FABRICATION AND CHARACTERIZATION OF POROUS POLYURETHANE
SCAFFOLD FOR APPLICATION IN THE FIELD OF TISSUE ENGINEERING

A Thesis

Presented to

The Graduate Faculty of The University of Akron

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

Manisha Shah

August, 2008
FABRICATION AND CHARACTERIZATION OF POROUS POLYURETHANE SCAFFOLD FOR APPLICATION IN THE FIELD OF TISSUE ENGINEERING

Manisha Shah

Thesis

Approved:  
Advisor  
Dr. Stephanie T. Lopina  
Committee Member  
Dr. Daniel B. Sheffer  
Committee Member  
Dr. Glen O. Njus  

Accepted:  
Department Chair  
Dr. Daniel Sheffer  
Dean of the College  
Dr. George K. Haritos  
Dean of the Graduate School  
Dr. George R. Newkome  

Date
In tissue engineering, a highly porous artificial extracellular matrix or scaffold is required to accommodate mammalian cells and guide their growth and tissue regeneration in three dimensions. Successful tissue engineering of soft tissue largely depends on synthetic scaffolds that support the survival, proliferation and differentiation of seeded cells. In this investigation of the use of L-tyrosine based polyurethane in soft tissue engineering, three dimensional and 90% porous biodegradable polyurethane scaffolds with highly interconnected pore structure were fabricated by solvent casting and particulate leaching technique. Scaffolds were fabricated using ground and sieved sodium chloride particles. The grinding and sieving of sodium chloride resulted in particulates of uniform particle sizes but irregular shapes. Biodegradable L-tyrosine polyurethane scaffolds fabricated from these particulates had highly interconnected channels and the pores size that could allow cellular infiltration and nutrient delivery. The scaffolds had anisotropic pore structure with pore diameter ranging from 144–250 μm in diameter. Studies were conducted to investigate the effect of sodium chloride particulates on scaffold porosity and mean pore diameter.

The study showed that scaffolds made using solvent casting and particulate leaching techniques demonstrated independent control of porosity and mean pore diameter of scaffold. Compression testing demonstrated mechanical anisotropy concomitant with the direction of the macro-pores. The porous architecture of these scaffolds reflected the
mechanical anisotropy which was congruent with the scanning electron microscopy investigation.

The results of this study showed that solvent casting and particulate leached (SPCL) L-tyrosine polyurethane scaffolds have great potential for use as a biodegradable tissue culture support devices. It is believed that, the porous polyurethane scaffold developed in this study will facilitate the construction of an implantable tissue engineered skin.
DEDICATION

This Thesis is dedicated to my parents and teachers
ACKNOWLEDGEMENTS

At the conclusion of my thesis it is very important for me to acknowledge that there have been many people along the way who have enriched and made this work possible. The nature of my research and thesis has been highly collaborative so there are a multitude of people, facilities, and resources that have made significant contributions.

I first wish to express my deepest gratitude to Prof. S.T Lopina, who has served as my advisor, master’s thesis committee chair, and mentor. She gave me the resources and freedom to explore this research topic and guided the development of my experimental, theoretical and analytical skills. Her experience, insight, and guidance were critical components of my development as an engineer and academic and I truly admire her positive energy and admiration and dedication towards research. I also appreciate her commitment she made to my intellectual and academic maturation.

I would also like to thank Prof. D.B Sheffer who served as the advisor of my master’s thesis and constantly helped me during statistical analysis of this study. The author would also like thank Dr. Newby and Dr. Chase, Department of Chemical and Biomolecular engineering for providing facilities to conduct contact angle measurement and sieve analysis. Additionally, I would like to thank Dr. Njus who served as the co-
advisor of my master’s thesis and for helping me with his immense knowledge in field of biomechanics.

I owe my special thanks to Senthilram Subramanian and Peter for their valuable friendly conversations, technical assistance, and invaluable support throughout my master’s thesis.

Most of all, I want to thank Rahul Patel, for being counselor and cheerleader. Thank you for your support, kindness, friendship and most of all, for standing by me in all odds. I would also like to thank Sheela Bandarkar for hours of fun and other adventure we had together.

I would also like to thank the members of the Biomaterials lab for their friendly conversations and Dr. S.C Jana (Chair of Polymer Engineering) and Sarah Thorley for their valuable suggestions.
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CHAPTER I
INTRODUCTION

The loss or failure of organ or tissue is one of the severe problems in the field of medical science [1, 2]. Although organ transplantation is considered as a standard therapy for organ or tissue loss, some of the challenges with this therapy are failure of transplanted organ or tissue, foreign body response, infections due to immunosuppression [3]. Over 95,000 US patients are currently waiting for an organ transplant; nearly 4000 new patients are added every year [4]. Other treatment alternatives for organ loss are surgical reconstruction and artificial prosthesis. Although these therapies are not donor limited as in transplantation, they are inefficient in replacing all functions of a lost organ or tissue.

Tissue engineering is a multidisciplinary field that combines the principles of engineering and life science in order to generate a biological substitute that can improve, restore, and maintain tissue function. This approach promises to be a better alternative in comparison to the above mentioned conventional approaches. One of the advantages of tissue engineering is lower chance of tissue rejection as the donor can be the patient himself / herself. This technique also overcomes other challenges such as immunosuppression, infection and remodeling. Therefore, over a period tissue engineering has emerged as a promising technique to treat loss or malfunctioning of an organ compared to other therapeutic techniques like organ transplantation and artificial
prosthesis. Tissue engineering is a field of tremendous promise, but one which requires a great deal of development in transition from demonstration-scale applications to commercial application.

One of the approaches in tissue engineering is scaffold-guided tissue regeneration. In this approach the tissue engineered scaffold acts as a porous three dimensional matrix that provides culturing cell with an artificial extracellular matrix so as to guide the cell’s growth and tissue regeneration. The porous structure of the scaffold also provides adequate nutrient movement, waste removal and gas diffusion, thereby mimicking an extracellular matrix function closely[5, 6]. Tissue engineering already has success in generating materials for repair of chronic wounds. Materials scientists and scaffold design engineers have the responsibility to create a scaffold, which can direct the arrangement of cells into the appropriate 3-D structure and provide the necessary molecular signals to make the cells form the desired tissue. In order to meet the goal of engineering organs and improving organ function further investigations into cell-material interactions are needed to determine which scaffold properties affects certain cellular behaviors.

Several natural polymers such as polysaccharides (e.g. cellulose, chitin) and proteins such as (collagen, elastin, fibrin) [2] have been used in field of tissue engineering. The common advantages of natural polymers are their biocompatibility and non toxicity while some of the disadvantages associated with natural polymers are reproducibility and mechanical strength. Several synthetic polymers such as poly (glycolide) (PGA), poly(lactide) (PLA), poly (lactide co glycolide) (PLGA) have been used in field of tissue engineering [2]. In contrast to natural polymer they show some promising
properties such as large scale production and reproducibility, while the major drawbacks related with them are inflammatory response, adverse effect on polymer erosion and limitation in structural modifications [7].

Polyurethanes are block copolymers consisting of aromatic or aliphatic isocyanates hard segments, and, polyester or polyether, soft segments. These polymers have unmatched combinations of physical, chemical and biological properties. Depending on the ratio of soft segments and hard segments, and molecular weight of the segments, one can vary the hardness, elastic modulus and many other properties [8]. Polyurethanes are extensively used biomaterials as by tuning its structure, engineers get wide range of properties.

Amino acid based synthetic polymers also named as poly-peptide polymer are developed and studied as biomaterials due to their biocompatibility and/or biodegradability. Polypeptides composed of amino acids, should have enhanced the biocompatibility over purely synthetic polymers for biomedical applications. However, studies also show that polypeptide polymers show certain disadvantages such as insolubility in common solvents, lack of moldability and lack of easy processability. To overcome these processing problems researchers modified the peptide bond of polypeptide polymer by replacing it with non peptide bond such as urethane linkage and therefore a new class of polymer has been developed which along with properties such as biocompatibility and/or biodegradability shows desirable physico-chemical and structural properties. In our laboratory setup, pseudo-polypeptide polyurethane has been developed. The use of amino acid (L-tyrosine) in our polyurethane offer advantage by
combining the desirable properties of commercially used polyurethanes (strength, processability) with desirable properties of polypeptides (biocompatibility, biodegradability) [7]. The goal of this project is to develop porous L-tyrosine based polyurethane biodegradable scaffold for application field of soft tissue engineering.

1.1 Objectives:

The primary objective of this project was to fabricate a porous polyurethane scaffold that would be used as an artificial three dimensional matrix for tissue engineering application. The L-tyrosine based polyurethane (PCL-HDI-DTH) developed in our laboratory was used to develop the scaffolds. Fabricated scaffold was characterized for:

1. **Pore Morphology**: The surface and pore morphology of scaffolds was observed using S.E.M photomicrographs.

2. **Pore Size**: The mean pore sizes of the scaffolds was calculated by analyzing the SEM pictures of different scaffolds having different salt weight fractions, using an image analysis software. A relation between the scaffold’s salt weight fraction and pore size was determined. Also, a relation between salt particle size and mean pore size was determined.

3. **Porosity Measurement**: The porosity of scaffolds was measured using the Archimedes’s liquid displacement principle. A relation between porosities and salt weight fraction was determined.
4. **Mechanical Characterization:** The compressive modulus for the porous scaffold was measured to characterize mechanical characteristics of the porous scaffold.

1.2 Hypothesis:

**Null Hypothesis I:** A biodegradable scaffold with a porosity of 88% can be achieved using the L-tyrosine based polyurethane.

$$H_{lo}: \mu = 0.88 \ (\text{Null Hypothesis})$$

**Alternate Hypothesis I:** A biodegradable scaffold with a porosity of 88% can’t be achieved using the L-tyrosine based polyurethane.

$$H_{la}: \mu < 0.88 \ (\text{Alternate Hypothesis})$$

where, $\mu$ is the mean of measured porosity for scaffolds.

**Null Hypothesis II:** There is significant difference between the mean pore size of scaffolds with salt weight fraction 0.7, 0.8 and 0.9.

$$H_{IIo}: \mu_{0.7} \neq \mu_{0.8} \neq \mu_{0.9} \ (\text{Null Hypothesis})$$

**Alternate Hypothesis II:** There is no a significant difference between the mean pore size of scaffolds with salt weight fraction 0.7, 0.8 and 0.9.

$$H_{IIa}: \mu_{0.7} = \mu_{0.8} = \mu_{0.9} \ (\text{Alternate Hypothesis})$$
where, $\mu_{0.7}, \mu_{0.8}, \mu_{0.9}$ are the mean pore size for each scaffolds having salt weight fraction as 0.7, 0.8 and 0.9 respectively.

**Null Hypothesis III**: There is significant difference between the mean pore size of scaffolds based on salt particle size (250 - 176 and 177-140 microns).

\[ H_{IIIo}: \mu_{250-176} \neq \mu_{177-140} \text{(Null Hypothesis)} \]

**Alternate Hypothesis III**: There is no a significant difference between the mean pore size of scaffolds based on two salt particle size (250 - 176 microns and 177-140 microns).

\[ H_{IIIa}: \mu_{250-176} = \mu_{177-140} \text{(Alternate Hypothesis)} \]

where, $\mu_{250-177}$ and $\mu_{177-140}$ are the mean pore size for each scaffolds having salt particles size as 250 - 176 microns and 177 - 140 microns.

