drug delivery, variety of tissue engineering applications, reinforced materials (Min et al. 2016) and energy generation (Nikmaram et al. 2017) (figure 1.8).

1.4.4.3 Gas Foaming

Gas foaming is a solvent-free method of synthesizing bio-artificial matrices. As a result, it is an effective approach for integrating sensitive compounds into matrices while maintaining their bioactivity (Lee, Cuddihy, and Kotov 2008). Gas foaming technology has been developed to prevent the use of high temperatures and organic cytotoxic solvents. This approach utilizes relatively inert gas foaming agents such as carbon dioxide or nitrogen to pressurize a modelled biodegradable polymer with water or fluoroform until it is saturated or filled with gas bubbles (Eltom, Zhong, and Muhammad 2019). For gas foaming, the polymers are molded into a solid disc and then penetrated with carbon dioxide under high pressure for several days in a confined chamber. The gas filters in the polymer during this phase and develops pores for tissue ingrowth (Gorth and J Webster 2011) (Figure 1.9). For example, Giannitelli et al. effectively constructed polyurethane-based scaffolds with a thick shell and a porous core for bone regeneration applications using gas foaming, allowing for tissue ingrowth and bone regeneration (Giannitelli et al. 2015). The disadvantage of this approach is that the product created may have a closed pore structure or a solid polymeric structure because of inadequate treatment in some cases, which is undesirable. This method usually results in structures that resemble sponges, with pores ranging in size from 30 to 700 microns in diameter and porosity of up to 85-93% (Abdelaal and Darwish 2013). Gas foaming produces high (up to 93%) porosity and may modify pore sizes by changing temperatures and pressures parameters (Garg et al. 2012).

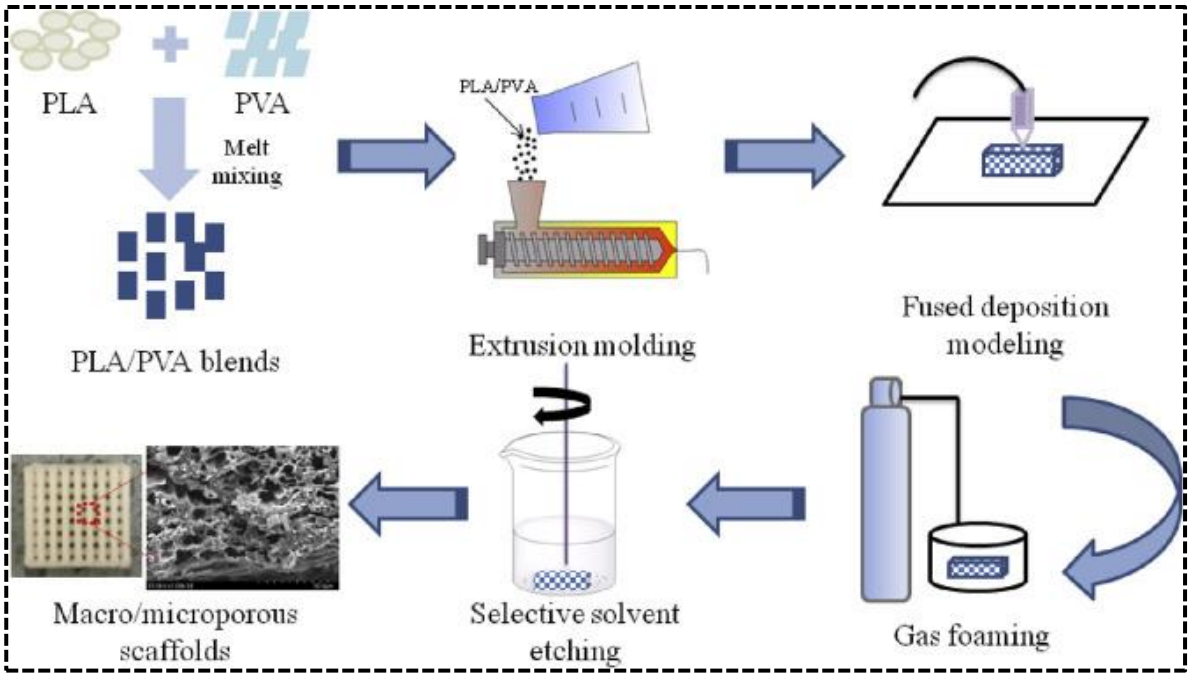

Figure 1.9 Schematic diagram for fabrication of hierarchical scaffolds with a tailored macroporous (3D printing) /micro-porous (gas foaming) architectures (Song et al. 2018).

