# Chapter 1
# Introduction

## 1.1 Tissue engineering

A persistent non-healing wound presents a challenging problem which exposes patients to a high risk of infection. A wound healing process that is both fast and efficient would drastically cut down on the need for medical treatment, wound care supplies, and hospitalization, which would dramatically improve the quality of patients' life. Failure in regeneration of organs or tissues, as well as a scarcity of tissue donors, are common and expensive challenges in human healthcare. Tissue engineering (TE) and regenerative medicine have emerged as alternate approaches to overcome these challenges. This is a continuously evolving multidisciplinary science that uses cell biology and biomaterials to support, repair, and restore the functionality of injured or defective tissues through viable replacements (El-Sherbiny and Yacoub 2013). Materials, or scaffolds, physiologically active substances such as growth factors, drugs, and cells such as stem cells or cells isolated from the patient, are the key components of TE. Together they constitute the TE triad (Figure 1.1).

The goal of skin TE is to build a replacement that can be generated quickly; can restore skin with its normal functional, mechanical, and cosmetic features. This comprises the regeneration of the extracellular matrix (ECM) in order to give guidance and support, the vascular network in order to promote graft acceptance, skin appendages in order to perform activities including sensitivity and temperature regulation, and the various cells necessary for protection.

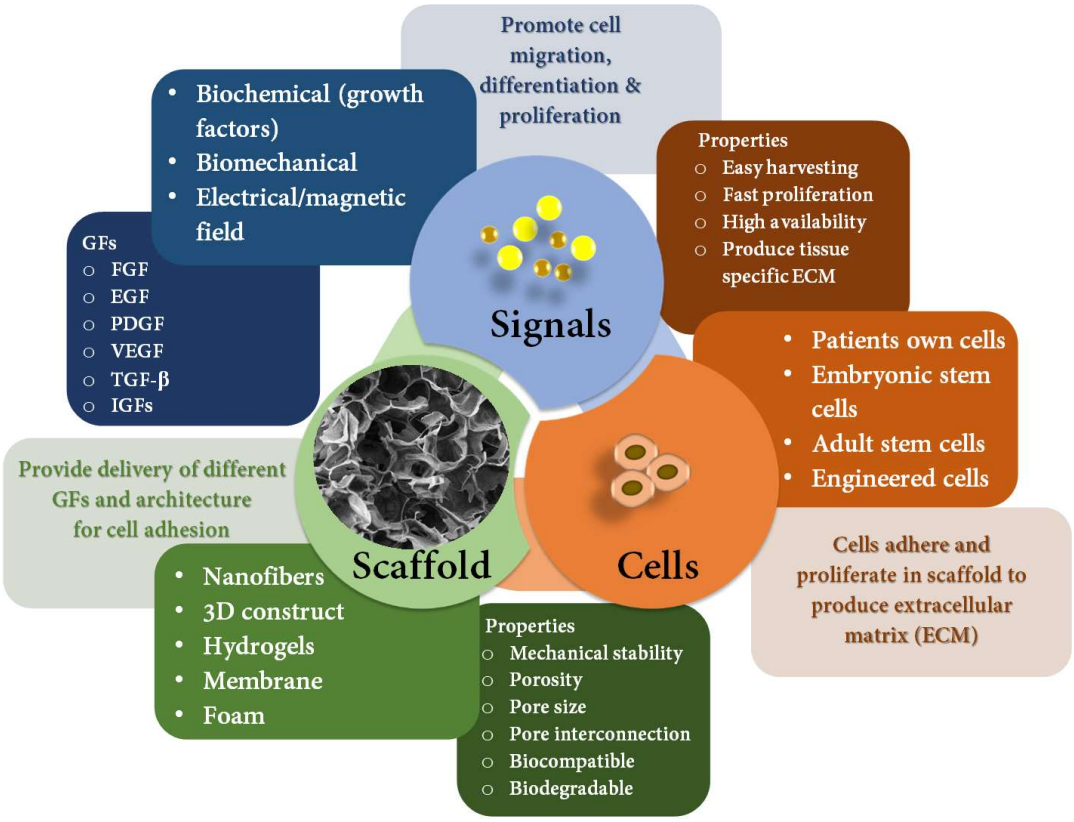


**Figure 1.1** Tissue engineering triad: scaffold, signals and cells.

### 1.1.1 Extracellular matrix

In native tissues, cells are held in an ECM which is a complex collection of extracellular molecules secreted by cells that provide structural and biochemical support to the surrounding cells. The ECM contains 3 classes of molecules: structural proteins such as collagen and elastin; proteoglycans/protein-polysaccharide complexes, e.g., hyaluronic acid and heparin; and adhesive glycoproteins such as laminins and fibronectin. The ECM is multifunctional and can influence multiple biochemical and mechanical processes simultaneously. Some functions of ECM include: 1) acting as an adhesive substrate, 2) providing structure and growth factors, 3) sensing and transducing mechanical signals. It differs significantly between tissues and even within tissues, as well as from one physiological state to another (Frantz, Stewart, and Weaver 2010). Cells create

intercellular connections with nearby cells and adhere to the ECM in a particular way during 3D tissue creation. These cell-cell and cell-matrix interactions influence tissue functions, permitting changes in the tissue's physiological state. Cell-matrix interactions are the binding of cell surface receptors, i.e., integrin, to particular regions on ECM molecules. When integrin on the surface of a cell bind to certain spots on ECM molecules, a chain of intracellular signalling events is triggered, which can influence how a cell acts or how a tissue is built.

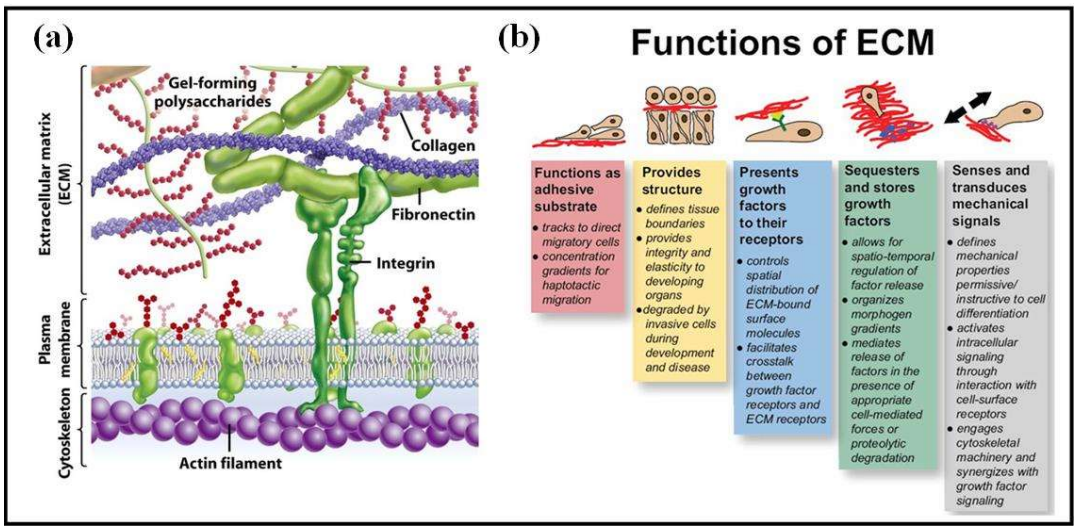


**Figure 1.2** Structural components of ECM and its various functions.

### *1.1.1.1 Significance of cell-ECM interaction*

The only route for cells to have a functional interaction with ECM proteins is through the process of cell-ECM interaction. However, in the absence of specific cell-ECM connections, the association between the two will be passive and won't be of any functional value to the cells. When cell surface integrin attach to certain ECM protein binding sites, a cascade of intracellular signalling is activated that affects cell proliferation and survival, transcription, protein synthesis regulation, cytoskeletal architecture and remodelling. Cell-matrix interaction is thus essential for proper cell behaviour,

phenotype, and function, and its absence results in aberrant cell behaviour and phenotype (Birla 2014).

Therefore, in TE, scaffold design/scaffolding is an attempt to mimic the in vivo ECM environment.

### 1.1.2 Advantages of three-dimensional (3D) cell culture

Most of our understanding of the biological processes involved in cell culture is based on trials performed on 2D plastic or glass substrates. Cells within the body, on the other hand, live in a 3D information-rich milieu of ECM that gives microenvironmental signals for appropriate cell activity. As a result, the reliability of the 2D substrates in reflecting the physiological activity of cells in vivo is debatable. Despite the fact that monolayer cell culture approaches have significantly contributed to our advancement in the field of basic cell biology, monolayer culture techniques are carried out in a 2D environment and lack in vivo 3D structure of tissue; hence, they do not provide an accurate representation of mammalian tissue. Accordingly, the benefits of 3D versus 2D cell culture are discussed in terms of cell attachment, mechanical factors, soluble substances, and so on (Figure 1.3). One of the most notable distinctions between 2D and 3D cell culture environments is the difference in cell shape. The cells' apical-basal polarity forces them to develop in a monolayer and spread easily in a horizontal plane when cultured on 2D surfaces whereas when cells are cultured on a 3D substrate, they acquire a stellate shape. Furthermore, because the polarisation is just from front to back, in 3D, they can spread vertically as well (Mseka, Bamburg, and Cramer 2007). It is crucial to note that flattening of cells on a 2D surface has a direct influence on cell function since it changes the surface-to-volume ratio, and in turn the membrane-to-cytoplasm ratio, implying greater signal propagation into the cells (Meyers, Craig, and Odde 2006). Cells can recognise the 2D or 3D

environment of the ECM based on integrin-mediated adhesions that are established on one side only, as in 2D, or in all directions, as in 3D.