**Null Hypothesis IV**: Salt weight fractions and porosities of scaffolds are not significantly related.

\[ H_{IVo}: b = 0 \text{ (Null Hypothesis)} \]

Model I: $Y = \alpha + \beta X + \epsilon$

where, $Y$ represents the dependent variable porosity and $X$ represents independent variable salt weight fraction, $\epsilon$ is assumed to be a normally distributed error term with a mean of zero, $b$ is the regression coefficient.
Alternate Hypothesis IV: Salt weight fractions and porosities of scaffolds are significantly related.

\[ H_{IVa}: b \neq 0 \text{ (Alternate Hypothesis)} \]

Research Hypothesis: The mechanical characteristics of porous tissue scaffolds are important for their biomechanical tissue engineering application. Therefore, it is hypothesized that the PCL-HDI-DTH porous scaffolds fabricated using solvent casting and particulate leaching techniques possess adequate mechanical characteristics to support cell growth and therefore can be potentially be used for soft tissue engineering.
2.1 Methods to treat organ loss function

Every year millions of surgical procedures are performed that require tissue substitute to repair or replace diseased or damaged tissues. Some of the approaches that restore some level of functionality to a damaged tissue or organ are transplantation, autografting, implantation of a permanent prosthetic device, use of stem cells, and in vitro synthesis of organs and are discussed below.

2.1.1 Organ Transplantation

Organ transplantation is widely used to replace complex tissues and organs, but is limited by two significant factors. A significant challenge with organ transplantation is the immunological barrier between donor and host. After transplantation, the donor organ is attacked by immune system and rejected by the host’s immune system. The primary clinical measure to avoid such rejection is the use of immunosuppressive drugs for the remaining of the host’s life to suppress the immune system.
However, immunosuppressions can make the host vulnerable to infection [9]. While the second major obstacle is the difficulty in finding immunocompatible donors and the shortness of supply of suitable organs [9]. For example, in 2005, there were a total of 28,111 organ transplants performed; but a total of 6,369 patients died while waiting for suitable donor organs. [10, 11]

2.1.2 Autografting

Autografting involves harvesting a tissue from one location in the patient and transplanting it into another part of the same patient. Autologous grafts usually produce the best clinical results since rejection is not an issue [10]. Example of a commonly used autografting procedure is coronary bypass i.e., veins grafts are removed from the leg (saphenous vein), and then transplanted to the heart as a conduit for blood flow around blocked coronary arteries. However, autografting, has several problems including additional surgical costs for the harvesting procedure, and infection and pain at the harvesting site. For example, of the 300,000 coronary bypasses / year and harvesting of a saphenous vein, 20% report wound infection and significant post operative pain in the leg. [10].

2.1.3 Artificial prosthesis and devices:

Engineers and scientists have tried to create biomimetic devices and materials to replicate, augment or extend functions performed by biological systems. Examples range from artificial hearts and valves to prosthetic hip and breast implants. Many of
these systems have had an enormous positive impact. The materials used in these therapies, however, are subject to fatigue, fracture, toxicity, inflammation, wear, and do not remodel with time (i.e., a metal bone implant can not grow with the patient and it cannot reshape in response to the loads placed upon the implant ). Also, they do not behave physiologically like true organs or tissues. Thus, devices like an artificial heart (or left ventricular assist device) are best suited as temporary solutions until a donor organ becomes available [10]. While all these therapies have a significant medical impact, some of the newer technologies like tissue engineering can seek to overcome the limitations of these conventional approaches.

2.1.4 Stem Cells:

The self – renew state of stem cells, and the ability to differentiate into many specialized cells offers multiple of therapeutic opportunities [12]. For many patients with extensive end -stage organ, a tissue biopsy may not yield enough normal cells for expansion and transplantation. In other instances, primary autologus human cells can not be expanded from a particular organ, such as pancreas. In these situation, pluripotent human embryonic cells serve as an alternative source of cells from which the desired tissue can be derived[ 13]. Human embryonic stem cells have been shown to differentiate into cells from all three embryonic germ layers in vitro. Skin and neurons have been formed, indicating ectodermal differentiation. Blood, cardiac cells, cartilage, endothelial cells, and muscle have been formed, indicating mesodermal differentiation. And pancreatic cells have been formed, indicating endodermal differentiation [13] Stem
cell technologies present a great deal of promise. However, improved understanding of stem cell behavior and development of stem – cell based technologies also raise a number of important ethical issues [14], consideration of which will play a significant role in development of stem cell – based tissue engineering solutions.

2.1.5 Induced In Vivo Organ Synthesis (Induced Regeneration)

Induced regeneration refers to recovery of the non-regenerative organ of the adult mammal. Organ regeneration is distinct from organ repair as an endpoint of a healing process following injury. Repair is physiological adaptation to loss of normal organ mass and leads to restoration of the interrupted continuity by synthesis of a scar tissue without recovery of the uninjured tissues. In contrast, regeneration restores the interrupted continuity by synthesis of the missing organ at the original site, yielding a regenerate organ. Regeneration restores the normal structure and function of the organ; repair does not. Induced organ regeneration is de novo synthesis of a physiological or nearly physiological, organ at the same anatomical site as the organ that is being replaced [14]. Being process of de novo synthesis it makes use of elementary reactants. These comprise cells of various types, soluble regulators (growth factors), cytokines or soluble signaling molecules and insoluble regulators of cell function (matrices or scaffolds). The adult organism responds spontaneously to a severe injury by making a healing response that spares the organism but condemns the injured organ [15]. Templates appear to function by interfering with normal myofibroblast function as
well by acting as temporary structure that guides cells for synthesis of new stroma that resembles that of the organ under replacement.

Induced organ synthesis *in vivo* relies on the processes inherently active in the wound site to regenerate lost or damaged tissue. A highly porous analog to ECM termed as scaffold, is utilized to induce regeneration at a wound site where the organism would normally respond via repair processes. Induced organ synthesis was made possible by the development of fabrication techniques to produce ECM analog with well defined pore microstructure, specific surface area, chemical composition, and degradation rate.[14]. Its first application was the use of a collagen-glycoaminoglycan, (CG) scaffold (termed dermal regeneration template- DRT) that induced skin regeneration following severe injury. The DRT displays high biological activity when implanted into a full thickness skin wound and was capable of inducing regeneration of the underlying dermal layer of skin, as well as the epidermal and basement layer [16].

2.2 Scaffold guided Tissue engineering

Many methods of organ replacement have been developed and are used successfully everyday, most of these techniques are imperfect, as they can not restore full organ function. Some of the common methods are organ transplantation, autografting or artificial prosthesis. Modern technology of cell biology and genetic manipulation now allow for the development of living tissue implant. National Scientific Foundation (NSF) [17] consensus has defined tissue engineering as “the application of biological,
chemical and engineering principles towards the repair, restoration or regeneration of living tissues using biomaterials, cells and factors alone or in combination [17].

To construct a tissue engineered organ the researcher must understand the components of the organ. Common organs, regardless of complexity and location, consist of four main components: cells, scaffolds, signals and nutrients. The cells are the building block of a tissue. They provide any active behavior or functionality of a tissue, such as the contraction of a muscle, the filtration of a kidney, or the metabolic behavior of a liver. Cells exist connected by a scaffold of solid and fluid. The solid is mostly extracellular matrix (ECM) and the fluid is mainly blood, while other solids and fluids exist in smaller quantities. Signals are molecules that communicate with the cells, and that the cells use to communicate with each other. Some signals are soluble and are transported between cells by fluid, and other signals are insoluble and may be attached to the surface of a cell or the scaffold. Nutrients are molecules needed to sustain the cell or are produced by the cell. The circulatory system exists to transport nutrients, especially oxygen, to and from all of the cells in the body [15].

The physical scaffold-guided tissue generation communication between cells and extracellular matrix directly and indirectly impacts cell shape and function, and these signals are all necessary cues for normal cellular activity. These concepts can be utilized in developing 3D cell culturing techniques as the next technological level in the development of substitute tissues and organs. These techniques involve placing cells and/or growth factors in synthetic scaffolds (which acts as temporary ECMs) in order to
help encourage the cells to organize into tissues or perhaps whole organs. This technique is much studied and is one enabling technology for the emerging field of tissue engineering [17].

The function of the scaffold is to guide the regeneration of new tissue and provide appropriate structural support i.e., to mimic the structure and functions of the natural extra cellular matrix (ECM)[1,2,6,17]. Bioreabsorbable polymers (both synthetic and natural polymers) are commonly used for fabricating scaffolds [1, 2]. During the tissue engineering process, the cells are seeded onto the bioreabsorbable scaffold. Over time, when the cells produce its own ECM, the scaffold degrades and reabsorbs resulting in the formation of remodeled native tissue [1, 2, 17] as shown in figure 2.1

![Figure 2.1: Scheme showing process of the tissue engineering](www.bme.utexas.edu/.../TissEng/index.html) cited on 11/20/2007, Copyright 2004

The technique of tissue engineering has led to the development of many tissues within the laboratory scale such as ligament, tendon, heart valves, blood vessels; myocardium, esophagus and trachea [17]. Tissue engineered skin was the first commercialized
product developed using the principles of tissue engineering e.g Dermagraft®, Apligraft®.

2.2.1 Native Extra cellular matrix (ECM):

ECM mainly composed of physical and chemical crosslinked network of a fibrous proteins and hydrated proteoglycans with glycoaminoglycans(GAG) side chains, which are collectively called physical signals that surrounds a cell [17]. A typical ECM structure is shown in Figure 2.2 below.

![ECM and its components](www.kentsimmons.uwinnepeg.ca/cm1504/Image122.gif) cited on 11/26/2007

Figure 2.2: ECM and its components [20]
With diversity of ECM, it performs many functions. One of the functions is to provide support and structure for the multicellular organism. ECM is a major component in tissues such as cartilage and blood vessels and therefore endows tissues with the requisite mechanical support and regulates the movement of water, nutrients and other solutes. It holds the cells and tissue together in a lattice and provides organizational matrix within which cells can migrate and interact with one another [21].

2.2.2 Scaffolds as extracellular matrix ECM:

Three general approaches have been utilized in the creation of new tissue (Langer and Vacanti, 1993) [22]. These include: (1) the replacement of only those isolated cells or cell substitutes needed for function; (2) production and delivery of tissue – inducing substances such as growth factors and signal molecules; (3) cells placed on or within matrix fashioned from synthetic polymers or natural substances such as collagen. As discussed in previous section, the ECM provides the cells with a three dimensional structure to organize cell-cell communications, and provides various biochemical and biophysical cues for cellular adhesion, migration, proliferation, differentiation and matrix deposition [22]. The use of three–dimensional system to create new tissues and organs by attaching parenchymal cells to biodegradable polymer scaffolds was based on several biological observations [22]. (1) Common tissues undergo continual renewal, remodeling, and replacement. (2) Dissociated cells will reform structures when provided with the cues from their environment as demonstrated by the formation of tubular structures by endothelial cells and ductal structures by biliary cells. (3) Organ
parenchymal cells are anchorage dependent with the cell matrix playing an important role in cell shape, division, differentiation, and function [22].