1.4.4.4 Solvent casting/Particulate leaching

Another process that produces highly porous foams is solvent casting/particulate leaching. A porogen is introduced to the polymer solution and leached out once the polymer has dried. A porogen is a substance that dissolves in the polymer's non-solvent. The porogen is leached simply by dissolving it in its solvent (majorly used solvent is water). As a result, the residual polymer structure has a large number of linked holes that the porogen left behind. This technique is chosen because the pore diameters may be adjusted by the porogen used as well as its size and quantity (Varshney et al. 2019; Iis, Toibah, and Toibah 2008) (figure 1.10).

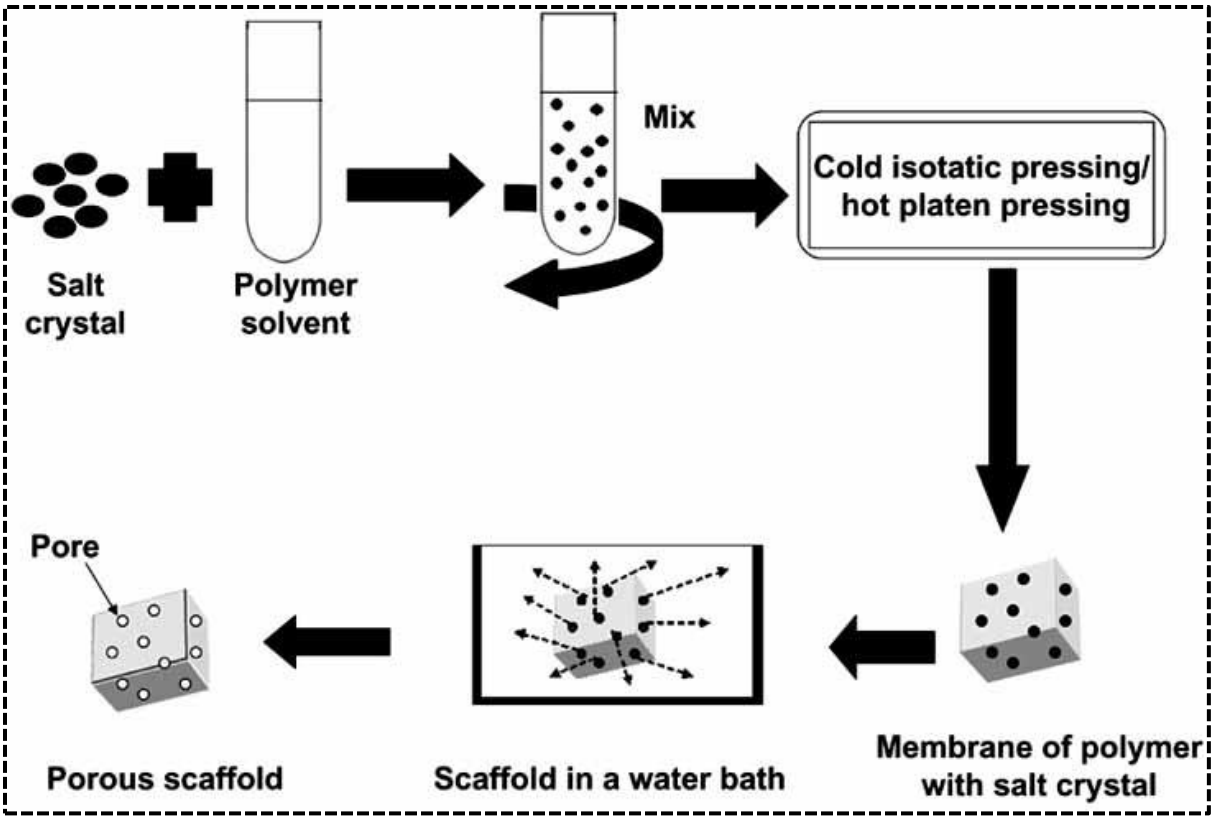

Figure 1.10 Schematic diagram for fabrication of scaffolds using solvent casting/salt leaching method (Iis, Toibah, and Toibah 2008).

1.4.4.5 Three-dimensional (3D) Bioprinting / Freeform fabrication technology

The advancement of additive manufacturing methods collectively referred to as solid freeform fabrication (SFF), has enabled the production of scaffolds with extremely thin structures and complicated geometries utilizing computer-aided design (CAD) data obtained from patient medical imaging. Solid freeform technology is transforming technology and holds tremendous promise for fabricating highly ordered biodegradable scaffolds for damaged tissues and organs (Shivalkar and Singh 2017). Stereolithography (SL), fused deposition modeling (FDM), selective laser sintering (SLS) and 3D printing are all SFF techniques that can be used to develop scaffolds for various tissue engineering applications (Hutmacher, Sittinger, and Risbud 2004; Melchels, Feijen, and Grijpma 2010).