When cells are cultured on a 2D system, they are exposed to a static mechanical environment that is supraphysiological with respect to rigidity and stiffness. However, cells cultured in a 3D environment are exposed to a range of stiffness. Integrin clustering is reported to be greater in 3D cell culture conditions than in 2D environments as a result of cell-exerted traction forces (Huebsch et al. 2010). On a 2D surface, cell stretching is smooth and homogenous, and cell deformation happens in a predetermined and related manner. Cell cultured in a 3D matrix, on the other hand, are fibrous, structurally diverse, and anisotropic. In a 2D culture environment, soluble substances diffuse readily throughout the media, resulting in rapid equilibrium. To analyse a cell's chemotactic events, temporary, and short-time gradients can be produced. 3D environments, on the other hand, provide continuous gradients ranging from hours to days, which are essential for examining morphogenetic processes. Controlling the spatial and temporal distribution of soluble components within a 3D cell culture environment is dictated not only by diffusion laws, but also by ECM structural properties including pore size, pore interconnectivity, gel dimensions, and so on (Baker and Chen 2012). Because cells are extremely sensitive to their surroundings, the cellular microenvironment is an important part of TE.

The above-mentioned constraints can be circumvented by using 3D scaffolds or artificial tissues that have been produced in the laboratory. These materials can replicate many of the attributes that are present in mammalian tissue. Therefore, TE approaches have many advantages over 2D culture systems, the most significant of which is their ability to replicate complex 3D structures of mammalian tissue, which support cell-cell and cell-

matrix interactions and can also improve our understanding of cell biology and physiology. Other analogous functions are listed below in Table 1.1.

**Table 1.1:** Analogous functions of ECM and TE scaffolds (Chan and Leong 2008).

| **Analogous functions** | |
|---|---|
| **ECM** | **TE scaffolds** |
| Facilitates structural support for cellular habitation | Facilitates structural support for the attachment, growth, migration, and differentiation of exogenously supplied cells in vitro and in vivo |
| Associated with tissue mechanical properties | Provides mechanical stability and shape to the damaged tissue; provides rigidity and stiffness to the engineered tissues |
| Bioactive cues are provided for cells to respond to their surroundings | Actively interacts with cells to support processes such as proliferation and differentiation through biological cues like cell-adhesive binding moieties and physical cues like surface topography |
| Serves as a reservoir for growth factors | It can act as a delivery system and reservoir for externally supplied growth stimuli |
| Provides a physically flexible environment for tissue remodelling in response to dynamic processes like wound healing | During remodelling, it provides a void space for vascularization and the development of new tissue by providing porous morphologies for the transport of nutrients and metabolites; matrix design with controlled degradation rate |

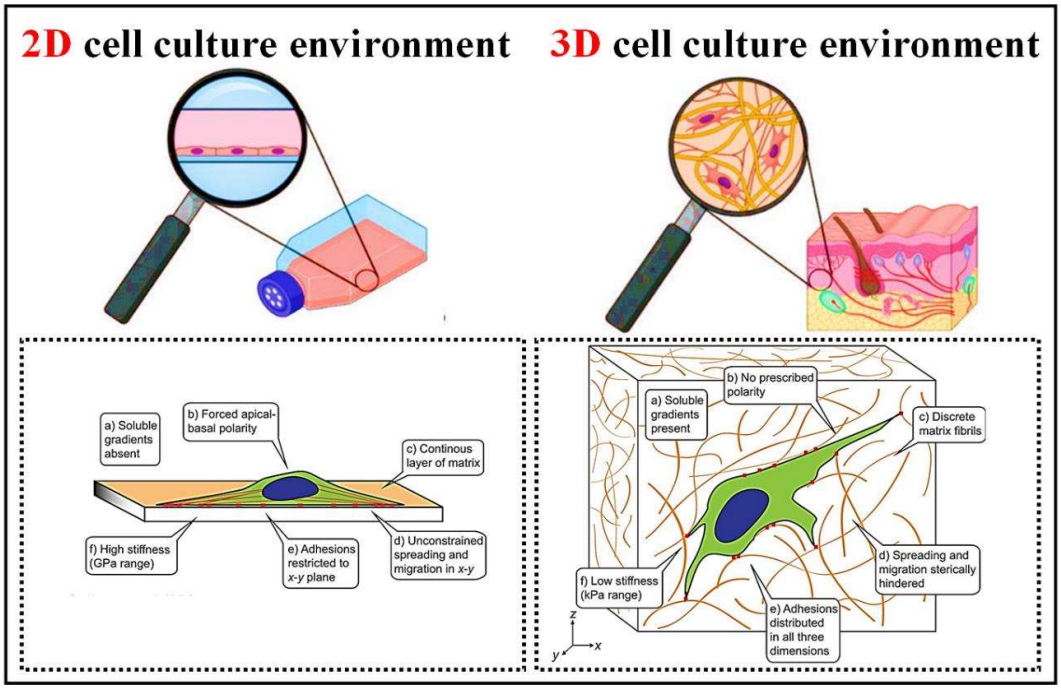


**Figure 1.3** Schematic representation showing the comparison between 2D and 3D cell culture environment (Baker and Chen 2012; Charwat and Egger 2018).

### 1.1.3 Scaffolds and their fabrication techniques

TE brings hope to patients who badly need tissue and organ substitutes in an era when organs for transplantation are becoming scarcer and there is a growing need for suitable alternatives. In the last three decades, scientists around the world have devised a slew of new approaches for shaping polymers into complex architectures with appropriate characteristics for desired TE applications. These manufacturing processes produce reproducible scaffolds for TE. Polymer scaffolds can control tissue regeneration by providing mechanical strength, porosity and surface area, variable surface chemistry, and different shapes (Dhandayuthapani et al. 2011). The scaffold should be biocompatible to elicit an adequate response in the host. It should be biodegradable, which means the degradation rate matches the new tissue growth rate. It should have adequate surface properties both chemically and topographically in order to promote cellular adhesion and

proliferation. It should have cell adhesion moieties to provide cell attachment and proliferation. It should have an interconnected open pore structure. Porosity is the most important structural scaffold requirement, which is useful for cell penetration, tissue ingrowth, and allows nutrient and waste metabolic product transport. Furthermore, for drug delivery purposes, porosity plays an important role in the loading and release of drugs or other biomolecules. It should have adequate pore size for facilitating cell infiltration, tissue growth, and vascularization. It should have sufficient mechanical strength to withstand the internal and external forces and maintain cell integrity. The functional performance of a tissue is closely related to its mechanical strength. Hence, the mechanical properties of scaffolds control many cellular characteristics such as the viability of the cell, cell–matrix interactions, cellular phenotype, differentiation, and magnitude of the focal adhesions. Hence, they play a crucial role in the development of a tissue-specific substitute (Figure 1.4).

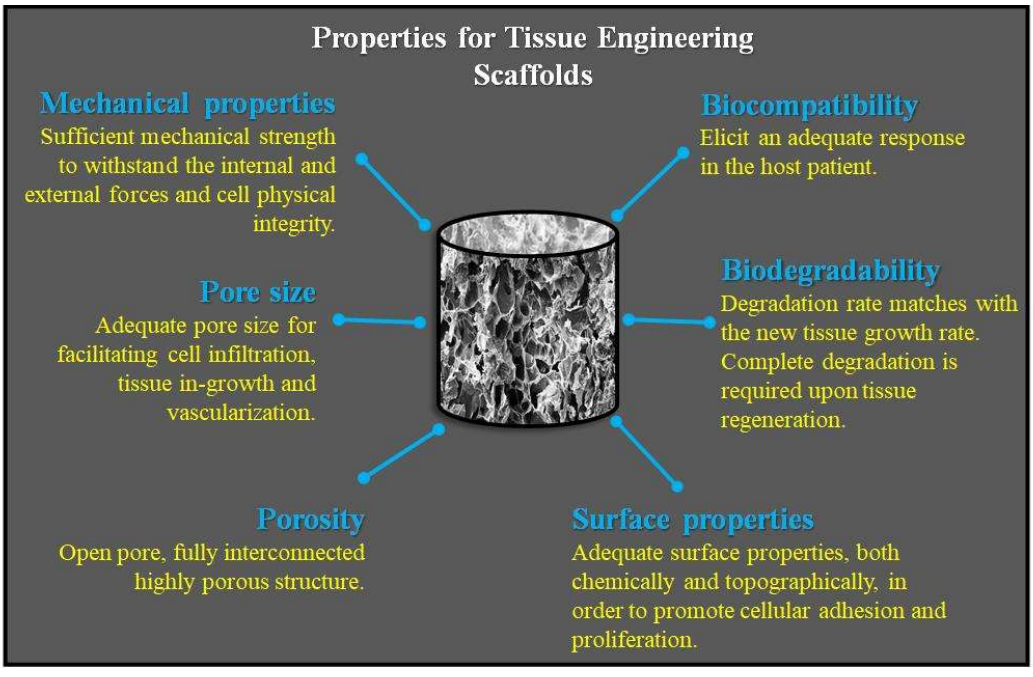


**Figure 1.4** Desired properties of tissue engineering scaffolds.

Scaffolding is an attempt to replicate the ECM and provide a three-dimensional (3D) template for tissue ingrowths (Freed et al. 1994). These fundamental scaffold features can be adjusted according to the application by selectively choosing the polymers, other support components, and fabrication process. Scaffold designs commonly use foams, sponges, fibres, meshes etc. These designs have been adopted because they facilitate homogeneous cell dispersion, nutrient diffusion, and the formation of structured cell communities (Vunjak-Novakovic and Freed 1998). Some of the most common techniques for producing and controlling overall porosity are casting/ porogen leaching, solvent phase separation, freeze drying, gas forming, cryogelation, electrospinning, 3D bioprinting, and soft lithography sintering (Mbundi et al. 2021) (Figure1.5). While each approach has its own set of benefits and drawbacks, suitability of the technique is primarily determined by the material's bulk and surface properties, needs of the specific tissue and/or the intended function of the scaffold. In the subsequent sections some scaffold fabrication techniques are briefly discussed.