Scaffolds are required to mimic the site where they will be implanted in vivo as closely as possible [1, 2, 23]. Studies have shown that when the cells are grown on 2-D surface such as a biomaterial membrane, they lose their characteristic behavior related to their specific tissues types [17]. This study, however, makes sense as when the cells are grown on 3-D synthetic matrix or scaffold the complex biochemical and biomechanical interplay between the cells and the matrix is addressed; and hence the probability of cells losing their characteristic behavior reduces [17]. The focus area in this research was to design an exogenous ECM that would use cell transplantation approach for creating integrated tissues instead of the induction of a new tissue formation by host cells or by immunoisolated –cell transplantation [21]. Apart from the 3-D structure the properties of materials for scaffold building play a vital role when designing a scaffold. The dynamics of different tissues vary significantly and hence appropriate materials selection is required in order to match functional requirements of a tissue.

2.2.3 Scaffold design requirement: Biomaterials:

- **Biocompatibility**: The scaffold should not transfer antigens [21] and should be immunologically inert. Also, the material should be reproducible into a variety of shapes and structures that retain their shape once implanted.

- **Biodegradability**: The scaffold should be able to degrade into smaller organic compounds that can be later consumed by the living organism. Also, the
degradation rate of scaffold needs to be in tune with the regeneration process of natural ECM [22].

2.2.4 Scaffold design requirement: Macro – Microstructure of scaffold

Apart from the properties of materials, the 3-D architecture of the scaffold is very important when attempting to mimic the structure of the natural ECM. Synthetic ECM provides an adhesion substrate for transplanted cells and serves as a delivery vehicle onto specific sites in the body. A large surface area to volume ratio is desirable in order to allow a delivery of high cell density, which dictates the use of highly porous matrices [17, 21, 22]. Below in Figure 2.3 is an example of scaffold with high surface to volume ratio made using the porogen leaching technique [24].

Figure 2.3: PEG/PBT scaffold fabricated by porogen leaching [24]

A large surface area favors cell attachment and growth, whereas a large pore volume is required to accommodate and subsequently deliver a cell mass sufficient for tissue repair. Highly porous biomaterials are also desirable for easy diffusion of nutrients to
and waste products from the implants and for vascularization which is major requirements for the regeneration of highly metabolic organs [25]. The surface area/volume ratio of porous materials depends on the density and average diameter of the pores. The diameter of cells in scaffold dictates the minimum pore size, which varies for one cell type to another. The usual effect in relation with the optimum pore size is shown below in Table 2.1[25, 26]

Table 2.1: Optimal pore size and its effect on tissue ingrowth[26]

<table>
<thead>
<tr>
<th>Pore Size (Microns)</th>
<th>Tissue Ingrowth / Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>For Neovascularization</td>
</tr>
<tr>
<td>5-15</td>
<td>Fibroblast ingrowth</td>
</tr>
<tr>
<td>20</td>
<td>Ingrowth of hepatocytes</td>
</tr>
<tr>
<td>20-125</td>
<td>Regeneration of adult mammalian skin</td>
</tr>
<tr>
<td>10-100</td>
<td>Osteoid growth</td>
</tr>
<tr>
<td>100 – 350</td>
<td>Regeneration of Bones</td>
</tr>
<tr>
<td>500</td>
<td>Fibrovascular tissues for rapid vascularization and survival of</td>
</tr>
<tr>
<td></td>
<td>cells</td>
</tr>
</tbody>
</table>

The scaffold should have the mechanical strength needed for the creation of a macroporous scaffold that will retain its structure after implantation, particularly in the reconstruction of hard, load-bearing tissues, such as bones and cartilages. The biostability of many implants depends on factors such as strength, elasticity, absorption
at the material interface and chemical degradation. The mechanical characteristics such as strength and mechanical properties such as modulus of a scaffold are determined by both its structure (macrostructure and microstructure) and its material properties [26]. Mechanical properties of some of the human tissues is given in Table 2.2 below.

Table 2.2: Mechanical properties of few human tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Young’ modulus , E</th>
<th>Reference</th>
<th>Ultimate Tensile Strength</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cartilage, Costal</td>
<td>5.0 MPa</td>
<td>[27]</td>
<td>2.8 MPa</td>
</tr>
<tr>
<td>Artery, Aorta</td>
<td>0.3-0.94 MPa</td>
<td>[27]</td>
<td>1.1 MPa</td>
</tr>
<tr>
<td>Bone</td>
<td>1-20 GPa</td>
<td>[27]</td>
<td>3.3 MPa</td>
</tr>
<tr>
<td>Skin</td>
<td>15- 150 MPa</td>
<td>[28]</td>
<td>5 – 30 MPa</td>
</tr>
</tbody>
</table>

Processability of the biomaterial is also required when the final shape of the repaired organ or regenerated tissue has a critical influence on its activity. The scaffold should be easily processed to acquire a variety of configurations. The reproducibility of scaffold or architecture is also vital in maintaining the dimensional stability of the scaffold [26].

2.3 Biopolymers for scaffolding

The first issue with regard to tissue engineering is the choice of suitable material. The desirable characteristics of these materials are biocompatibility (i.e., not to provoke any unwanted tissue response to the implant, and at the same time to possess the right
surface chemistry to promote cell attachment and function) and biodegradability (i.e., degradable into nontoxic products, leaving the desired living tissue). Each type of material has its own distinct properties that can be advantageous for specific tissue engineering. The choice of materials depends on the type of tissue to be reconstructed. For example, metals and ceramics are widely used for hard tissue replacement [25]. In this chapter we will briefly focus on a few natural polymers and synthetic polymers which are widely used, for tissue engineering applications. Some of the synthetic polymers used in field of scaffold tissue engineering are summarized in Table 2.3.

Table 2.3: Common polymers used in tissue engineering [29]

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biodegradable Polymers</td>
<td></td>
</tr>
<tr>
<td>Poly(α-Hydroxy esters)</td>
<td>Poly 3-hydroxybutyrate</td>
</tr>
<tr>
<td>Poly(ε-caprolactone)</td>
<td>Polycaprolactone</td>
</tr>
<tr>
<td>Poly(ortho esters)</td>
<td>Poly( L-lactide)</td>
</tr>
<tr>
<td>Poly(phosphorous)</td>
<td>Poly(phosphazene)</td>
</tr>
<tr>
<td>Poly(amino acids)</td>
<td>Poly(l-Lysine)</td>
</tr>
<tr>
<td>Non-Biodegradable Polymer</td>
<td></td>
</tr>
<tr>
<td>Poly(ether glycols)</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>Poly(oxytetramethylene glycol)</td>
<td></td>
</tr>
</tbody>
</table>

2.3.1 Natural polymers

There are two major classes of natural polymers used in developing the scaffolds: polypeptides and polysaccharides. Natural polymers are typically biocompatible and
enzymatically biodegradable [25, 30, 31, 32, 33, 34]. The main advantage with natural polymers is that they contain bio-functional molecules that aid in cell attachment, proliferation, and in cell differentiation. The disadvantage with natural polymers is the rate of degradation, which may not be easily controlled since the enzymatic activity varies between hosts which therefore vary the degradation rate too. Another disadvantage of a natural polymer may be its poor mechanical integrity [26, 30, 31, 32]. Some good examples of natural polymers are collagen and chitosan.

2.3.1.1 Collagen

Collagen is a major component of structural mammalian tissues. The most abundant form of collagen is Type I collagen [31]. Collagen is composed of three polypeptides chains which intertwine with one another to form a triple helix. The chains are held together through hydrogen bonding of the peptide bond in glycine and an adjacent peptide carbonyl group [31]. Collagen is enzymatically biodegradable, but has a tendency to degrade quickly, which limits its mechanical properties [31]. Collagen has been isolated and used as collagen gels and when fabricated into tubular structure, these have provided the basic conduit for a vascular graft [1].

2.3.1.2 Chitosan

Chitosan is derived from chitin, a polysaccharide that is present in the hard exoskeletons of shellfish like shrimp and crab. Some desirable properties like minimal foreign body reaction, mild processing conditions, availability of chemical side groups for
attachments to other molecules has already made this material as one of the most investigated material for its use in field of tissue engineering [31]. However, current difficulties with using chitosan as a polymer-scaffold for tissue engineering includes low strength and inconsistent behavior with seeded cell. It’s been observed that pure chitosan shows low potential for cell-attachment [30].

2.3.2 Synthetic Polymer

A major advantage with synthetic polymers over natural polymers is that they can be tailored to suit specific functions and thus exhibit controllable properties. Some of advantages related with synthetic polymers are: large scale production with reproducibility, variable micro- and macro- structure, and controllable physicochemical properties. However, several drawbacks e.g. inflammatory response, aberrant cellular response, adverse effect of polymer erosion are associated with these polymers [30, 31, 32, 33, 34].

2.3.2.1 Polyglycolic acid and Polylactic acid

PGA is a highly crystalline polymer, with crystallinity typically reported in the range of 35–75% [26]. Because of its high degree of crystallization, PGA is not soluble in most organic solvents. Owing to its hydrophilic nature, PGA tends to lose its mechanical strength rapidly (50%) over a period of 2 weeks and is absorbed in about 4 weeks after implantation. It can be completely absorbed in 4–6 months [26]. Although structurally very similar to PGA, PLAs (polylactic acid) are quite different in chemical, physical,
and mechanical properties. Poly(L-lactide) (PLLA) is a semicrystalline and relatively hard materials with glass transition temperature at about 65°C and melting temperature of about 170–180°C[26]. PLLA is generally less crystalline than PGA, with crystallinity reported in the range of 35% [26]. Some of limitations associated with polyesters are the release acidic degradation products that can adversely affect biocompatibility. These polyesters tend to be relatively stiff materials. While this may be an advantage in load-bearing applications, it becomes a disadvantage when mechanical compliance with soft tissue or blood vessels is required. Finally, none of these polyesters provides a chemically reactive pendant chain for the easy attachment of drugs, crosslinkers, or biologically active moieties [26].

2.3.2.2 Poly-peptide based polymer

The problems associated with synthetic and natural polymers as discussed above have led to the development of polymers from naturally occurring nutrients and metabolites as monomer. Amino acids are monomeric units of proteins and are major part of natural metabolite. The polymers of amino acid linked by peptide linkages are called poly (amino acid) or peptide polymers. Several amino acids are used for this technique: serine, hydroxyproline, tyrosine, cysteine, glutamic acid etc.
L-Tyrosine is one of the several amino acids. Its structure (Figure 2.4) shows the presence of phenolic hydroxyl group. This feature makes it possible to use derivatives of tyrosine dipeptides as a motif to generate monomers used to build polymers. The most common dipeptides used are the desaminotyroyl tyrosine alkyl esters (DTH) [7, 36, 37, 38]. Figure 2.5 shows structure of desaminotyrosyl tyrosine hexyl ester.