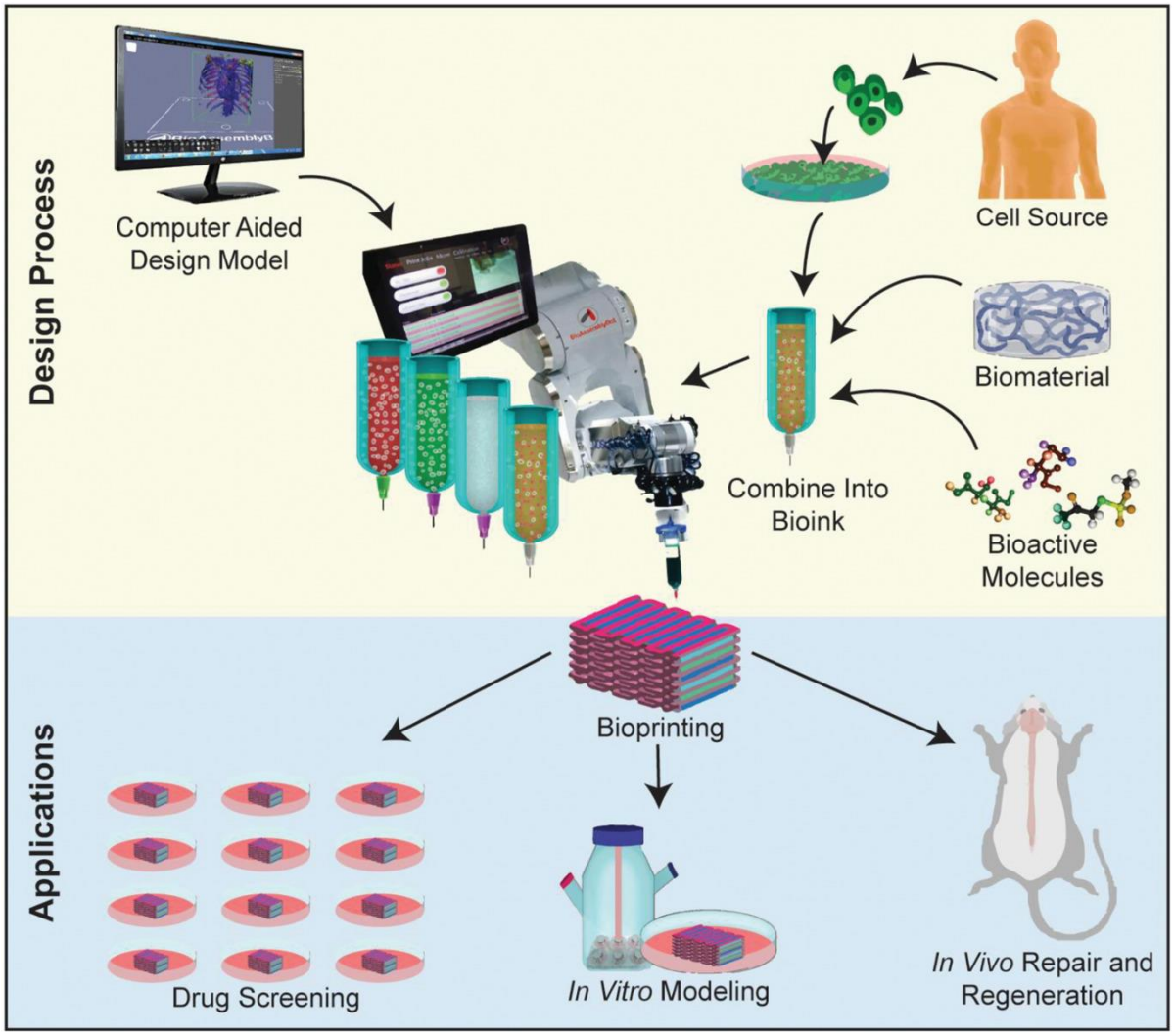

Figure 1.11 Overview of 3D bioprinting for tissue engineering. Computer aided design models use patient derived images to mimic the specific geometry of tissues of interest. The printing bioink may contain a combination of biomaterials, bioactive molecules, or cells to create functionalized and personalized scaffolds. Scaffolds are then printed using the computer aided design and desired bioink (s) (Murphy and Atala 2014).

Three-dimensional (3D) bioprinting is the process of combining cells, growth factors, and/or biomaterials to create biomedical components, frequently with the goal intending to mimic the properties of real tissue. In general, 3D bioprinting uses a layer-by-layer approach to deposit materials called bioinks to build tissue-like structures that may be utilized in a variety of medical and tissue engineering applications (Roche et al. 2020). Three-dimensional

bioprinting encompasses a diverse variety of bioprinting methods and biomaterials and by using these approaches; researchers may manipulate the characteristics of the scaffold such as the pore size, porosity and interconnectivity. In comparison to non-biological printing, 3D bioprinting entails extra complexity, including material selection, cell type selection, growth and differentiation factors and technological problems associated with the sensitivities of live cells and tissue building. Three-dimensional bioprinting has been utilized to generate and transplant a variety of tissues, including multilayered skin, bone, vascular grafts, tracheal splints, cardiac tissue, and cartilaginous structures (Murphy and Atala 2014) (Figure 1.1). Bioprinting applications fall into two broad categories: 1) tissue regeneration, which includes printing blood vessels, heart valves, musculoskeletal tissues, liver, nerves, and skin; and 2) biomedical applications, which include drug development and screening (Derakhshanfar et al. 2018).

1.4.4.6 Decellularization technology

Several studies have demonstrated that decellularization of tissues and organs is an efficient approach for removing potentially immunogenic components while retaining critical growth hormones and extracellular matrix (ECM) proteins required for normal cell function (Brown, Buckenmeyer, and Prest 2017). Ex vivo, decellularization of mammalian tissues is used to create ECM scaffolds, which include natural biological signals and factors that facilitate in the regeneration of functional tissue at appropriate locations. Maintaining the native ECM elements (i.e., three-dimensional ultrastructure and biochemical composition) during decellularization would potentially result in the perfect scaffold for tissue regeneration (Keane, Swinehart, and Badylak 2015). It is extremely desired to preserve the original ultrastructure and composition of the ECM during the process of tissue decellularization (Crapo, Gilbert, and