#### *1.1.3.1 Solvent casting /particulate leaching (SCPL)*

SCPL is one of the most basic and low-cost methods for creating a porous biomaterial scaffold for a variety of TE and medical applications. In this technique, the polymeric biomaterial solution is cast onto evenly distributed salt particles or a porogen-filled Petri dish or mold. After the evaporation of solution, the salt composite matrix is leached in water for two days to remove porogen. This is the most appropriate method to attain controlled pore size, porosity, crystallinity, and highly interconnected thin porous scaffolds or membranes and foams. However, residual solvent and salt particles may cause adverse effects such as cell and surrounding tissue damage, denature the bioactive molecules of the scaffold. This technique is extensively used to develop three-dimensional porous scaffolds.

#### *1.1.3.2 Freeze drying*

Freeze drying is commonly known as the lyophilisation technique. The freeze-drying process involves three main stages. Firstly, the biomaterial solution is frozen at very low temperature (-70 °C to -80 °C) followed by placement of frozen samples in a freeze-drying chamber to allow very low vacuum pressure to dry the polymeric solution and in the final stage of drying process, the residual water from polymeric material is removed by sublimation phenomenon. Freeze drying process works on the sublimation principle and is majorly used in various applications such as food science, enzyme stabilization and pharmaceuticals. It is one of the easiest and cost-effective processes to fabricate porous functional scaffolds for TE. However, the fabricated scaffolds have certain limitations such as smaller pore sizes of the scaffolds which create complexity in the vascularized system and more processing time is required to fabricate a scaffold. Moreover, some times the technique also involves the use of toxic solvents during fabrication.

#### *1.1.3.3 Gas foaming*

The gas foaming scaffold fabrication technique is primarily used to produce controlled porosity scaffolds. In this process, unlike other fabrication techniques, organic solvents and high temperatures are not required during the fabrication process. Therefore, this is the safest method to fabricate a porous scaffold due to absence of any toxic organic solvents. The porous scaffolds are obtained through nucleation and development of gas bubbles. This process usually works on high-pressure carbon dioxide gas ($CO_2$) (800psi). The polymeric solution is exposed to $CO_2$ gas till the point $CO_2$ gas becomes thermodynamically unstable and phase separation takes place within the polymeric solution. Furthermore, the $CO_2$ molecules get transformed into a cluster to reduce the free energy which results in nucleation and development of porous gas. The 3D porous structure of the scaffold can be determined through the amount of gas suspended in the

polymer solution. Controlling the pore connectivity and pore size is a bit difficult while fabricating a scaffold using this process. However, to overcome these issues various porogen materials including wax, sugar and salt particulates are used.

#### *1.1.3.4 Cryogelation/freeze-thawing*

Cryogelation (freeze-thawing) is comparable to salt leaching in that both entail the stability of the polymer matrix surrounding solid particles that are later removed to create pores. Cryogelation involves the incubation of monomers or polymers with or without cross-linker premixed in an aqueous solvent at sub-zero temperatures such as -12°C, followed by the elimination of formed solvent crystals (ice crystals) by thawing to develop polymer-based superporous sponge like elastic scaffolds. The ice crystals formed during cryogelation also offer pore walls surfaces on which the cross-linking processes of cryoconcentrated monomers (unfrozen phase) occur. Because ice crystals operate as porogen, they must develop prior to polymerization. Cryogelation is a time and resource economical approach when compared to other approaches for synthesizing macroporous scaffolds. Other benefits of cryogels include their ease of fabrication and the use of water as the most common solvent, making this form of biomaterial more cost-effective and ecologically friendly (Memic et al. 2019).

Cryogenic methods are progressively being used to generate new polymeric materials to address issues primarily in the biomedical, environmental, and food technology fields (Rogers and Bencherif 2019). Freeze-thaw method provides certain advantages over the other existing methods such as radiation induced, enzymatic and chemical crosslinking (Hassan and Peppas 2000) such that it is a simple process that does not require high temperature or any chemical treatment.

#### *1.1.3.5 Electrospinning*

Electrospinning is a versatile spinning technique used to generate interconnected porous fibrous scaffolds similar to in vivo architecture of ECM. It uses electrostatic forces to generate fibers from micro to nanoscale dimensions. A relatively simple equipment system is needed to control the parameters such as electric field, flow rate, rotation per minute, voltage, diameter of the needle, distance between the collector and the needle and viscosity. Also, the concentration and conductivity of the solution play a key role to eject polymeric fibers. The complete setup is controlled by an electric field between two electrodes, which are connected to the tip of the needle and collector drum. A polymer solution is loaded into the syringe and when a high voltage is applied, polymeric fibers are ejected which get deposited on collector drum. The solution parameters such as viscosity of the solution, concentration and conductivity need to be optimized for the formation of nanofibrous scaffolds. Some of the natural and synthetic polymer materials used in the electrospinning technique are silk fibroin (SF), gelatin, poly (vinyl alcohol) (PVA), collagen, chitosan, poly-lactic acid (PLA) and polycaprolactone (PCL). This technique is widely suitable due to its fibrous structure, promoting cell growth/adhesion and facilitating the transport of nutrients to the cells. By adjusting the orientation, controlled pore geometry and fine fibers can be obtained. Electrospinning has evolved as an attractive technique due to its advantages over the others methods, particularly for nanofibrous structure formation. The nanofibrous structure provides a large surface to volume ratio and high porosity that together offers enough space for drug loading, cell incorporation, migration and proliferation (Ye et al. 2019). Electrospinning based scaffold fabrication is a promising approach for various TE applications such as wound dressing, drug delivery, artificial skin substitute, microengineered platform etc. (Thenmozhi et al. 2017; Bhardwaj and Kundu 2010). In addition, it has found applications

in diverse areas such as nanosensors, cosmetics, separation, filtration membrane, water treatment and purifications, environmental remediation, and smart textiles (Barhoum et al. 2019). Recently, Ke et al. have developed SPI-functionalized nanofibers (SPNF) made up of SPI and poly (L-lactic acid) (PLLA) using an electrospinning technique. They showed that SPNF exhibits good potential as a wound dressing because of its enhanced biocompatibility and hemostatic effect (Ke et al. 2021).

#### *1.1.3.6 3D Bioprinting*

3D printing (biological/non-biological printing) is an emerging field and has gained a significant interest for TE applications. Over the past few years, there has been a huge demand for 3D printing models. 3D printing is also referred to as additive manufacturing (AM). It is a prominent and widely acceptable technique in diverse fields such as automobile, food, manufacturing, mechanical engineering, architecture and ceramics etc. There are various 3D printers available such as melt extrusion/fused modelling method, stereolithography, inkjet printing, laser printer and selective laser sintering. The AM method prints the scaffolds layer-by-layer using computer-aided design (CAD) models or from the data which is acquired from pre-existing scans of MRI, CTs. A 3D bioprinter can print the cells while fabricating 3D tissue constructs using a top-down printing method. For this, the material should have adequate mechanical strength, physicochemical and biological properties to restore the functionality of tissue and organ. However, 3D bioprinting has some major limitations, the most significant of which are its high cost, limited availability of suitable material, and its lack of accuracy in droplet size and placement.

In this thesis work, we have explored salt-leaching, freeze-thaw, cryogelation, and electrospinning techniques to design 3D scaffolds for TE applications.