DTH is a commonly used dipeptide, used to make different polypeptide polymers with improved physical and chemical properties [38, 39] over standard poly(amino acids) with only peptide bonds. Poly-peptide based polymer show various advantages such as
biocompatibility, biodegradability, enzyme specificity and non-immunogenecity. Moreover, the side chain modification offers the chance to attach small molecules, small peptides or pendant groups. Poly(amino acids) have found applications in different biomaterial applications including suture material, artificial skin substitutes and as drug delivery systems[37]. However, some of the unfavorable synthesis and processing properties of poly(amino acids) have limited the use of these materials commercially. Apart from being highly expensive polymers, some of the other disadvantages with these polymers are: insolubility in most of the organic solvents; extensive reactivity; moisture sensitivity; degradability before reaching their melting temperature; and, absence of chances of structural modifications. All these limitations have restricted the use of poly(amino acid) polymers as a biomaterial [2,7]. The major difficulties with poly (amino acid) polymers are related to their structure. Structural modification by adding non-amide linkage instead of peptide linkages in the polymer backbone by using ‘pseudo –polypeptide’ chemistry is a tool to new class of polymer called pseudo-polypeptide.

2.3.2.3 Polyurethanes

Polyurethanes are widely used as biomaterials for different applications due to excellent properties and good biocompatibility [7, 34]. The polyurethanes have structures consisting of polyol, which constitutes the soft segment, and the poly functional isocyanate (mainly diisocyanate) and the chain extender (or crosslinker) which constitutes the hard segment [31]. The general structure of polyurethane is shown in
A wide range of properties can be obtained by tuning the structure of polyurethane.

Figure 2.6: Structure of polyurethane

The versatility of polyurethanes lies in its phasic behavior; elastomeric as well as in its thermoplastic nature [7, 34, 35]. Biodegradable polyurethane is synthesized using different polyols, polyisocyanates and chain extenders. The selection of ingredients depends on the biocompatibility and non-toxicity of the component material, and the degradation products as well [7]. Poly (ester) urethanes are widely used biomedical application, and have been shown to degrade under hydrolytic condition, and in oxidative condition [7, 39]. The use of polyurethane for tissue engineering application emerged due to degradation properties of polyurethane [7]. The use of polyurethanes as biomaterials has been exploited for various implants, including pacemakers, vascular graft etc. Since the polyurethane structure may be tailored to have degradable linkages and a range of chemical, physical and mechanical properties, polyurethanes have been studied as an alternative material for tissue engineering [35].
2.3.2.4 Pseudo –Polypeptide polyurethane

In this study, a new class of polymer called pseudo –polypeptide polyurethane was developed. Backbone structure modification of conventional poly (amino acid) by non-amide linkages in general improves the physico-chemical and mechanical properties e.g. solubility, thermal property, moldable property etc. while preserving desired properties e.g. biocompatibility, non-toxicity [38, 39]. Introduction of easily hydrolysable non-peptide moieties in an L-tyrosine based peptides backbone provide a way to obtain a natural amino-acid based polymer with customizable degradation properties. The L-tyrosine based polyurethanes were found to be soluble in a variety of common organic solvents, thereby emphasizing their potential for chemical processability. The polymers were found to be readily hydrolytically degradable in vitro and the degradation products had negligible effect on local pH. All these were considerable improvements compared to pure poly-L-tyrosine, which was mainly insoluble and hardly degradable. Hence, in the light of developing potentially biodegradable novel polymeric biomaterials from naturally obtained amino acids, the L-tyrosine based polyurethanes hold significant promise.[39].

In our laboratory, an approach is followed to design and synthesize L-tyrosine based polyurethane with DTH as the chain extender.[36,38,39] L- Tyrosine is introduced to the polymer structure using DTH dipeptide. The hard segment of the polyurethane is made using hexyl diisocynate (HDI) due to its biocompatibility, and poly (caprolactone)
(PCL) is used as polyol to make the soft segment of polyurethane. This structure gives PCL unique properties because of its high olefinic content, while the presence of hydrolytically unstable aliphatic-ester linkage causes the polymer to be biodegradable. This polymer has been regarded as tissue compatible and used as a biodegradable suture in Europe. Because the homopolymer has a degradation time in the order of 2 years, copolymers have been synthesized to accelerate the rate of bioabsorption [26].

2.4 Scaffolding in Tissue engineering

Plethoras of processing techniques are available in the literature for producing 3-D scaffolds from various biodegradable polymers. These include fiber bonding[29,40], solvent casting and particulate leaching[23,29,40], membrane lamination[29], phase separation, co-extrusion, gas foaming[40], and electrospinning[17,28,29]. In this chapter, we examine the fabrication of 3D scaffolds using solvent casting and particulate leaching. Solvent casting and particulate leaching is one of the technologies to make polymer scaffolds. In this technique there is minimal use of equipment and therefore, it is also one of the least expensive techniques.

2.4.1 Solvent casting and Particulate Leaching (SCPL)

With the advances in processing techniques for tissue engineering scaffolds there is still a need especially for a laboratory technique that can process polymers into porous cell scaffold with controllable porosity, pore size, and outer shape. For example, solid free
form scaffolds for cell culturing experiments in tissue engineering are currently introduced in field of tissue engineering [29, 40]. Accordingly to produce small, polymer scaffolds with adequate porosity for use in cell culturing; the technique of porogen leaching is often undertaken. A suitable porogen is added to a solution of polymer in an appropriate mold for the fabrication of a solid porogen constructs. The porogen is leached out to form highly porous sponges for the cell culturing [29, 41]. Different modifications are introduced in solvent casting and particulate leaching technique to create salt leached scaffolds. In this study, Mikos et al [5, 24, 27] protocol was used to manufacture salt leached scaffold using PCL-HDI-DTH as the polymer and sodium chloride (NaCl) as porogen. Mikos et al [5, 23, 27] first introduced this technique in the early 1990’s. Since then this technique has become by far the most commonly used technique for fabricating 3D polymeric scaffolds. The method dictates dispersing formerly calibrated minerals particles (such as sodium chloride [24], ammonium bicarbonate [24] or organic particles (such as paraffin, gelatin [41]) in a polymer solution. The solvent in this dispersion is then removed either by air drying or by vacuum–drying. The particles are leached out by selective dissolution to produce a porous polymer matrix as shown in Figure 2.7 [23, 24, 40, 41].
2.4.1.1 Polymer mixing

During this step, the sieved and sized salt particles are mixed with the viscous organic solution of polymer. The mixture is then cast into a Teflon mold, antiadhesive petri dishes or troughs made using release paper. Alternatively, the polymer solution can be cast over a salt bed and the resulting suspension dried in fume hood. The aim of this technique is to fabricate scaffold with an evenly distributed pore size throughout the scaffold height. However, sometimes, salt sedimentation in the polymer solution and the formation of a thin film on the surface of the scaffold often prevent uniform scaffold on drying. Also, a rapid surface drying causes the crack formation on the surface of the scaffold. To avoid such imperfections, the quantity and viscosity of the polymer used in the making scaffold process must be optimized for every process. Also, to avoid salt
sedimentation, optimized sized salt particle must be used considering the interplay between the polymer solution viscosity and salt particles.

2.4.1.2 Polymer/ Salt construct drying

After using one of the methods for combining polymer and salt particles to give three dimensional structures, the resulting polymer/salt constructs are then air dried and later vacuum dried usually for 48 hours to remove any residual organic solvent to avoid any cytotoxicity when conducting cell experiments.

2.4.1.3 Porogen leaching

After drying the polymer/salt constructs, the incorporated salt is finally leached out using distilled water to leave behind water-insoluble polymer foam. Construct is submerged into water to dissolve the incorporated salt. The water is exchanged every 6 h for 48 h to ensure complete leaching of the incorporated salt. During this step, the exchanged water must be gently removed to avoid breaking of the polymer scaffolds, which sometimes occurs due to excessive swelling in water [40].

2.4.1.4 Scaffold drying and Shaping

The scaffolds are dried extensively to remove water from the swollen scaffold to prevent a further decrease in the polymer’s molecular weight [40]. To obtain the required shape, scaffolds are often cut with cork borers, razor blades or scissors. The shape of the scaffold is mainly determined by the desired application. To achieve larger
scaffolds, lamination of several layers has been proposed to achieve the dimensions while maintaining scaffold porosity [29, 40].

2.4.2 Scaffold Properties

Once scaffold is fabricated, the second issue to be addressed in tissue engineering is the macro- and microstructures of the scaffolds. From materials engineering point of view, tissues are considered to be cellular composites representing multiphase systems. Cellular composites are then seen as consisting of three main structural components: (1) cells that are organized into functional units, (2) extracellular matrix, and (3) scaffold architecture. This architecture is increasingly believed to contribute significantly to the development of specific biological functions in tissues and is thought to provide appropriate nutritional conditions and spatial organization for cell growth. The regeneration of specific tissues aided by synthetic materials has been shown to be dependent on the porosity and pore size of the supporting three-dimensional structure [24]. A large surface area favors cell attachment and growth, whereas a large pore volume is required to accommodate and subsequently deliver a cell mass sufficient for tissue repair. Highly porous biomaterials are also desirable for the easy diffusion of nutrients to and waste products from the implant and for vascularization.

2.4.2.1 Porosity and Pore sizes

The microstructure of the scaffold plays a vital role in tissue regeneration. Most of the cells are anchorage dependent. The scaffold should possess properties that aid cell
growth and facilitate attachment of a large cell population [17, 21]. A large pore volume is required to accommodate and subsequently deliver a cell mass sufficient for tissue repair. Highly porous biomaterials are also desirable for the easy diffusion of nutrients to and waste products from the implant and for vascularization which are major requirements for the regeneration of highly metabolic organs such as liver and pancreas. Highly porous scaffolds allow an abundant number of cells to infiltrate the scaffold’s void space. Porosity of approximately 80%-90% is reliably appropriate for cell attachment and matrix deposition [1]. Whang et..al.1999 claims that PLLA scaffold designed using solvent casting and particulate leaching with porosity of 90% promotes osetoinduction. Also, scaffolds with controlled microarchitecture show the ability to incorporate and deliver proteins.

2.4.2.2 Mechanical Characteristics

Tissue engineered scaffolds needs to closely match the mechanical properties of the natural organs. The mechanical characteristics such as strength, toughness, ductility and mechanical properties such as modulus of a scaffold are determined by its structure (macrostructure, microstructure, and nanostructure) and polymeric material characteristics [17, 26]. For example, polyurethanes show elastic characteristics and therefore were used for vascular applications [1]. But its been also observed that once smooth muscle cells are seeded onto the polyurethane scaffolds, modification in mechanical properties of scaffold was transformed from elastic to viscoelastic is observed [1]. For successful tissue reconstruction the mechanical properties of the
polymeric scaffold should be retained until the regenerated tissue resumes its structural role [1, 21, 26].

Scaffolds engineers are required to design scaffold that could provide adequate mechanical properties to support cell culturing during initial artificial organ development. Works from some of the researchers shows that a high modulus somewhere around 0.1 – 4 Mpa [26] is required for hard tissue replacement whereas modulus of 40 – 100 Kpa [26] is good for soft tissue replacement. It’s been observed by researchers that increased in porosity leads to effect the mechanical properties of scaffold [26]. In view of this contrary case, scaffold engineers are required to optimize the value of mechanical characteristics and porosity of scaffold considering the nature of organ in mind to be reconstructed. Therefore, the choice of appropriate biomaterial and optimized mechanical characteristics plays a vital role especially during initial *in-vitro* set up for artificial organ reconstruction.