Badylak 2011). Each tissue and organ have variable morphology, therefore the most efficient agents for decellularization will rely on several parameters, including cellularity (e.g. liver vs. tendon), density (skin vs. fat), lipid content (e.g. brain vs. urine bladder) and thickness (e.g. dermis vs. pericardium). Every cell removal chemical procedure is known to produce some degree of ultrastructure disturbance; therefore, it is critical to appreciate this before beginning treatment (Crapo, Gilbert, and Badylak 2011). A wide range of applications is available for decellularized materials and matrices in tissue engineering and regenerative medicine, including medical implants. The first scenario can be important to understand the procedure, in which entire decellularized parts are usually utilized as scaffolds for transplantation reasons. Decellularized extracellular matrix processed to create sheets and patches, which are then employed in soft tissue and heart repair, is the second example of our services. Bone matrix powder can be reconstituted with water to form injectable hydrogels with regenerating characteristics. ECM generated scaffolds can be utilized as cell carriers for in vitro modelling or in vivo regeneration purposes (figure 1.12).

Freeze-thaw, pressure gradient, supercritical fluid dissolution, electroporation, and mechanical oscillations are all examples of physical techniques that are often employed to decellularize aorta, bladder, cornea, blood vessels and some other organs or tissues (Crapo, Gilbert, and Badylak 2011; Sasaki et al. 2009; Sawada et al. 2008). Physical techniques provide several benefits, including ease of operation, the absence of chemicals to mitigate the body's unfavourable reaction and a relatively low immunological response.

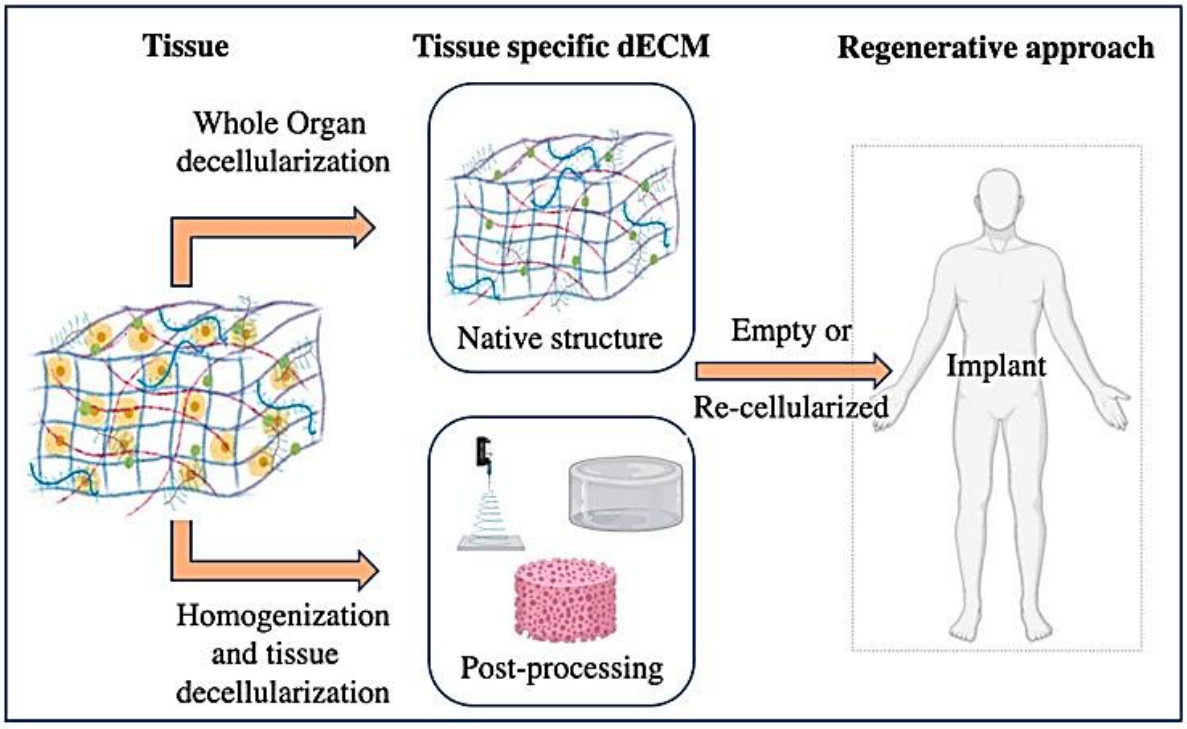

Figure 1.12 Schematic of organ decellularization and tissue decellularization approaches. (Mendibil et al. 2020)