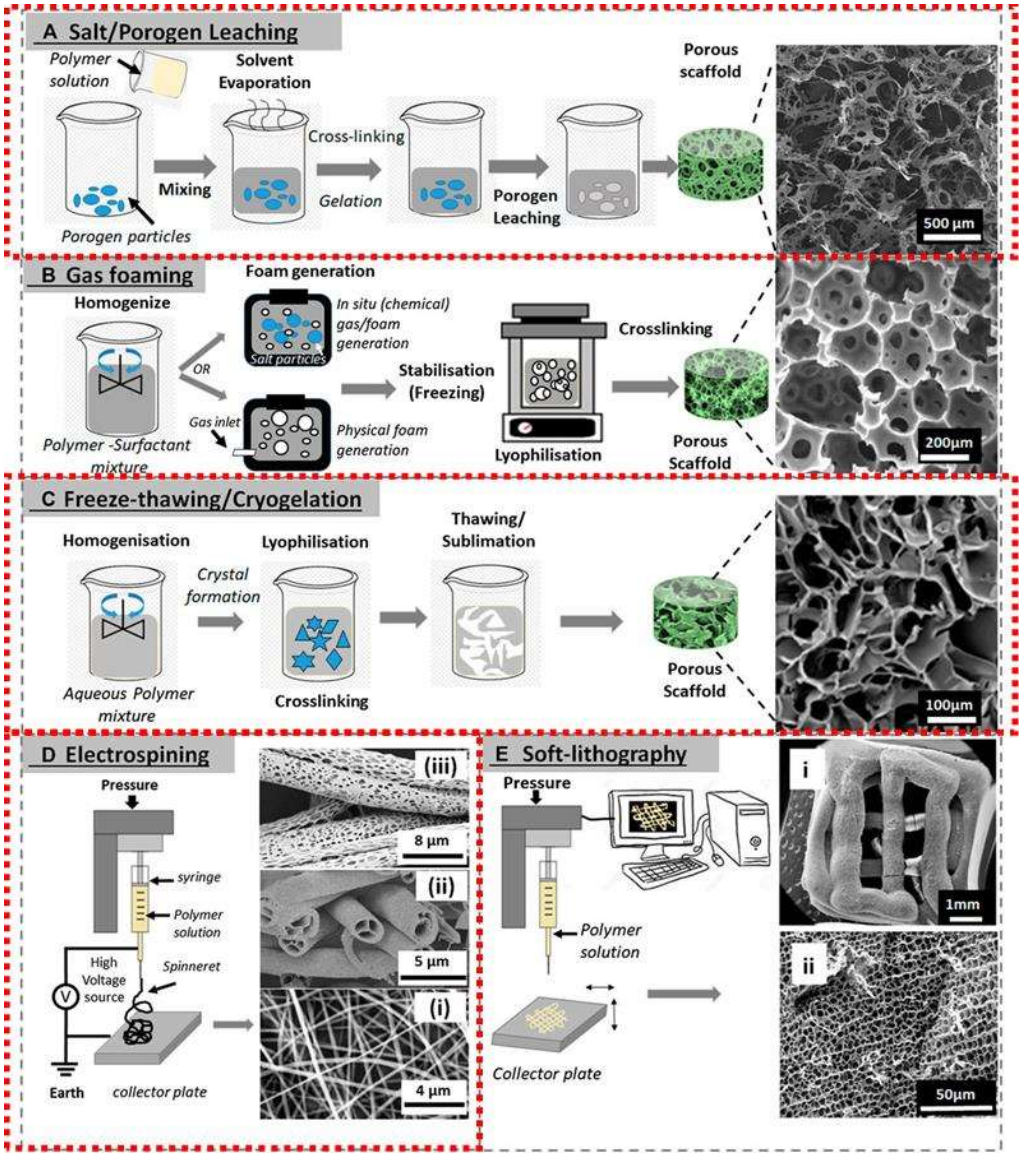


**Figure 1.5** Various porous scaffold fabrication techniques: **(A)** Porogen leaching **(B)** Gas foaming **(C)** Freeze-thawing **(D)** Solution electrospinning **(E)** Soft lithography (3D printing) (Mbundi et al. 2021).

### 1.1.4 Scaffold-based tissue engineering approaches

Scaffolds are one of the most essential components in TE. Scaffolds, which are primarily constructed using polymeric biomaterials, offer structural support for cell adhesion and eventual tissue growth. Either a single or a combination of various techniques can be employed to fabricate scaffolds with desired shape, size and properties. Thereafter, the prepared scaffolds are investigated for physiochemical properties and their

biocompatibility towards mammalian cells. As per the requirement, growth factors or drugs are loaded into them. Subsequently, their loading efficiency and release kinetics are examined. Scaffolds have a very critical role in TE since they can direct the growth of cells. This could be either due to the cells that have already been seeded or due to the cells migrating from a nearby region. In the first approach, an acellular construct i.e., a scaffold without cells is directly used as an implant. After implantation, cells from the surrounding tissues migrate towards the scaffold for tissue regeneration. In the second approach, cell seeded scaffolds are directly used as a tissue engineered construct. In the third approach, the cell seeded scaffolds are first incubated in laboratory to grow tissue. The matured cell seeded scaffolds are then implanted as bioengineered tissue constructs. Therefore, the goal of TE is to develop complex living constructs as functional substitutes that can be implanted to facilitate cell attachment, proliferation, and differentiation, thereby providing a biological microenvironment through ECM synthesis. This thesis has focused on the development of various types of acellular constructs using various fabrication techniques.

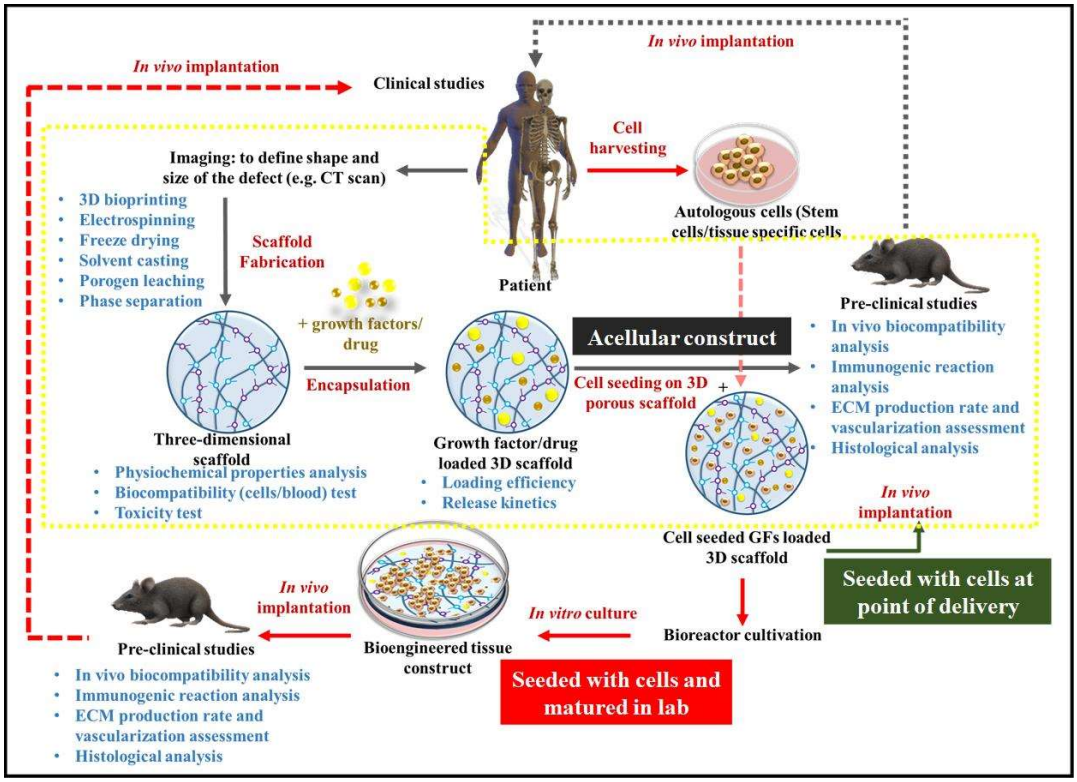


**Figure 1.6** Schematic representation of various scaffolding tissue engineering approaches: acellular and cellular constructs.

## 1.2 Skin tissue

### 1.2.1 Skin tissue anatomy

Skin is one of the largest organ in the body, accounting for around one-tenth of the total body mass (Metcalfe and Ferguson 2007). The skin is made up of two primary cell layers; the upper stratified epidermis and the lower dermis, which are coupled with the subcutaneous fatty layer that separates the skin from the remainder of the body (Figure 1.7). A basement membrane (BM) made up of BM-specific ECM proteins such as laminin and collagen IV separates the two layers. The epidermis is comprised of four fine layers of gradually differentiated keratinocytes: the stratum basale or stratum germinativum, the stratum spinosum, the stratum granulosum, and the stratum corneum. Among them, the stratum corneum is highly differentiated, being the first to shed off. Keratinocytes (KC)

are the most abundant cell type in the epidermis, accounting for roughly 95% of the whole cell population. Melanocytes, which release melanin and are responsible for skin pigmentation, are also found throughout the epidermis. The dermis is comprised of fibroblasts, which provide tensile strength to the skin tissue (McGrath, Eady, and Pope 2004). Collagens account for 70 to 80% of dermal dry weight.

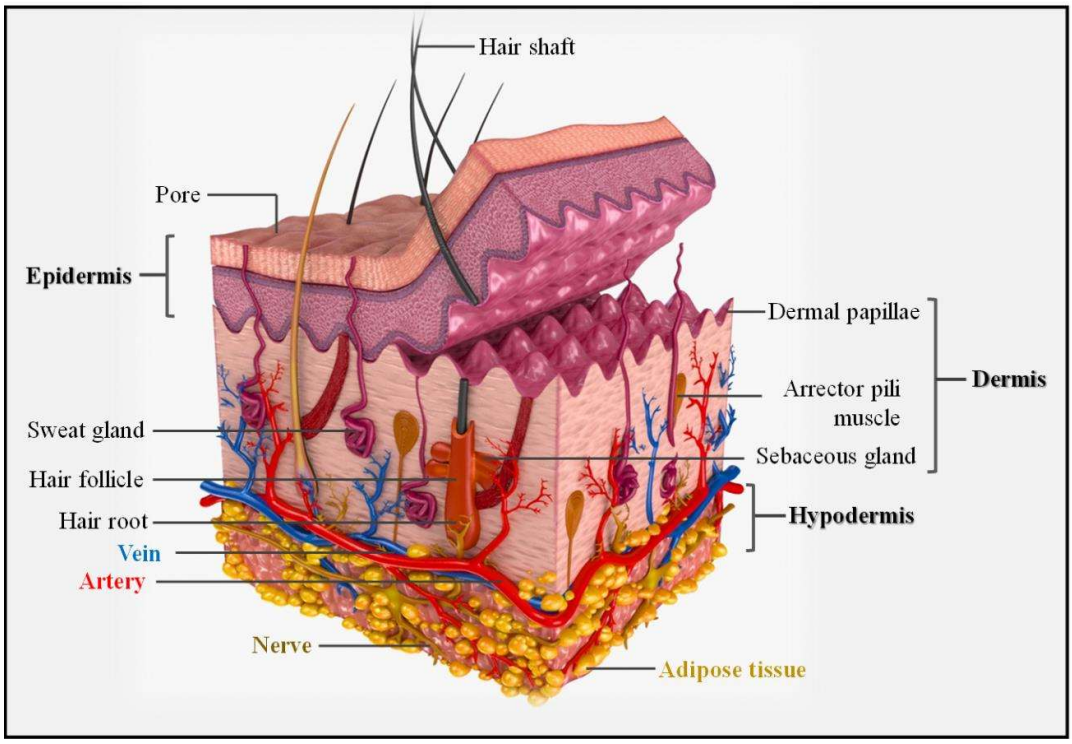


**Figure 1.7** Skin anatomy: skin consist of three main layers, namely- 1) epidermis 2) dermis and 3) hypodermis or subcutaneous layer.