2.5 Commercial application of scaffold guided tissue engineering

Tissue engineering is a field of tremendous promise, but one which requires a great deal of development in the transition from demonstration-scale applications. Tissue engineering has to strike a balance between tissue culture, which is a resource-intensive activity, and business considerations that are concerned with minimizing cost and maximizing customer convenience. Allowing cells to form tissues in a reliable manner is the essential engineering design problem that must be accomplished under the
classical engineering constraints of reliability, cost, government regulations and societal acceptance [42]. Even though less than five engineered tissues have been approved by Food and Drug Administration (FDA), more than seventy companies are spending total $600 million per year to develop new products [42]. Some of the skin substitutes being developed are discussed below.

2.5.1 Integra (Integra Lifesciences, NJ)

Integra artificial skin is currently a widely accepted skin substitute for use in certain burn patients and was originally described by Yannas et.al. [14]. Integra has a bilaminar structure, made of cross-linked bovine collagen and glycoaminoglycan, coated on one side with a silicone membrane that provides epidermal function. The pore size has been designed at 70 – 200 μm in order to allow migration of the patient’s own endothelial cells and fibroblasts [14].

![Integra Components and Estimated Cost](image)

Figure 2.8: Components and estimated cost of Integra, Biological skin substitute [43]

Some of the advantages associated with Integra are such as, it provides thick dermal analog, reasonable shelf life. However, some of the disadvantages associated are it requires epidermis from the patient, dermal cells must come from the patient requiring product corporation and it requires extensive two stage procedure.[44]
2.5.2 Epicel (Genzyme Corp, MA)

Genzyme has developed epithelial skin replacement and introduced it as the product Epicel. Epicel consists of a polyurethane sheet upon which, keratinocytes, the cells that compose the living portion of the epidermis, may be cultured to form small colonies or fully confluent sheets [44]. The advantages related with Epicel are such as small skin biopsy, used in large burns whereas some of the disadvantages are 2 to 3 week lag time for production, provides only epidermal layer, epithelial layer can be fragile for sometime, and needs to be used immediately on delivery[44].

2.5.3 Alloderm

This product is basically treated human allograft with the epidermis removed. The dermis is treated to produce a co preserved lyophilized allogermis, which incorporates. The product is used as a dermal implant. Therefore application of a thin epithelial autograft is required. Primary indication is for use in the replacement of soft tissue defects. This product is not commonly used in large burns. A period of incorporation is required before the epithelial skin graft can be applied. The product has a long shelf life in its lyophilized form. It requires re-hydration prior to use. Some of the advantages associated with Alloderm are such as off-shelf product, does not require skin bank. However some of the disadvantages are requires thin skin graft to provide epidermis, two stage procedure required to achieve bilayer skin. [44]
CHAPTER III
MATERIALS AND METHODS

3.1 Materials
Polyurethane (PCL –HDI- DTH) was prepared under laboratory conditions following the understated methodology. The chemicals and solvents were purchased from Sigma Aldrich and used as received, unless otherwise stated. Distilled water was used for all reactions. For the scaffold fabrication salt (Sodium chloride), 60 gm was used as porogen, chloroform was used as the solvent purchased from Sigma Aldrich.

3.2 Polyurethane Synthesis
The synthesis of DTH has been described in details in the literature [7, 36, 39, 45]. The reaction used follows:

i. The carboxylic acid group of the L-tyrosine (0.05 mole) was esterified by 1-hexanol (50 mL) in presence of thionyl chloride (0.05 mole) at 0 °C initially, followed by reaction at 80 °C for 12 hours. The reaction product was obtained after cooling down the reaction to room temperature and was completely precipitated in cold
ethyl ether. The product was then filtered and washed with cold ether to obtain white solid, which was the chloride salt of hexyl ester of L-tyrosine.

ii. The white solid was re-dissolved in distilled water (50 ml) and subsequently neutralized by 0.5 M sodium bicarbonate solution till the pH of the solution was slightly basic (pH~7.5). At this point solution turned turbid due to formation of TH (Hexyl ester of L-tyrosine). TH was extracted in ether, and the ether was evaporated to complete dryness to obtain tyrosine Hexyl ester (TH) as an off-white solid.

iii. Coupling of TH with DAT was mediated through hydrochloride salt of N-Ethyl-N’-dimethylaminopropyl carbodiimide (EDC.HCl). TH, DAT and EDC.HCl were added in equimolar proportion in 99% pure tetrahydrofuran (THF) as solvent at 0°C. After that, the reaction was allowed to continue at room temperature for 12 hours. At the end of 12 hours, the reaction mixture was diluted into four times its volume of distilled water and is extracted in the organic phase by dichloromethane (DCM).

iv. The organic DCM phase was washed with 0.1 N HCl solution, 0.1 N sodium carbonate solution and concentrated sodium chloride solution to remove the by products. The organic DCM phase was dried, and the solvent was evaporated under vacuum to obtain desaminotyrosyl tyrosine hexyl ester (DTH) as yellow, viscous oil.

The synthesis of polyurethane has been described in detail in literature [45] and described following:
i. The polycaprolactone diol (PCL) was reacted with HDI at a 1:2 molar ratio in anhydrous DMF as solvent and using 0.1% stannous octoate as catalyst to form prepolymer under dry and inert N\textsubscript{2} atmosphere with continuous stirring for 3 hours at 110\textdegree C.

ii. After 3 hours, the temperature was lowered to room temperature (~ 25\textdegree C) with continuous stirring. DTH was added in this step at a 1:1 molar ratio with prepolymer. Typically, 5 mmol of DTH in 10 mL of anhydrous DMF was added.

iii. The temperature of reaction was gradually increased to 80\textdegree C and the reaction was allowed to continue for 12 hours. After 12 hours the reaction was quenched by pouring the reaction into cold concentrated aqueous solution of sodium chloride. At this point, solid polyurethane polymer precipitates out from the reaction mixture.

iv. The polymer was suspended as solid polymer. The final polymer was filtered out and washed with water. The washing was continued at least 3 times to remove impurities and unreacted materials. The final polymer was then dried in vacuum at 40\textdegree C for 48 hours. The polymer was yellowish white solid. The nomenclature used for polymer was PCL-HDI-DTH.
3.3 Scaffold fabrication

This project aims to design and fabricate porous polyurethane scaffold for application in field of tissue engineering. To fabricate 3D scaffolds solvent casting and particulate leaching technique was used. This technique is one of the simple technologies to make polymer scaffolds. In this technique there is minimal use of equipment and therefore, it is also one of the least expensive techniques. To fabricate scaffold, in this study pseudo–polypeptide polymer (PCL-HDI-DTH) polyurethane was used, whose synthesis mentioned above.
3.3.1 Optimization setup – Scaffold fabrication

In order to develop processable and tailorable polyurethane scaffold it was important to consider certain parameters which affects the macro and microstructure of fabricated scaffold. Some of the parameters that are discussed in detail later are solvent, polymer solution concentration and porogen particle size.

a) Parameter: Solvent

Factors considered to choose right solvent were the solvent’s evaporation rate, viscosity, solvent retention, and toxicological properties as these factors influence how the solvents interact with the polymer, and they affect the solvent power and retention. Another factor that was considered was the solubility parameter of the solvent. Solvents will more readily dissolve polymers with like solubility parameters. Considering all the factors chloroform was considered as the appropriate solvent for PCL-HDI-DTH polyurethane to conduct further experiments. Table 3.1 shows chloroform’s evaporation rate, retention and power.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Dispersion Forces</th>
<th>Evaporation rate</th>
<th>Vapor pressure(kPa at 20ºc)</th>
<th>Solubility Parameter (J/cm³)½</th>
<th>Viscosity (cP at 20ºc)</th>
<th>Polar forces</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform</td>
<td>Moderate/High</td>
<td>2.5</td>
<td>20.0</td>
<td>19.4</td>
<td>0.57</td>
<td>High</td>
</tr>
<tr>
<td>Tetrahydrofuran</td>
<td>Moderate</td>
<td>8.0</td>
<td>17.2</td>
<td>18.5</td>
<td>0.48</td>
<td>Moderate</td>
</tr>
</tbody>
</table>
b) Parameter: Concentration

During optimization step, PCL-HDI-DTH polyurethane scaffolds were prepared by the solvent casting and particulate leaching (SPCL) technique. Crushed salt particles were added to PCL-HDI-DTH solution in chloroform using different concentration of 6.25%, 10% and 25% as shown in Table 3.2. These concentrations were chose to get low viscous polymer solution to promote uniform thickness of scaffold. The mixture was later vortexed for about 2 – 3 minutes to get homogenous dispersion.

<table>
<thead>
<tr>
<th>Volume of Solvent (ml)</th>
<th>Concentration (w/v)%</th>
<th>Polymer weight (gm)</th>
<th>Salt weight (gm)</th>
<th>Total weight(gm)</th>
<th>Salt weight fraction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>6.25</td>
<td>0.75</td>
<td>1.75</td>
<td>2.5</td>
<td>70</td>
</tr>
<tr>
<td>7.5</td>
<td>10</td>
<td>0.75</td>
<td>1.75</td>
<td>2.5</td>
<td>70</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td>1</td>
<td>5</td>
<td>6</td>
<td>70</td>
</tr>
</tbody>
</table>

It was observed that both 6.25% w/v and 25% w/v gave a non-homogenous mixture of polymer solution and salt particles (shown in results and discussion section). Therefore, it was suggested to use 10% w/v concentration for further studies.

C) Parameter: Porogen size - Sieve analysis test / Particle size measurement

Sieve analysis technique is a procedure used to assess the particle size distribution of a granular material [47]. To obtain particle size distribution for salt particles, the following steps were followed.

1) Salt particles were crushed using a mortar and pestle.
2) The salt sample was weighed 60gm.

3) Stack of sieves was prepared as shown in figure 3.2. Sieves having larger opening sizes (i.e. lower number) were placed above the ones having smaller openings (i.e. higher numbers). The very last sieve is # 400 and a pan was placed under it to collect the portion of salt particles passing # 400 sieves.

<table>
<thead>
<tr>
<th>Mesh #</th>
<th>Sieve size (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>48</td>
<td>0.295</td>
</tr>
<tr>
<td>60</td>
<td>0.248</td>
</tr>
<tr>
<td>80</td>
<td>0.1778</td>
</tr>
<tr>
<td>100</td>
<td>0.1496</td>
</tr>
<tr>
<td>120</td>
<td>0.12446</td>
</tr>
<tr>
<td>170</td>
<td>0.0889</td>
</tr>
<tr>
<td>400</td>
<td>0.038</td>
</tr>
</tbody>
</table>

4) All sieves and pan were weighed separately.

5) Salt particles from step 2 were poured into stack of sieves from the top and cover was placed. Stack of sieves were placed on vibrating sieve shaker and clamps were affixed. The shaking time was 10-15 minutes.

6) Following the sieving process, the mass of the salt sample in each sample and the remaining salt was measured.

7) A particle size distribution curve was obtained.
3.3.2 Scaffold fabrication:

i) Crushed and sieved salt particles were added to PCL-HDI-DTH solution in chloroform. For each composition, sieved salt particles of two different sizes, $d$ were employed: $250 < d > 176 \, \mu m$; and, $177 < d > 140 \, \mu m$ as shown in Table 3.4. The compositions were stirred for about 2-3 minutes to get homogenous dispersion. The vortexed dispersion was poured in troughs made using release paper.