Cells, lipids, antibodies, soluble proteins, and other substances from tissues and organs are removed using chemical reagents, while insoluble matrix components with their complete appearance, histology, and ultrastructure are retained. These include collagen, elastin, proteoglycans, glycosaminoglycans, and non-collagenous glycoproteins; that are retained using the chemical method (Yi et al. 2017). Chemical techniques are being utilized to extract required components from organs or tissues such as adipose tissue, blood vessels, heart valves, sciatic nerve, rat brain tissue, and corneal tissue. In biological techniques, tissues or organs are primarily decellularized by the action of particular enzyme reagents such as nuclease, collagenase, trypsin, lipase, dispersible enzyme, thermolysin, and alpha-galactosidase (Nakamura, Kimura, and Kishida 2017; Lumpkins, Pierre, and McFetridge 2008). Decellularized matrices are often used in several forms i.e. sheet or tube, powder, solution,

hydrogels, 3D printing bioink, and electrospinnable biomaterials, among others, as seen in figure 1.13.

Figure 1.13 Schematic diagram displaying applications of decellularized materials. (Liao et al. 2020)

1.5 Literature Review

An extensive assessment of the literature was conducted to situate the research findings of this thesis within the existing knowledge. The review has been carried out while keeping in mind the implemented electrospinning for scaffold fabrication.

Electrospinning has been used in a wide variety of applications in tissue engineering as a versatile nanofiber-producing technique. Very recently, Farasatkia et al. in 2021 have proposed transparent silk nanofibrils (SNF)/gelatin methacryloyl (GelMA) based hybrid films for ocular tissue engineering. Among various combinations of SNF/GelMA, the 30/70 ratio exhibits excellent transparency (around 85% light transmittance in the wet state), optimum in terms of

hydrophilicity and mechanical characteristics comparable to those of the native corneal stroma (Farasatkia et al. 2021).

Based on the research, it has been demonstrated that electrospun nanofiber reinforcement may significantly improve the mechanical characteristics of compliant hydrogels. Tonsomboon et al. in 2013 fabricated electrospun gelatin nanofibers infiltrated with alginate hydrogels, resulting in transparent (in the visible spectrum) fiber-reinforced hydrogels. They claimed that electrospun gelatin nanofibers increased the tensile elastic modulus of hydrogels from 78 ± 19 kPa to 450 ± 100 kPa without the need for previous crosslinking. The crosslinking of gelatin fibers with carbodiimide hydrochloride in ethanol prior to the infiltration procedure resulted in stronger hydrogels with an elastic modulus of 820 ± 210 kPa, albeit at the price of transparency (Tonsomboon and Oyen 2013).

Lately, Forouzideh et al. 2020 prepared an anti-angiogenesis silk fibroin scaffold loaded with epigallocatechin gallate (EGCG) for corneal tissue engineering and the results anticipated that with further development the EGCG-loaded scaffold has the potential for use as a delivery system for corneal tissue engineering (Forouzideh et al. 2020).

Kim et al. in 2018 have developed a unique electrospinning technique based on the use of a single nonconductive hemispherical device and a metal pin. A custom-designed peg-top collector (a hemispherical nonconductive device with a metal pin in the middle and copper wire creating a circle around the perimeter) was added to a standard conductive collector. With the proposed peg-top collector, a 3D hemispherical transparent scaffold with radially aligned nanofibers was successfully produced. The fabricated 3D electrospun scaffold was predicted to be useful for the treatment of ocular tissue injuries due to its hemispherical form and radially aligned nanofibers that may drive the major collagen and cellular actin filaments in the

extracellular matrix (Kim, Kim, and Park 2018). Along the same lines, Wu et al. developed aligned polyvinyl acetate (PVA)/collagen (PVA-COL) scaffolds to enhance the mechanical stability of the electrospun scaffold to meet the requirement for surgery suture, which limits its clinical applications to a large extent. Aligned polyvinyl acetate (PVA)/collagen (PVA-COL) scaffolds were electrospun by combining collagen and PVA to increase the mechanical strength of the collagen electrospun scaffold (Wu et al. 2018).