### 1.2.2 Functions

Skin has many functions. It acts as a tough barrier between the human body and the outside environment. The tight arrangement of corneocytes, which are anucleated and flattened cells and lipids in the epidermis's uppermost layer, restrict the water loss from the skin, also called transepidermal water loss (TEWL). The dermal layer is important in wound healing because it produces new collagen and some proteolytic enzymes that are

needed during the remodelling of healed tissue. Furthermore, the subcutaneous layer of vascularized adipose tissue beneath the dermis provides mechanical strength to the skin tissue. It protects against radiation, gases, chemicals, diseases and a variety of other destructive forces. However, if the skin is damaged, it can result in serious complications. It serves as a barrier between the human body and the external environment, preventing pathogens from entering the body. Moreover, the skin regulates a number of body functions, including protection, excretion, temperature regulation, and perception of external stimuli (Metcalfe and Ferguson 2007).

### 1.2.3 Wound healing

Healing is a natural process with four overlapping stages. The first stage is haemostasis, which occurs shortly after wounding and includes blood clotting. The second phase is inflammation, which begins with invading immune cells releasing proteolytic enzymes and proinflammatory cytokines into the wound region, as well as producing reactive oxygen species (ROS) to defend the wound from microbial invasion. Neutrophils and macrophages remove all foreign substances and tissue debris from the wound bed at this stage, preventing infection. Furthermore, the release of cytokines and enzymes promote the formation of fibroblasts and myofibroblasts, and wound exudate provides the moisture required for healing. The third phase is proliferation, which involves the formation of new granulation tissue and the production of new ECM. Remodelling is the fourth and final phase. The matrix composition changes at this stage, and collagen III is replaced by collagen I, resulting in increased tensile strength of new tissues (Figure 1.8).

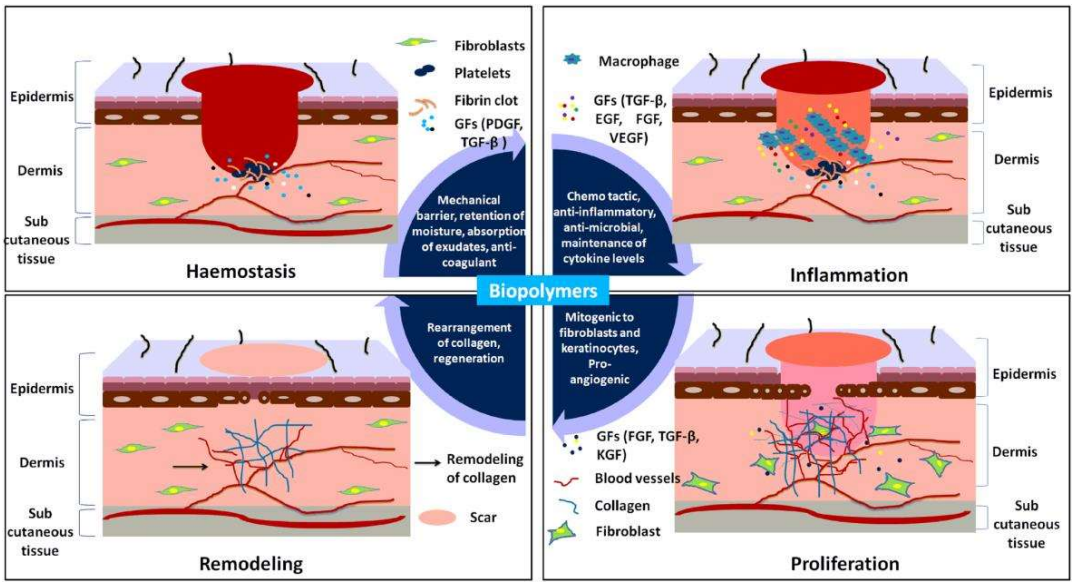


**Figure 1.8** Wound healing phases and the function of biopolymers at each stage (Sahana and Rekha 2018).

According to the depth of a wound, it can be classified into three categories: the first one is the superficial wound, which includes the loss of epidermis; the second is the partial thickness wound, which includes the loss of epidermis and dermis; and the third is full thickness wounds, which includes the loss of epidermis, dermis, subcutaneous fat, and sometimes bone also (Figure 1.9).

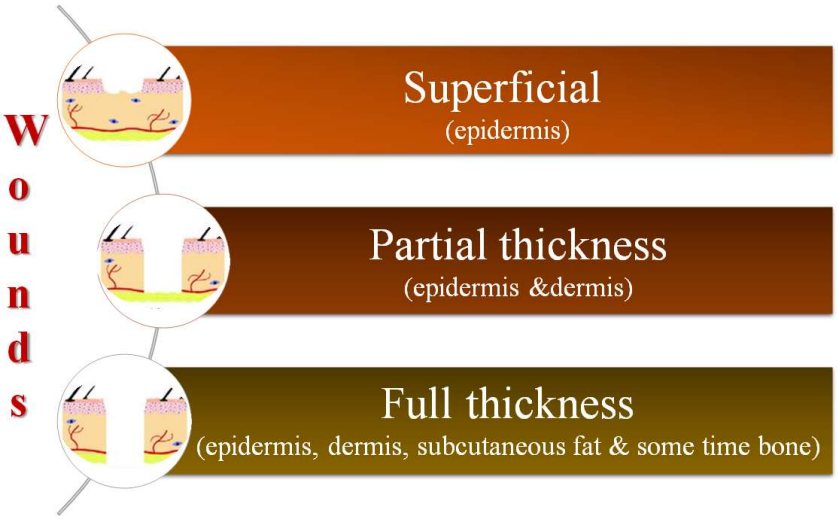


**Figure 1.9** Wounds classification on the basis of wound depth.

### 1.2.4 Wound dressing

Skin wound repair is a complex physiological process that is frequently affected by unknown factors. Because wound healing is slow and susceptible to external infections, wound dressing is required to promote and guide the healing process. An optimal dressing should be biocompatible; capable of preventing water loss to maintain high humidity at the wound site, removing excess exudates, preventing microbial invasion; non-toxic and non-allergic; allowing oxygen exchange to withstand wound hypoxia condition; easy to prepare; flexible in thickness; comfortable; easy to secure and apply; able to provide long-term wound stability and coverage, withstand shear forces; easy to store and have long shelf life. Moreover, biodegradability and capability of recreating dermal and epidermal components are the other desired properties in the wound dressing.

In 1960, British researcher George D. Winter described the benefits of moist wound healing. His research found that moist environments promote wound healing. The ideal wound healing environment is the one that is clean, insulated, safe from further trauma, and, most importantly, moist. However, while moist wound healing is beneficial, an overly moist wound environment is not. This has the potential to cause periwound maceration. Macerated skin is prone to breakdown, which can result in an increase in wound size and a delay in healing. Moist wound healing is thus a delicate balancing act between avoiding maceration and maintaining adequate levels of tissue hydration. There are several advantages of moist wound healing over dry wound healing, including: reduced pain, a physical barrier against further trauma and infection, easier autolytic debridement, increased vapour transmission rate, lower wound infection rate, retention of growth factors at wound site, promotion of dermal/wound bed healing responses (e.g., cell proliferation, ECM synthesis), faster wound healing, reduced scarring, and promotion of epithelialization. As a result, moist wound healing with adequate moisture level is the

best option for complete wound care and quick healing process and hence, has been the focus of this thesis.

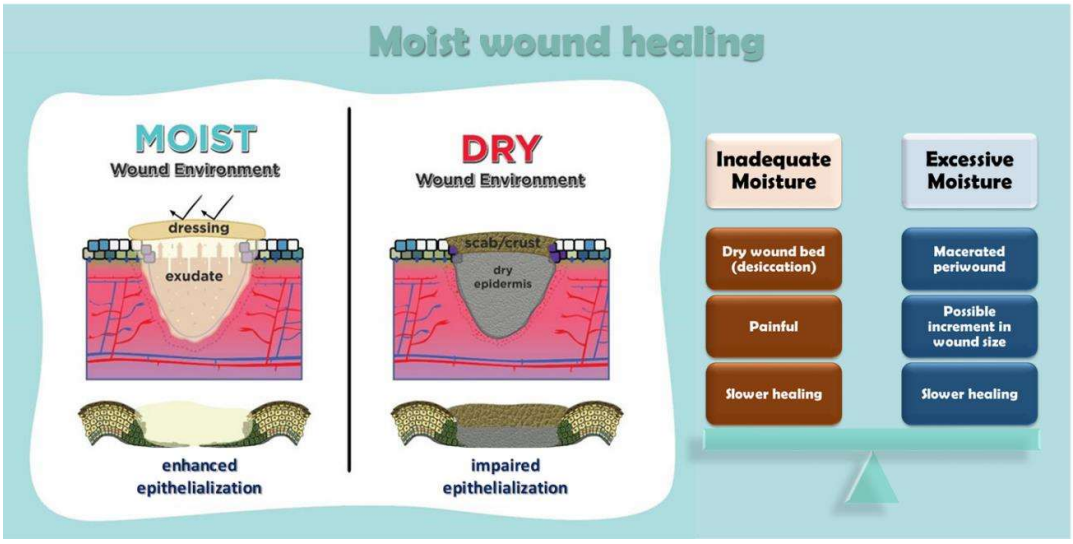


**Figure 1.10** Advantages of moist wound environment over the dry wound environment.

Biopolymers of various origins are among the most widely used bioactive materials for TE applications. Because of their outstanding biocompatibility, capacity to promote cell development, regenerative potential, biodegradability, and durability, biopolymers have a wide range of applications as wound care materials. According to Davison-Kotler et al., skin substitutes can be classified on the basis of 1) replaced region 2) cellularity, 3) permanence, 4) materials used, and 5) layering (Davison-Kotler et al. 2018) (Figure 1.11).