Table 3.4: Different composition used to make scaffolds

<table>
<thead>
<tr>
<th>Concentration (w/v)%</th>
<th>Polymer weight (gm)</th>
<th>Salt weight (gm)</th>
<th>Total weight (gm)</th>
<th>Salt weight fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1.0</td>
<td>2.3</td>
<td>3.4</td>
<td>0.7</td>
</tr>
<tr>
<td>10</td>
<td>1.0</td>
<td>4</td>
<td>5.0</td>
<td>0.8</td>
</tr>
<tr>
<td>10</td>
<td>0.5</td>
<td>4.5</td>
<td>5.0</td>
<td>0.9</td>
</tr>
</tbody>
</table>
ii) The solvent was allowed to evaporate from the loosely covered troughs to avoid excessively fast solvent evaporation to avoid macrocracks formation on the surface of scaffold. The mixture was dried first 24 hours in a fume hood and next 24 hours in vacuum.

iii) The polyurethane/salt composite was then immersed in 250 ml distilled, deionized water on a shaker table at around 100 rpm for 48hrs (water was changed every 6hrs) to leach out the salt.

iv) Following the leaching process, the polyurethane scaffolds were air dried for 24 hrs and were stored in desiccators for further use. The schematic for scaffold fabrication is shown in figure 3.3.

![Figure 3.3: Schematic diagram of scaffold fabrication](image)
3.4 Scaffold Characterization

Once fabricated polyurethane scaffolds were characterized for pore morphology, porosity, pore size and mechanical characteristics. This architecture is increasingly believed to contribute significantly to the development of specific biological functions in tissues and thought to provide appropriate nutritional conditions and spatial organization for cell growth.

3.4.1 Scanning Electron microscopy

In order to observe the microstructure and pore morphology of scaffolds scanning electron microscope was used. The specimens were coated with silver (50 mtorr, 45 mA, 90 s) using a sputter coater. A Hitachi (model –S 2150) scanning electron microscope was used in our studies and was operated at 20kv.
3.4.2 Pore size measurement: 2-D image analysis

Figure 3.4: SEM photomicrograph of porous polyurethane scaffold with 90% salt weight fraction

Various sections of different scaffolds were viewed under SEM to measure the pore size and shape. The SEM micrograph acquired, figure 3.3, was a 256 grayscale monochrome image with tonalities ranging from pure black (grayscale value 0) to pure white (grayscale value 255). In an ideal situation level 0 regions corresponds to the pores within the scaffold and the nearly white regions approaching level 255 to the walls. The challenge with determining threshold of histogram for micrographs was to segment this type of grayscale image into regions associated with the pores and walls respectively. The intensity of the grayscale image ranges from dark grey pixels to light grey pixels depending on whether a pore or wall edge was being viewed. The histogram of Figure 3.4 is shown below in Figure 3.5.
Figure 3.5: Image histogram of SEM image of polyurethane scaffold with 90% salt weight fraction with the number of pixels in the image (vertical axis) with the particular brightness value (horizontal axis)

Figure 3.6: Equalized and normalized SEM image of scaffold with 90% salt weight fraction was obtained using users toolbox provided in Image J software
The contrast in an image can be artificially enhanced by expanding the histogram as can be seen in figure 3.6 and 3.7 so that it covers the full range from level 0 to level 255. An inevitable consequence of this action was the introduction of periodic gaps in the histogram which are cosmetically unsightly but don’t influence subsequent analyses.

To determine threshold ‘by eye approach’ technique was used discussed elaborately in the stated reference [48]. The figure 3.8 shown below was obtained after adjusting the threshold for figure 3.4.
Once a threshold value was defined the grayscale image was changed to binary black and white. The binary image which was unambiguously divided was analyzed for the pore shape and size, Figure 3.9 and with a histogram, Figure 3.10.

Figure 3.9: Binary image of threshold SEM image for scaffold with 90% salt weight fraction

Figure 3.10: Image histogram for threshold binary image.
Image analysis software, Image J, was used to measure the Equivalent Circle Diameter of irregular shaped pores using the formula [48]:

Equivalent Circle Diameter, \( ECD = \left(\frac{4A}{\pi}\right)^{1/2} \), where \( A \) = area of the pore, was obtained from the software [47].

A reliability test was conducted for 2-D image analysis in order to test the validation of 2-D image analysis technique. A set of equivalent circle diameter (ECD) for 3 different pores, viewed from single SEM photomicrographs, were measured for span of five days. Statistical analysis (\( p > 0.05 \)) proves that there was no significant difference between mean pore diameters measured on day 1, day 3 and day 5. This proved that 2-D image analysis technique was a reliable technique to perform mean pore diameter measurement.

3.4.3 Porosity measurement test / Void fraction measurement

Porosity of the scaffold was measured using liquid displacement method. Measurement technique is discussed as follows.

3.4.3.1 Wetting properties: Contact Angle measurement

The porosity studies were conducted using Archimedes’s principle. Therefore, to ensure the uniform wetting and regular filing of interconnected pores while performing porosity studies, it was important to choose a solvent which would show good wetting properties with PCL-HDI-DTH film. Therefore, it was suggested to measure the contact angles using solvents such as hexane (non-polar, hydrophobic) ethanol (polar, hydrophilic) and water (polar) with PCL-HDI-DTH polyurethane thin films to check for their wetting properties. A thin film of PCL-HDI-DTH was formed using spin coating
method. An excess amount of polyurethane solution was placed on the substrate, which then was rotated at high speed (~ 3000 rpm) in order to spread the fluid by centrifugal force. Rotation was continued while the fluid spins off the edges of the substrate, until a thin film was achieved. The substrate with thin films was vacuum dried for 24 hrs. Hexane and ethanol contact angle was measured using sessile drop method using a Ramè – Hart goniometer[49], figure 3.11 at room temperature in air both in advancing and receding mode. The average of five readings from 3 different parts of the film was taken for each sample.

![Figure 3.11: Schematic diagram of a contact angle measuring goniometry apparatus][49]

3.4.3.2 Void fraction measurement

An Archimedes’s principle was used to measure the porosity of the scaffold. The initial mass of dry scaffold, $W_s$ was measured. The dry scaffold was then immersed in water with known weight ($w_w$) in a round bottom flask (15ml). The sample was kept in water
for 5 minutes and then series of brief evacuation – repressurization cycles were conducted to force water into the pores of the scaffold by repeatedly applying vacuum to the setup. Cycling was conducted until no air bubbles are seen emerging on the surface of the scaffold. The total weight of water and water – impregnated scaffold was recorded as, \( w_g \). The water-impregnated scaffold was removed from the round bottom flask and the residual water weight was recorded as \( w_{g1} \). The quantity \( \frac{w_g - 2w_s - w_{g1}}{\rho_w} \) was the volume of the water held in the scaffold and was determined as void volume of the scaffold. The total volume of the scaffold was determined as\([50, 51, 52]\).

\[
V = \frac{(w_g - w_s - w_{g1})}{\rho_w} \text{ Equation 3.1}
\]

The porosity was calculated as the ratio of volume of void scaffold to total volume (bulk volume) of scaffold

\[
\text{Porosity} = \frac{(w_g - 2w_s - w_{g1})}{(w_g - w_s - w_{g1})} \text{ Equation 3.2}
\]

where \( \rho_w \) was density of water which was 1.0.

3.4.4 Mechanical characterization: Compressive modulus

A set of experiments was run to determine the compression modulus of the scaffold with 88% porosity using protocols as stated below according to standard ASTM guidelines \([53, 54]\).

1) The Instron (5567) machine was setup as:
• An appropriate load of 1 KN
• A slow cross – head speed 1 mm/min.

2) Width (W), thickness (T) and length (L) of test-piece were measured as 10mm × 3.5mm × 5mm. The ratio of width, W to thickness T was 3.

3) The test-piece was mounted between the end blocks (steel platens).

4) The cross head was activated to deform the specimen along the z-direction.

5) The cross-head was stopped on completion of the test, i.e. when the specimen is deformed to 30% of its length (L).

6) Compressive modulus was measured from force vs. displacement graph

\[
\text{Compressive modulus} = \frac{\left( \frac{\partial F}{\partial x} \times L \right)}{(W \times T)}
\] ................................. .... Equation 3.3

Where, \( \frac{\partial F}{\partial x} \) is the gradient of force vs. displacement curve and L, length of the specimen, W, is the width of the specimen and T, thickness of the specimen. Figure 3.11; shows schematic for compression testing apparatus.
3. 5 Statistical analysis and data analysis

The statistical analysis to measure the void fraction measurement (porosity) of the scaffold, and also to study the linear regression model showing relation between salt weight fraction and porosity of scaffolds were performed using twelve replicates. The statistical significance and regression coefficient for Model I, showing linear relation between porosity and salt weight fraction of scaffold was determined using one-way ANOVA.

The mean pore diameter studies were performed using seventeen replicates. The significant relation between mean pore diameter based on salt particle size and salt weight fraction was determined using two–way ANOVA. Values of p < 0.05 were
considered statistically different for both void fraction measurement studies and mean pore diameter determination.

The data on the plots corresponding to all experiments conducted were expressed as mean values with the error bars reflecting the standard errors. To verify the normal distribution of the data obtained in void fraction measurement of scaffold, the ANDERSON – DARLING test for normality was performed on each sample groups. Each groups composed on 12 samples was considered normally distributed when p \geq 0.05.

The statistical analysis were performed using MINITAB15, a computer based statistical analysis system and MICROSOFT EXCEL. The results of the statistical analysis are presented in APPENDIX.
CHAPTER IV
RESULTS AND ANALYSIS

The creation of an autologous implant involves processes such as individual cell isolation from donor tissue and the cell attachment on proper substrates, which is then followed by cell growth and proliferation in \textit{vitro} and \textit{in vivo}. It is believed that primary organ cell are anchorage dependent and require a specific environment that often includes the presence of supporting material to act as a template for cell growth. The success of tissue engineering principles \textit{in vitro} and \textit{in vivo} therefore relies on the development of suitable substrates commonly known as scaffolds. A biopolymer scaffold with adequate architectural parameters (porosity, pore size and mechanical characteristics) is believed to act as a suitable substrate for cell seeding; therefore this project aimed on developing a porous L-tyrosine polyurethane scaffold using the solvent casting and particulate leaching techniques with 88% porosity.

4.1 Polymer synthesis

The final polymer obtained after following polymer synthesis protocol as stated previously was yellowish white solid. The molecular weight of polymer was measured using gel permeation chromatography (GPC) as follows in table 4.1
Table 4.1: Molecular weight of the polyurethane

<table>
<thead>
<tr>
<th>Polymer</th>
<th>$M_n (10^3)$</th>
<th>$M_w (10^3)$</th>
<th>Polydispersity index</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCL-HDI-DTH</td>
<td>150</td>
<td>246</td>
<td>1.64</td>
</tr>
</tbody>
</table>

4.2 Scaffold Fabrication

PCL-HDI-DTH polymer was used further to fabricate porous scaffold using solvent casting and particulate leaching technique.