1.6 Research Objectives

The foremost aim of this research work is to develop nanofibrous three-dimensional scaffolds as corneal stromal equivalents using biopolymers such as silk fibroin and gelatin through electrospinning techniques. To achieve this, several sub-objectives need to be accomplished. Therefore, the objectives may be divided into the following parts:

1. Fabrication of gelatin-based nanofibrous scaffolds using gelatin A and gelatin B.
2. Improvement of gelatin nanofibrous scaffolds in terms of stability, degradation, transparency and mechanical strength through silk fibroin permeation.
3. Fabrication and characterization of gelatin permeated silk fibroin based nanofibers.
4. Evaluation of biocompatibility of all the prepared scaffolds for corneal stromal cells in vitro.
5. Comparative evaluation of all the prepared nanofibrous scaffolds for their suitability towards corneal stromal equivalents.

1.7 Thesis Outline

This thesis is organized into four chapters.

Chapter 1 (this chapter) provides a general overview of the cornea i.e., its anatomical macro and microstructure, basic layers of the cornea and their role. This chapter also presents the detailed literature review of the enabling technologies that are employed in the development of three-dimensional (3D) scaffolds for recreating cellular microenvironments on an in vitro platform. The research objectives of this thesis towards the goal of developing functional 3D micro/nano-scale structures as corneal stromal equivalents are also explained in this chapter.

Chapter 2 describes the comparative study of acid and alkaline hydrolyzed gelatin nanofibers. This chapter also refers to the characterization of nanofibers obtained by acid hydrolyzed gelatin (type A) and alkaline hydrolyzed gelatin (type B), in terms of porosity, functional groups, stability at physiological conditions and degradation behavior. This chapter also demonstrates strategies to stabilize gelatin A nanofibrous scaffolds with help of silk fibroin to fabricate a composite scaffold. To enhance the stability of the silk permeated gelatin nanofibrous scaffold and to avoid use of harsh chemical crosslinkers, a scaffold is treated with ethanol vapor to crosslink them physically. The chapter also refers to various characterizations such as spectroscopic analysis to ensure the presence of characteristic function of both the constituents, and physical cross-linking and beta sheet formation within the silk fibroin constituents. Morphological analysis of the fabricated scaffolds has been performed by scanning electron microscopy (SEM) images. Prolonged stability and degradation of the scaffolds have been evaluated in the cell culture medium until 5 days of incubation. Furthermore, transparency, degradation and solubility of the scaffolds have been evaluated to validate their potential applications in the field of corneal tissue engineering.

Chapter 3 focuses on the successful development of a new nanocomposite gelatin hydrogel system reinforced with mechanically robust silk nanofibers. The scaffolds have been characterized for their physicochemical and mechanical properties. Mechanical testing of the scaffolds is carried out in order to verify the strength and degree of cross-linking of the scaffolds. Additionally, the degradation and solubility of the scaffold have been determined in order to verify their prospective uses in the area of corneal tissue engineering. The MTT analysis is also carried out to evaluate the in vitro vitality and proliferation of corneal stromal cells inside the scaffolds. The permeation of gelatin into the silk nanofibrous scaffold is expected to improve the proportion of visible light transmission, increasing its usefulness in a variety of applications where transparency is critical, such as skin wound dressing and, most significantly, ocular tissue constructions. Ethanol vapor treatment of silk nanofibers has been employed to change the less stable random helical conformation to the more stable sheets structure.

Finally, the key conclusions and future scope of work of the thesis are summarized in **Chapter 4.** An overview of the comparison between both the fabricated nanofibrous scaffolds for a variety of factors has been presented in Chapter 4.

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