***1) Region replaced:***

This might be either the epidermis or the dermis. The precise layer that needs to be substituted by the skin substitute will influence the number of layers, the materials utilized, and the functionality of the product. Materials such as CEA6 are simple epidermal replacements. When employed alone, they frequently produce negative clinical

results. In contrast, dermal or full-thickness replacements give higher stability, resulting in significantly more successful wound healing and less scar tissue development.

***2) Cellularity:***

Depending on whether or not they have cellular components, the skin substitutes can be either acellular or cellular. The existence of cells in a skin substitute has significant impact on its clinical use, storage, cost, and availability. Cellularized products are also more likely to be rejected by the host, particularly if the cells are not autologous. Production and regulatory complexity are also increased by cellularity.

***3) Permanence:***

Even though the relative durability of a skin substitute is often thought to be important, the actual time it takes for a material to break down is not an adequate method to classify it. Instead, skin substitutes can be classified according to whether they are biodegradable (temporary) or non-biodegradable (permanent). All natural materials like collagen, and other biological proteins, as well as decellularized matrices, are considered to be biodegradable.

***4) Material used:***

Skin substitutes can be made up of natural or synthetic materials. Accordingly, they are put into three groups: natural skin substitutes, which are made from natural biomaterials including proteins (e.g. collagen, gelatin, elastin, fibrin, SF, soy protein), polysaccharides (e.g. hyaluronic acid, alginate, chondroitin sulphate, chitosan) and decellularized matrices; synthetic skin substitutes, which are made from synthetic materials such as polyesters (e.g. PLA, PCL, poly-glycolic acid (PGA)), nylon or polyglactin meshes, and silicone membranes; and composite skin substitutes, which are made from a mix of

natural and synthetic materials to obtain specific biological, chemical, physical and mechanical properties.

***5) Layering:***

Skin substitutes can be single layer or bilayer. Bilayer substitutes replace both the epidermis and dermis components of the skin tissue, while those with a single layer replace only one of them.

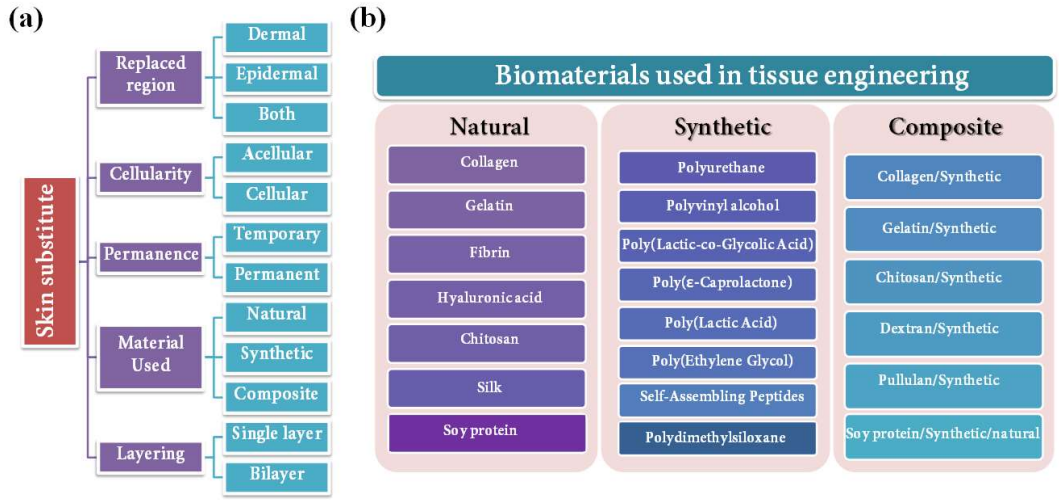


**Figure 1.11 (a)** Classification of skin substitutes on the basis of replaced region, cellularity, permanency, material utilized and layering. **(b)** Some examples of commonly used biomaterials in tissue engineering.

## 1.3 Review of the existing literature

A detailed assessment of the existing works has been conducted in order to present the research findings of this thesis within the context of current literature. Because of the complexity of the dermal layer, injury to this area might result in lifelong impairment of skin function. Deep burns and chronic lesions like diabetic ulcers or pressure sores can cause partial or total skin loss. The problems associated with conventional skin transplants have prompted extensive exploration in skin TE and biomaterials field to generate a wide range of viable skin substitutes (Davison-Kotler et al. 2018).

Developing an appropriate biomaterial that allows cells to grow and proliferate toward the regeneration of new tissues continues to attract significant research efforts worldwide in the TE research community. Several biomaterials, including natural and synthetic polymers, are being used for tissue engineered scaffolds in the literature. Natural polymers are highly attractive owing to their similarities to the chemical diversity as well as outstanding biological performance of the ECM components (Mano et al. 2007). On the contrary, after degradation, residues of synthetic polymers like polyesters can reduce the local pH, triggering cell and tissue necrosis and inflammatory and immunological reactions in the body (H. Liu, Slamovich, and Webster 2006).

Owing to their high abundance in the body, protein-based materials have gained significant interest for a plethora of applications such as TE, controlled drug delivery systems, filtration, and food sector uses (Jao et al. 2017; DeFrates et al. 2018; Varshney et al. 2020). They have molecular structures and biological functions similar to those seen in natural tissue proteins, which aid in promoting the recovery and repair of human tissues and organs. Commonly used proteins such as fibrin, collagen, gelatine, silk fibroin, keratin etc., provide various advantages over synthetic materials such as high biocompatibility, biodegradability, and exhibit excellent cell adhesion properties (Reddy and Yang 2011). Several studies on the use of different protein-based scaffolds for various TE applications have been reported in the literature. In particular, Nagarajan et al. have developed a chemically cross-linked gelatin-based nanofibrous scaffold for bone TE (Nagarajan et al. 2017). In another study, Jiang et al. have developed a shape-memory scaffold based on collagen for cartilage regeneration (Jiang et al. 2018). However, animal-derived proteins have been shown to suffer from drawbacks such as immunogenicity and the potential risk of disease transmission (Cooperman and Michaeli 1984a; Keefe et al. 1992; Cooperman and Michaeli 1984b; DeLustro et al. 1986). Plant-

derived proteins are renewable, abundant, and inexpensive. They are a non-animal-derived alternative to animal-derived proteins, such as collagen. Because of these benefits, plant-derived proteins-based materials are gaining significant interest both in academia and industry for possible applications in wound dressing and as vehicles for drug delivery.

Soy protein isolate (SPI) is a globular plant-origin protein-polymer isolated from soybeans. Due to its remarkable properties, such as high abundance, excellent biocompatibility, biodegradability, and hydrophilicity, it has emerged as a potential alternative to synthetic and animal-derived polymers. It is used in various applications such as edible films, packaging, biomedical, and so on. It also contains RGD (arginine, glycine, aspartic acid) sequences that facilitate cell adhesion (Chatterjee, Gleddie, and Xiao 2018). Additionally, SPI, like keratin, includes leucine, aspartic acid, and valine (LDV), which are also present in fibronectin and other ECM proteins. As a result, SPI, similar to keratin, has the ability to promote cell adhesion and proliferation. In addition, SPI, like gelatin, includes considerable levels of glycine and proline, which have the ability to speed up soft tissue repair and enhance wound healing (Yao et al. 2017). It also contains isoflavones like genistein and daidzein; these phytoestrogens have been proven to enhance wound healing by promoting collagen deposition at the location of the wound. Furthermore, by suppressing oxidative stress in diabetic wounds, genistein can shorten the delay of wound closure and healing (Tie et al. 2013). Furthermore, Shingle et al. have observed that soy protein and poly (ethylene glycol) hydrogel dressings enhance wound healing in partial and full thickness wounds due to the anti-inflammatory activity of soy protein isoflavones (Shingel et al. 2006).

Silk fibroin (SF) is one of the most versatile naturally occurring biopolymers. This polymer has been utilized in various fields including food, cosmetics, pharmaceutical,

and biomedical applications (Carrasco-Torres et al. 2019; Marcolin et al. 2017; Catto et al. 2015; Alessandrino et al. 2008). It is a highly abundant natural protein fiber produced by larvae of silkworm to form cocoons. It constitutes 25-30% sericin and 70-75% fibroin proteins. The biocompatibility, low immunogenicity, adequate permeability to oxygen and water vapor, non-toxicity, low cost as well as its robust mechanical properties make SF a biomaterial of choice for biomedical applications (Dadras Chomachayi et al. 2018; Marelli et al. 2012). SF-based electrospun scaffolds have received a lot of interest as wound dressing materials because they induce greater collagen spreading and cell attachment on their surface than a basic SF film (Min et al. 2004). SF is highly beneficial in wound dressings and skin substitutes due to its great biocompatibility, low inflammatory response, and high flexibility (Mogoşanu and Grumezescu 2014). SF-based scaffolds including nanofibers, sponges, and porous SF films or membranes have demonstrated encouraging outcomes in skin tissue regeneration and wound healing. According to Liu et al., SF film has no negative effect on the proliferation and functioning of vascular endothelial and fibroblasts cells. As a result, SF can be a good biomaterial with high biocompatibility (T. Liu et al. 2010). However, its use in TE is limited because of its very slow mass loss rate. Therefore, several studies have been conducted to improve the properties of SF such as degradation, cell attachment, etc. by blending it with other polymers, for example, chitin (Park et al. 2006), elastin (Vasconcelos, Gomes, and Cavaco-Paulo 2012), gelatin (Yin-Guibo et al. 2009) and poly(lactide-co-glycolic acid) (Shahverdi et al. 2014).