4.2.1 Optimization Results

Biomaterials play a vital role in the development of a scaffold as discussed in previous literature review section. The material used in this project to make scaffolds was pseudo-polypeptide polyurethane (PCL-HDI-DTH) which was expected to show non-cytotoxic and tailorable biodegradation properties [7]. It has been observed that during scaffold fabrication using solvent casting and particulate leaching along with the choice of right material, the choice of solvent and concentration of polymer solution plays important role [29]. To check for right concentration, during preliminary studies scaffolds were made using 6% w/v, 10% w/v and 25% w/v polyurethane solutions as mentioned previously. It was observed that 25% w/v concentration caused a non-homogenous mixing of salt (porogen) in polyurethane solution which can be explained due to high viscosity of the solution. Also, with a 6% w/v concentration, the salt sedimentation to the bottom of the mold was observed which can be explained due to low viscosity of the solution. A more homogenous mixture of salt and polyurethane
solution was obtained using the 10% w/v. Therefore, further studies were carried out using 10% w/v as the concentration. It was also observed that after pouring the homogenous mixture of salt (porogen) and polymer solution into Teflon mold, solvent evaporation played an important role in deciding the scaffold macro surface property. The solvent evaporated from the mold in two stages. In the initial stage the solvent evaporates at a rate which is related to the evaporation rate of the pure solvent and in the next stage evaporation occurred based on the structural property of the polymer [29]. It was observed that fast solvent evaporation due to an open system during the initial stage of evaporation leads to formation of macro sized cracks onto the surface of the scaffold, Figure 4.1, affecting scaffold robustness. Therefore, a closed system was used for future scaffold fabrication.

![Figure 4.1: PCL –HDI –DTH scaffold with macro sized cracks on surface due to fast solvent evaporation](image)

Figure 4.1: PCL –HDI –DTH scaffold with macro sized cracks on surface due to fast solvent evaporation

It was further observed that along with polymer solution concentration and solvent another factor that effected the formation of homogenous mix of salt (porogen) and
polymer solution was the salt particle size used. The uncrushed salt particles, with size equal or greater than 250 microns, showed salt sedimentation and formation of polymer film onto the layer of salt. Therefore, to conduct future fabrication, salt particles were crushed using mortar and pestle.

4.2.2 Sieve analysis /Particle size analysis

In order to fabricate scaffold with calibrated salt particles, the sieve analysis method (section 1.1.2 of Material and Methods) was used to get two different range of salt particle size: 250 – 176 µm and 177 – 140 µm. It was hypothesized that the salt particle size affected the mean pore diameter of the scaffold. It was also hypothesized that salt weight fraction (weight of salt / (weight of salt + weight of polymer)) did not affect the mean pore diameter of the scaffold. The differential distribution of salt particles obtained from the sieve technique was determined, figure 4.2.

![Particle size distribution](image)

**Figure 4.2: Differential distribution of salt particles**
4.2.3 Scaffold fabrication:

PCL-HDI-DTH (Mw: 150 K) porous scaffold were made following the protocol as described in the Material and Methods chapter. Two batches of scaffold with different salt particle size (250 -176 microns and 170-144 microns) were made. In each batch scaffolds were made using three different salt weight fractions (70%, 80%, and 90%). One batch of scaffolds made using 250 -176 µm salt particle size as illustrated in Figure

Figure 4.3: PCL-HDI-DTH scaffold made using 250 – 176 microns salt particle size and different salt weight fractions. Different scaffold shapes show the polyurethane’s processability behavior.
4.3 Scaffold Characterizations

Fabricated scaffolds were characterized for its pore morphology, porosity and mechanical characteristics.

4.3.1 SEM photomicrographs

Scanning electron microscopy was used to investigate the anisotropy and homogeneity of the foam/scaffold microstructure and to access the porous architecture in different regions within the structure. Foams/scaffolds prepared with salt weight fraction of 70 wt%, 80 wt% and 90 wt% showed evenly distributed pores as may be seen from Figure 4.4a, Figure 4.5a and Figure 4.6a respectively. The pores are interconnected thus yielding an open –cell polymer foam. It was apparent that as the salt weight fraction is increased the uniformity in the pore morphology increased as shown in Figure 4.4a, Figure 4.5a and Figure 4.6a. It was also observed from the SEM photomicrographs that with increasing salt weight fraction, the apparent porosity of the foam also increased. Also, by utilizing sieved salt particles of different sizes we prepared foams of the same porosity but with different mean pore diameters.
Figure 4.4 (a) SEM (20 KV, 500 microns) photomicrographs of PCL-HDI-DTH foams prepared with 70 wt% for all NaCl particles in the size range from 250 to 176 μm. (b) 177 to 140 μm.
Figure 4.5 (a) SEM (20 KV, 500 microns) photomicrographs of PCL-HDI-DTH foams prepared with 80 wt% for all NaCl particles in the size range from 250 to 176 μm b) 177-140 μm.
Figure 4.6 (a) SEM (20 KV, 200 microns) photomicrographs of PCL-HDI-DTH foams prepared with 90 wt% with NaCl particles in the size range from 250 to 176 μm. (b) (20 KV, 500 microns) 177 - 140 μm.

To investigate the porous morphology throughout the height of scaffold the transverse cross section of the scaffold was also viewed under scanning electron microscope. The
cross section view for 70 wt% foam/scaffold made using 250-176 microns sized salt particles is shown in figure 4.7. Continuous pore morphology all through the thickness of scaffold was observed. However, it was observed that asymmetric foams were formed as seen in Figure 4.7 showing cross section view of 70 wt% scaffold, with three distinctive regions which are classified as region1 (24μm), region 2 (0.38 mm – 0.45 mm) and region 3 (0.8 -1.5mm). The top layer (Region1) had dense impermeable skin at the surface. Here the amount of polymer was much larger than that required for filling crevices between the salt particles (which precipitated at the bottom of the Petri dish upon casting of the particle suspension). Region 2 comprised the anisotropic region of foam which at macro scale forms the fluffy layer at the top of foam. The formation of such layer can be explained due to preferential cooling direction of solvent (chloroform) which is through the air or top side of the foam/scaffold. It is apparent that due to effective insulation the foam structure becomes less ordered with increasing thickness as the foam cooled down during solvent sublimation [55]. Region 3 showed a more ordered pore structure and constituted the majority thickness of the scaffold. In Figure 4.7 the variation in the depth of the homogeneous region (Region 3) may be explained due to the shape of the vessel base (slightly convex) used during fabrication [55].
Figure 4.7 SEM (20 KV, 500 microns) photomicrographs of cross section of PCL-HDI-DTH foams prepared with 70 wt% with NaCl particles in the size range from 250 to 176 μm.

It can be observed from Figure 4.8 and Figure 4.9, transverse cross section view, that the scaffold made using 80 wt% and 90 wt% salt weight fraction only consisted of an anisotropic Region2 (fluffy region) and homogenous a region (region 3) and Region1 as discussed for 70 wt% was absent. This might have happened due to smaller polymer particles. The variation in depth of homogenous region for scaffold made using 80wt% and 90wt% salt weight fraction in comparison to 70 wt% salt weight fraction was also not observed as the vessel used to make these scaffolds was flat at the base and not convex.
Figure 4.8 SEM (20 KV, 500 microns) photomicrographs of cross section of PCL-HDI-DTH foams prepared with 80 wt% with NaCl particles in the size range from 250 to 176 μm.

Figure 4.9: SEM (20 KV, 200 microns) photomicrographs of cross section of PCL-HDI-DTH foams prepared with 90 wt% with NaCl particles in the size range from 250 to 176 μm.
4.3.2 Void fraction measurement / porosity measurement

Porosity test was conducted using the Archimedes’s principle of buoyancy, where a dry scaffold sample was displaced in a wetting fluid. This technique is however plagued with a defect of irregular filling of the pores [48]. This defect can affect the porosity value calculated for scaffold. Therefore, to get regular filing of pores; a choice of appropriate wetting fluid played a pivotal role.

In this project, the contact angle method was used to measure the wettability of liquid such as water, ethanol and hexane on PCL-HDI-DTH film. Water and ethanol are two common wetting fluids used in most of the void fraction measurements.

4.3.2.1 Wetting properties: Contact angle measurement

Contact angle measurement of liquid droplets on a solid substrate was used to characterize surface wettability, surface cleanliness and the hydrophilic/hydrophobic nature of surface. Contact angle is defined as angle between the substrate support surface and the tangent line at the point of contact of the liquid droplet with the substrate as shown in Figure 4.10. The value of contact angle of the liquid droplet was dependent on the surface energy of the substrate and the surface tension of the liquid as given by the Young’s equation below:

\[
\cos \theta = \frac{\gamma_{sv} - \gamma_{sl}}{\gamma_{lv}} 
\]

where \( \theta \) is the measured contact angle , \( \gamma_{sv} \) is denoted as surface vapor interfacial energy, \( \gamma_{sl} \) is denoted as surface liquid interfacial energy and \( \gamma_{lv} \) is denoted as liquids vapor energy.
The contact angle $\theta$ of water with PCL-HDI-DTH film was calculated as $70^\circ$ by other researchers [7]. Using sessile drop method the contact angle of ethanol and hexane with PCL-HDI-DTH films is measured as $16^0 \pm 4.1$ and $15.8^0 \pm 3.8$ respectively, Figure 4.11 a and b. It was observed that even though ethanol and hexane are polar and apolar in nature respectively still they show same contact angle with PCL-HDI-DTH film. This phenomenon can be explained using Young’s Equation as stated above. The surface tension value of ethanol and hexane is 22 dynes/cm and 20 dynes/cm respectively which make the value of $\theta$ very low irrespective of type of solid surface.

Ethanol was not used in further void fraction studies as it shows thermodynamic reaction with the PCL-HDI-DTH polymer and therefore, degrades the scaffold.
structure. Hexane was also excluded because of its highly volatile nature. As the solvent is highly volatile it was very difficult to measure the stable solvent weight while conducting porosity studies. Therefore, water was chosen as the best solvent among these solvents to conduct further porosity studies.

Figure 4.11: Contact angle of a) ethanol and b) hexane with PCL-HDI-DTH film
4.3.2.2 Void fraction measurement / porosity test

Mass transport is one of the significant challenges in tissue engineering. Large-scale cell transplantation in open structures is dependent on an adequate nutrient delivery. Scaffold engineers especially who work with load bearing tissue regeneration have cautioned that higher porosity of scaffold might affect the mechanical integrity of the scaffold[26]. In view of this contradictory statement it was assumed (based on literatures as shown in table 4.2 below) that to achieve adequate mass transport through the scaffold, a porosity of 88% was adequate [26] for this study. This claim was supported as others previously conducted studies shows that porosity ranging from 80 – 97% [26] is adequate enough to support cell growth for soft tissue engineering applications.