PVA is one of the most commonly used synthetic polymers capable of forming hydrogel through various methods such as physical and chemical crosslinking. PVA has various properties like biodegradability, stability, mechanical strength, biocompatibility, hydrophilicity etc. and it is widely used in several biomedical applications. However, the

suitability of pure PVA for cell adhesion and protein adsorption is still debatable (S. Gupta, Webster, and Sinha 2011; Nuttelman, Henry, and Anseth 2002). Therefore, as a scaffold biomaterial for TE applications, PVA is generally used in combination with natural polymers such as gelatin (Choi et al. 2013), chitosan (Vrana et al. 2008), starch (Bursali et al. 2011) etc.

In literature, PVA hydrogels fabricated through the freeze-thaw process have been studied by various researchers. In particular, Fathi et al. have fabricated the PVA and dextran blend hydrogel using repeated freeze-thaw cycles (Fathi et al. 2011). Vrana et al. have prepared elastic PVA/chitosan hydrogels for vascular tissue engineering by employing freeze thaw cycles (Vrana et al. 2008). Cho et al. have developed a hybrid alginate/PVA porous scaffold to enhance the cellular compatibility of the PVA based scaffold (Cho, Oh, and Lee 2005). Testing of polyvinyl alcohol-tetraethylorthosilicate-alginate-calcium oxide (PTAC) biocomposite cryogels as healing aids for critical-sized cranial bone lesions in Wistar rats for 4 weeks has been reported by Mishra et al. This study's findings highlight the importance of cryogels in bone repair and even osteoblastic development (Mishra et al. 2014).

Because of the difficulties caused by co-morbidities like diabetes or infection, the wound has become one of the primary causes of concern over the patient's health. According to the numbers provided by the WHO, there are 58 million individuals who are impacted by fatal injuries, among whom about 4.4 million people pass away annually. In addition, there are still several million individuals who require treatment and care that is adequate (“Injuries and Violence” 2022). If we look at the national data, for every thousand people, there are approximately five people who are suffering from chronic wounds (N. Gupta et al. 2004). This puts a lot of burden on the healthcare economy. Even though more than 3000 different products have been produced to heal wounds, it is still a burden for

healthcare community as a whole as well as on the individual (Sahana and Rekha 2018). Moreover, to date, no single material has been referred to as an ideal biomaterial that can be employed as a skin substitute. Thus, demand for less expensive products for skin tissue engineering remains high. Furthermore, the fabrication of an ideal tissue-engineered skin dressing is still an open problem, and many efforts have been made over the past many years to investigate a range of biomaterials that could be potentially useful for designing such constructs. Hence, this thesis attempts to develop cost-effective, tissue-engineered scaffolds for skin wound healing applications by exploring different biomaterials and techniques. The research objectives of this thesis are detailed next.

### 1.4 Research objectives

The primary aim of this thesis is to develop novel, acellular, polymer-based scaffolds for skin tissue engineering and wound healing applications. Several sub-goals must be met in order to achieve this. As a result, the objectives have been divided into the following sections.

1. Fabrication and Characterization of SF- Soy protein based Nanofibrous Scaffolds for Skin Tissue Engineering.
    1.1. Optimization of electrospinning parameters and polymer concentration.
    1.2. Fabrication of SF and SPI nanofibrous scaffolds with a range of formulations using electrospinning technique.
    1.3. Physio-chemical characterization of the fabricated nanofibrous scaffolds.
    1.4. Biocompatibility evaluation of fabricated nanofibrous scaffolds.
    1.5. Evaluation of the applicability of the fabricated nanofibrous scaffolds as a wound dressing material by conducting in vivo wound healing assay.

2. Fabrication and Characterization of PVA- Soy Protein Composite Hydrogels for Skin Tissue Engineering.
    2.1. Fabrication of PVA and SPI composite hydrogels with a range of formulations using freeze-thaw technique.
    2.2. Physio-chemical characterization of the fabricated composite hydrogel scaffolds.
    2.3. Biocompatibility evaluation of obtained composite hydrogel scaffolds.
    2.4. Evaluation of applicability of fabricated composite hydrogel scaffolds as a wound dressing material by conducting in vivo wound healing assay.
3. Fabrication and Characterization of Highly Porous Soy protein Cryogel for Skin Tissue Engineering.
    3.1. Studying the effect of polymer concentration in cryogelation of GA cross-linked SPI cryogels.
        3.1.1. Selecting and optimizing the polymer and cross-linker concentration.
        3.1.2. Fabrication of SPI cryogels using cryogelation technique.
        3.1.3. Physio-chemical characterization of the fabricated SPI cryogels scaffolds.
    3.2. Biocompatibility evaluation of obtained SPI cryogel scaffolds.
    3.3. Hemocompatibility evaluation of obtained SPI cryogel scaffolds.
    3.4. Evaluation of applicability of fabricated SPI cryogel scaffolds as a wound dressing material by conducting in vivo wound healing assay.
4. Fabrication and Characterization of Macroporous PDMS based Scaffold for Tissue Engineering.
    4.1. Fabrication of PDMS porous scaffolds using salt-leaching method.
    4.2. Physio-chemical characterization of the fabricated PDMS porous scaffolds.
    4.3. Biocompatibility evaluation of obtained PDMS porous scaffolds.

## 1.5 Thesis outline

This thesis is organised into six chapters. Chapter 1 (the present chapter) provides a general overview of TE and its different approaches to regenerate damaged tissue by developing complex living constructs as functional substitutes. This chapter also includes a comprehensive review of the enabling technologies used in the development of 3D scaffolds for TE applications. Furthermore, a brief description of skin tissue, including its anatomical structure, basic skin layers and functions, has been presented in this chapter. This chapter also explains the research objectives of this thesis in terms of exploring different polymers, both natural and synthetic, for the development of functional 3D scaffolds.

Chapter 2[1] of the thesis describes the fabrication of SPI and SF blend nanofibrous scaffolds using an electrospinning technique. This chapter also demonstrates the effect of ethanol (EtOH) vapour treatment on the obtained nanofibers. By scanning electron microscopy (SEM), nanofibrous scaffolds have been characterised for their morphology and microstructure. ATR-FTIR and Raman spectroscopy analysis have been carried out to investigate the molecular structure and interactions. Thermal stability and degradation have also been studied to see the effect of the mixing of these two polymers and of the EtOH vapour treatment. An in vitro degradation study, MTT assay, and long-term cell culture have been performed to analyse the potential of the electrospun nanofibers for tissue regeneration. An in vivo wound healing study has also been performed to evaluate the potency of the electrospun nanofibers as a wound dressing. A graphical representation of this chapter is presented in Figure 1.12.

[1] Varshney, N., Sahi, A.K., Poddar, S. and Mahto, S.K., 2020. Soy protein isolate supplemented silk fibroin nanofibers for skin tissue regeneration: Fabrication and characterization. *International Journal of Biological Macromolecules*, 160, pp.112-127.

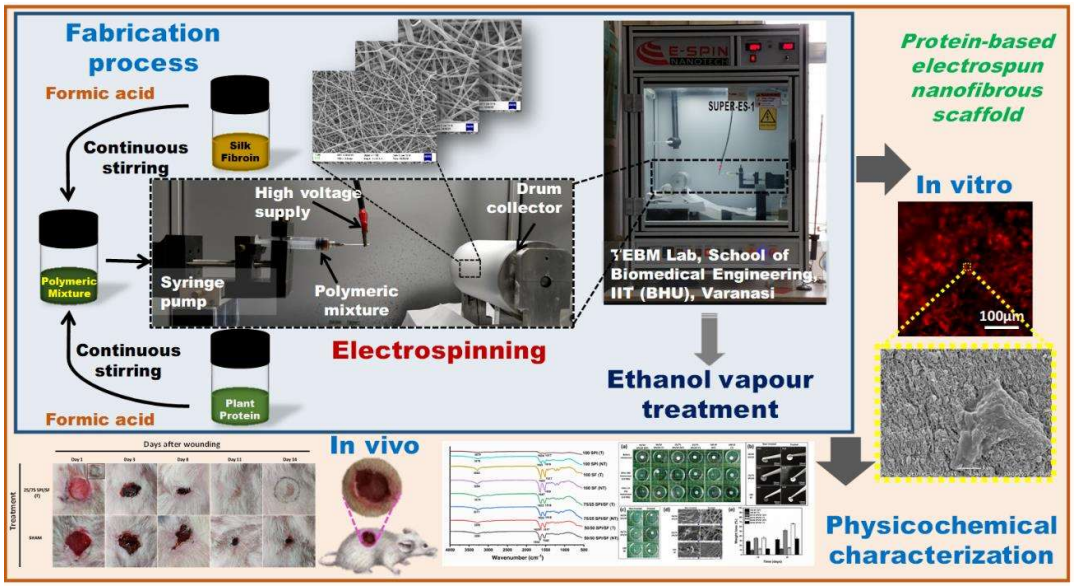


**Figure 1.12.** Schematic representation of Chapter 2 (Fabrication and Characterization of SF- Soy protein based Nanofibrous Scaffolds for Skin Tissue Engineering).

Chapter 3[2] of the thesis focuses on the development of physically cross-linked SPI and PVA based composite scaffolds by employing the facile freeze-thaw technique. The fabricated scaffolds have been characterised for their morphology and microstructure analysis by SEM. ATR-FTIR and XRD analysis have been performed to study the molecular interactions of the formed structures. TGA and DSC analysis have been performed to investigate the thermal properties of the obtained scaffolds. Mechanical testing is carried out on the scaffolds to validate the strength and extent of cross-linking of the fabricated scaffolds. Porosity, swelling study, in vitro degradation study, MTT assay, and long-term cell culture have been performed to analyse the potential of the fabricated scaffolds for tissue regeneration. An in vivo wound healing study has been

[2] Varshney, N., Sahi, A.K., Poddar, S., Vishwakarma, N.K., Kavimandan, G., Prakash, A. and Mahto, S.K., 2022. Freeze–Thaw-Induced Physically Cross-linked Superabsorbent Polyvinyl Alcohol/Soy Protein Isolate Hydrogels for Skin Wound Dressing: In Vitro and In Vivo Characterization. *ACS Applied Materials & Interfaces*, 14(12), pp.14033-14048.

performed to evaluate the potency of the scaffolds as a wound dressing. A graphical representation of this chapter is presented in Figure 1.13.

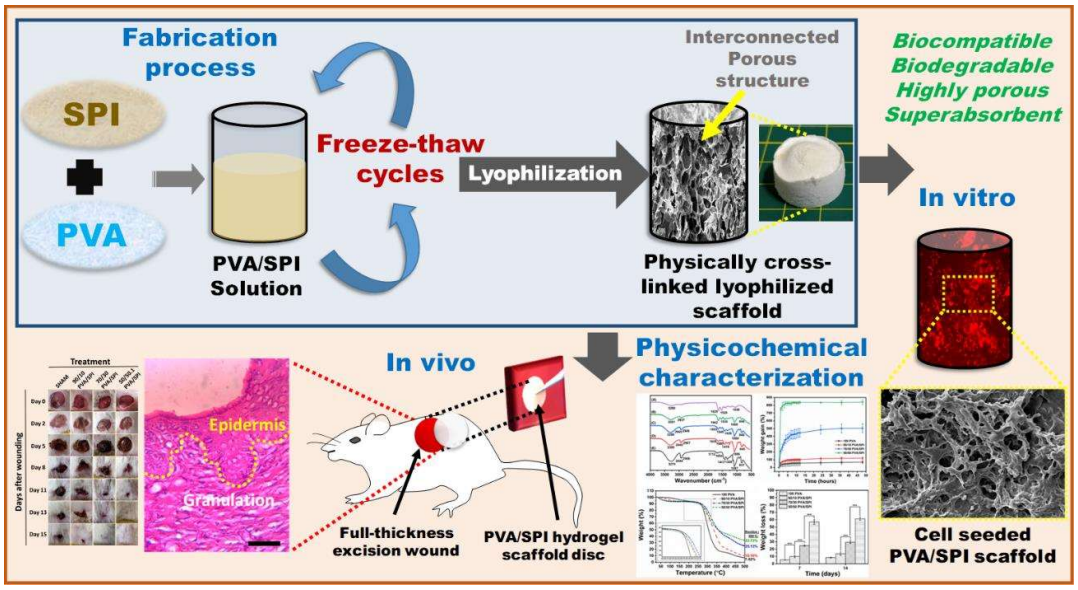


**Figure 1.13.** Schematic representation of Chapter 3 (Fabrication and Characterization of PVA- Soy Protein Composite Hydrogels for Skin Tissue Engineering).

Fabrication of chemically cross-linked superporous SPI scaffolds using the facile cryogelation technique has been presented in Chapter 4[3]. The fabricated scaffolds have been characterised for their morphology and microstructure analysis by SEM. An ATR-FTIR analysis has been performed to study the molecular interactions of the formed structures. TGA and DSC analyses have also been performed to investigate the thermal stability. Mechanical testing of the scaffolds has been performed to validate the strength and shape recovery property of the prepared scaffolds. Porosity, swelling study, MTT assay, and long-term cell culture have been performed to analyse the potential of the

[3] Varshney, N., Singh, P., Rai, R., Vishwakarma, N.K., and Mahto, S.K., 2022. Chemically Cross-linked Superporous Soy Protein Isolate Cryogels for Skin Wound Dressing: In Vitro and In Vivo Characterization. *(Under preparation for possible publication in an international research journal).*

fabricated scaffolds for tissue regeneration. Furthermore, the hemocompatibility of the prepared scaffolds has also been investigated. An in vivo wound healing experiment has been carried out to assess the scaffolds' efficacy as wound dressing. A graphical representation of this chapter is presented in Figure 1.14.

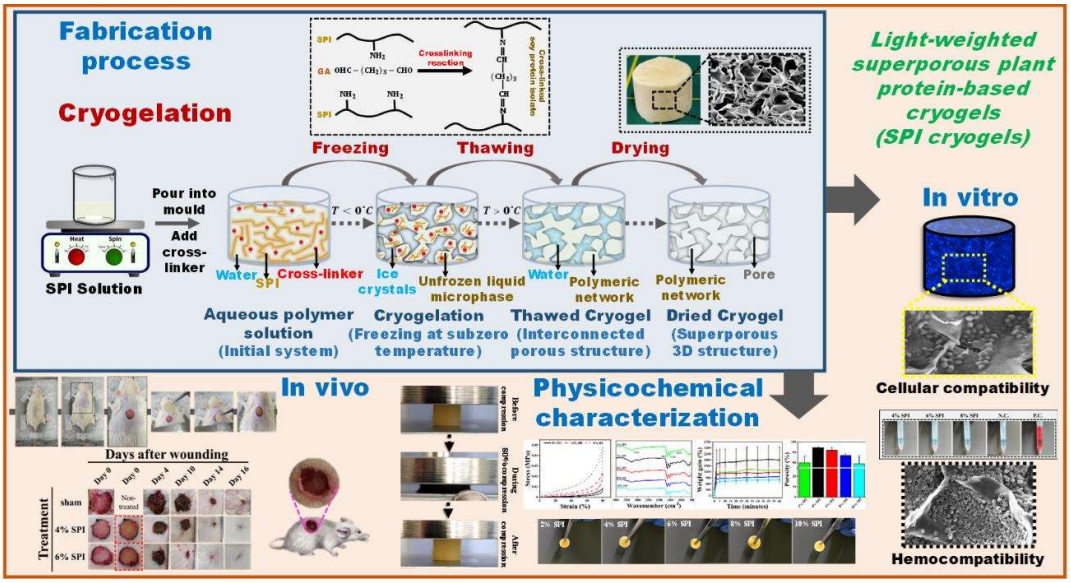


**Figure 1.14.** Schematic representation of Chapter 4 (Preparation and Characterization of Highly Porous Soy Protein Cryogels for Skin Tissue Engineering).

In Chapter 5[4], the salt-leaching method has been used to fabricate porous PDMS scaffolds with varying porosities. SEM has been used to characterize the morphology and microstructure of the fabricated salt leached PDMS scaffolds. Porosity, pore interconnectivity, liquid retention study, mechanical strength, surface wettability, MTT assay, and long-term cell culture was employed to assess the PDMS porous scaffold's potential for tissue regeneration. A graphical representation of this chapter is presented in Figure 1.15.

[4] Varshney, N., Sahi, A.K., Vajanthri, K.Y., Poddar, S., Balavigneswaran, C.K., Prabhakar, A., Rao, V. and Mahto, S.K., 2019. Culturing melanocytes and fibroblasts within three-dimensional macroporous PDMS scaffolds: towards skin dressing material. Cytotechnology, 71(1), pp.287-303.

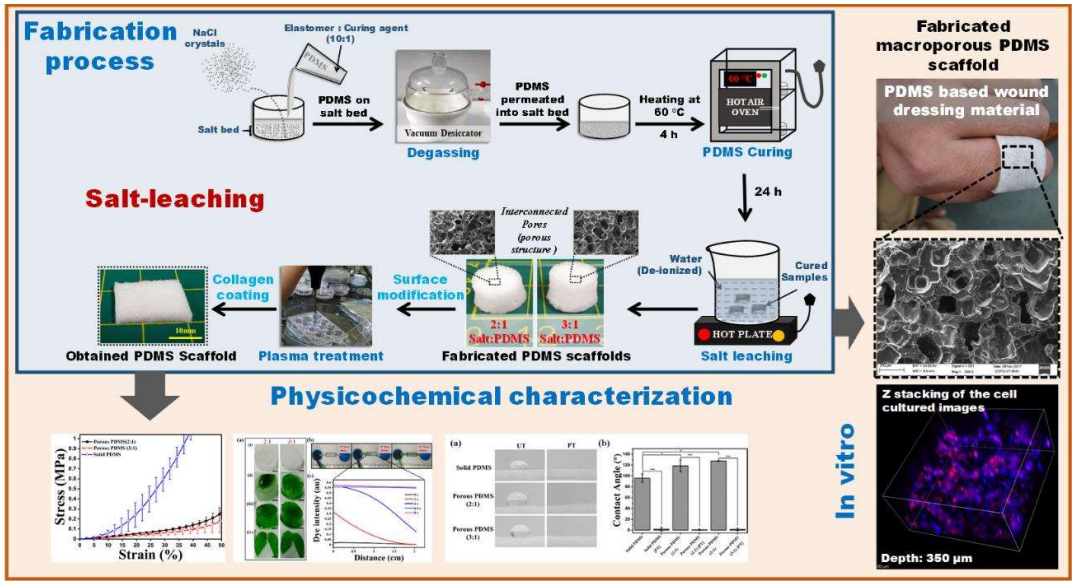


**Figure 1.15.** Schematic representation of Chapter 5 (Fabrication and Characterization of Macroporous PDMS based Scaffolds for Tissue Engineering).

Finally, Chapter 6 concludes the thesis' principal findings and future scope of the study. Overall, all the studies performed in this thesis evidence that the fabricated scaffolds have the potential to improve the wound healing process.

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