Table 4.2: Porosity and pore size of previously designed scaffolds (continued)

<table>
<thead>
<tr>
<th>Scaffold Material</th>
<th>Channel/pore size, Porosity</th>
<th>Claims/conclusion</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(D,L-lactide-co-glycolide matrix</td>
<td>23 - 52μm, 85%</td>
<td>Scaffolds are osteoconductive</td>
<td>56</td>
</tr>
<tr>
<td>PLG, Polylactic acid</td>
<td>100-300μm, 60 – 90%</td>
<td>Useful for regeneration of tissue like blood vessel, intestine</td>
<td>57</td>
</tr>
</tbody>
</table>
Table 4.2: Porosity and pore size of previously designed scaffolds (continued)

<table>
<thead>
<tr>
<th>Material</th>
<th>Porosity Range</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(L-Lactide-co-DL-Lactide)</td>
<td>58-80%</td>
<td>Initial mechanical properties of scaffold comparable to trabecular bone</td>
</tr>
<tr>
<td>Polyurethanes</td>
<td>150 - 500μm</td>
<td>Cell scaffolds in cardiovascular tissue or other soft tissue.</td>
</tr>
<tr>
<td></td>
<td>80-97%</td>
<td></td>
</tr>
</tbody>
</table>

Porosity for a scaffold was represented by the ratio of volume of pores to the volume of the scaffold. The void fraction or porosity of scaffold was measured using Archimedes’s principle as described in the Materials and Method chapter. The foam or scaffold porosity was calculated using equation 3.2. The porosity / void fraction results are shown Figure 4.12; The porosity of the scaffold varied from 0.66 – 0.88 by varying the salt weight fraction from 0.7 – 0.9. It was statistically demonstrated using one way ANOVA (p<0.05) that there was a linear relationship between salt weight fraction (i.e. ratio of weight of salt and sum of weights of polyurethane and salt) and porosity of the scaffold.
4.3.3 Pore size measurement

Depending on envisioned applications, the pore size of the scaffold needed to be controlled. The effect of pore size on tissue regeneration is emphasized by experiments that are explained in literature review section in detail. The minimum pore size required was decided considering factors such as vascularization and the suspension size of the cell [25]. Also, it can be observed from table 4.2 that researchers had conducted cell studies with scaffolds showing pore size ranging from 100 – 300 µm and they claimed that pore size ranging from 100 - 300µm is adequate to support vascularization and cell growth.

Using 2-D image analysis as explained in Material and Method chapter the mean pore diameter of the each scaffold was measured. In this experiment to control the mean pore diameter of scaffold; two different size ranges of salt particles were 250 – 176 microns

\[ y = 1.35x - 0.3267 \]
\[ R^2 = 0.9887 \]

Figure 4.12: Linear relationship between porosity of scaffold and salt weight fraction
and 177 – 140 microns to make two different sets of scaffolds. In each set of scaffolds 0.7, 0.8 and 0.9 salt weight fraction were used. This was to check for the affect of salt weight fraction on mean pore diameter of the scaffolds. Using Two Way ANOVA, it was observed that there is statistically no significant relations between salt weight fraction and mean pore diameter of scaffold, Figure 4.13 but there was a significant relation between salt particles and mean pore diameter size, Figure 4.14.

Figure 4.13: Graph showing no significant relation (p<0.05) between salt weight fraction and mean pore diameter of the scaffold.
4.3.4 Mechanical Characteristics

In tissue engineering applications, porous scaffolds must have sufficient mechanical strength to restrain their initial structures after implantation *in vivo*. The ASTM terminology for porous materials is classified into three groups: interconnecting pores (open pores), non-connecting pores (closed pores) or a combination of both [54]. When pores are open, the foam material is usually drawn into struts forming the pore edges through open faces forming a low density solid. When the pores are closed, a network of interconnected plates produces a high density solid. The closed pores are sealed off from the neighboring pores. The interconnecting pores are critical parameter in designing a tissue engineering scaffold. The interconnecting pores should be large enough to support cell migration and proliferation in the initial stages [54].
interconnection means a low density solid, and therefore low mechanical structure. There is often a compromise between porosity and scaffold mechanical characteristics. Therefore, the biomechanical challenge in designing a scaffold is to achieve sufficient stiffness and strength in a highly porous structure to provide mechanical integrity [52]. The biostability of many implants depends on factors such as strength, stiffness, absorption at the material interface and chemical degradation [54]. The review found by Gibson and Ashby (1997) [54], found the mechanical characteristics of a porous solid depended mainly on its relative density, the properties of the material that made up the pore edges or walls and anisotropic nature cause of processing technique. Also, to get the maximum structure information all through out the height of the scaffold compression testing plays a significant role. Therefore, the investigation of compressive properties is of primary importance in determining the suitability of the designed scaffold. In this study, three samples (10mm × 15 mm × 3 mm) of PCL-HDI-DTH scaffolds with 88% porosity were tested. They were tested uniaxial testing system (Instron 4205) and 1kN load cell. The specimens were compressed in z – direction of scaffold fabrication process at cross speed of 1 mm/min between two steel platens up to a strain level of approximately 70%. The force –extension curve is shown in Figure 4.13 for scaffold samples tested in air.
Gibson and Ashby (1997) [54] classified porous material into two general groups: foams and honeycombs [54, 55]. The curve shown in figure 4.15 demonstrates the typical behavior of foam undergoing deformation. It comprises of three distinct behavior regions: a linear – elastic region followed by a plateau region and a final region of steeply rising stress. As the stress increased the 3-D pres of the scaffold were crushed and undergo densification process. Due to anisotropic nature of our polyurethane scaffold, a shorter linear region and a longer period of plateau region is observed in comparison to force-extension curve obtained after compression of
honeycomb structure [54]. The plateau region was followed by densification region where stress rises steeply. The modulus of elasticity $E \ 30 \pm 20$ kPa was calculated as the slope of initial linear portion of force-extension curve neglecting toe region due to the initial settling of the specimen shown in figure 4.16 [54, 55]

![Figure 4.16: Initial linear region for PCL-HDI-DTH scaffold under compression](image)

Previous studies by other researchers shows that scaffolds porosity ranging from 70 – 97% and compressive modulus of 40 kPa have adequate mechanical support to be used for soft tissue regeneration such as meniscus or to culture chondrocytes and smooth muscle cells (Table 4.3). The compressive modulus, $E$, for 88% porous PCL-HDI-DTH scaffold measured was $30 \pm 20$ kPa. Therefore, it was assumed that our PCL-HDI-DTH scaffold with 88% porosity and 30kPa compressive has adequate mechanical characteristics to support cell growth for future cell studies for soft tissue regeneration.
Table 4.3: Compressive modulus of scaffolds obtained by other researchers

<table>
<thead>
<tr>
<th>Scaffold Material</th>
<th>Compressive modulus</th>
<th>Claims</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyurethane</td>
<td>compression moduli of the scaffolds were between 40kPa and 400kPa</td>
<td>Scaffolds are very suitable for the use as meniscus replacement materials.</td>
<td>59</td>
</tr>
<tr>
<td>PGA Fibers</td>
<td>Compression moduli 40.86± 4.1 kPa</td>
<td>Cultured Chondrocytes and smooth muscle cells</td>
<td>60</td>
</tr>
</tbody>
</table>
CHAPTER V
CONCLUSION AND FUTURE WORK

5.1 Conclusion

This project was successful in generating a reproducible and processable L-tyrosine based polyurethane scaffold. Studies by other researchers suggest that a porosity of 88% ± 3% [1] was considered as optimum porosity to conduct cell culturing studies onto the porous scaffold. As we fail to reject null hypothesis I, that PCL-HDI-DTH scaffolds shows porosity of less than 88% (p>0.05). Therefore, the scaffold developed in this study with 88% porosity with pore size range of 300 – 150 is expected to support regeneration of adult mammalian skin as mentioned in literature review section [26]. Other than architectural property, mechanical characteristics are important in considering the immediate application of developed polymeric scaffold. As we fail to reject null hypothesis III (p>0.05) and reject null hypothesis II (p <0.05) stating that there is significant relation between mean pore diameter of scaffold made using different two different salt particle sizes (i.e. 250 – 177 μm and 176 -140 μm) and salt weight fractions of 0.7, 0.8 and 0.9. Therefore in this study, the combination of L-tyrosine based polyurethane and SCPL (Solvent casting and particulate leaching) technique the L-tyrosine based polyurethane scaffold was developed with porosity of
88% ± 1% and pore size of 250 – 140 μm was achieved which is independently controlled using calibrated salt particles, thereby making our scaffolds highly predictable and tailorable. Also as we reject null hypothesis IV (p > 0.05) stating that there is no linear regression between salt weight fraction used to fabricate scaffold and the porosity of scaffold, therefore this study was successful in showing linear relation between salt weight fraction used and porosity of scaffold. In comparison, to other researchers work that were able to develop scaffold with limited thickness to 1-2 mm our study was able to develop scaffold with higher thickness of 4 mm [5] thereby making the combination of L-tyrosine and SCPL technique a successful combination for scaffold development.

Our biodegradable porous polyurethane made using SCPL technique with compressive modulus of 30 ± 20 kPa in air, with 88% porosity, and with pore size of 250 -176 μm would be expected to be a potential candidate to conduct in vitro and in vivo cell studies for application in the field of regeneration of adult mammalian skin and smooth muscle cell seeding [59, 60].

5.2 Future work

The pore morphology in our work was observed to be anisotropic. In order to make it more isotropic, certain fabrication parameters can be varied such as the solvent evaporation rate can be controlled [55], and the shape of the porogen can be varied from irregular to regular shape (spherical)[41] to observe its effects on the pore morphology.

Also, the mechanical characteristics of L-tyrosine based polyurethane scaffold are
suggested to improve when pore morphology is made more isotropic [54, 55]. The next phase of work will be to evaluate the potential application of scaffold in regeneration of adult mammalian skin. In order to do so, firstly an *in vitro* cell culturing studies on the scaffold needs to be performed. Later this device could be placed in a bioreactor and observed over a period of time as cells regenerate their own natural extracellular matrix.
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APPENDIX

STATISTICAL ANALYSIS

1) Statistical porosity studies using MINITAB version 15 and Microsoft Excel.

1.1) Normalization results

![Probability Plot of C1](image_url)
Normalization results achieved using Anderson- Darling’s Test, \( P > 0.05 \). Hence, fail to reject null hypothesis \( \mu_0 = \) Data is normally distributed.

1.2) T- test for Null Hypothesis I

<table>
<thead>
<tr>
<th>Variable</th>
<th>N</th>
<th>Mean</th>
<th>StDev</th>
<th>SE Mean</th>
<th>Bound</th>
<th>T</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>11</td>
<td>0.88182</td>
<td>0.01991</td>
<td>0.00600</td>
<td>0.89270</td>
<td>0.30</td>
<td>0.616</td>
</tr>
</tbody>
</table>

As \( p(0.616) > 0.05 \). Hence we fail to reject null hypothesis. L- Tyrosine scaffold show 88% porosity

1.3) One – way ANNOVA for Null Hypothesis IV

\( H_{IV0}: b = 0 \) (Null Hypothesis)  \( \text{Model I: } Y = \alpha + \beta X + \varepsilon \)

<table>
<thead>
<tr>
<th>SV</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>Ft</th>
</tr>
</thead>
<tbody>
<tr>
<td>Am G</td>
<td>12</td>
<td>0.432956</td>
<td>0.03608</td>
<td>2.474155</td>
<td>2.18</td>
</tr>
<tr>
<td>With G</td>
<td>23</td>
<td>0.3354</td>
<td>0.014583</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>35</td>
<td>0.768356</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

As \( F_t < F \), hence groups with different salt weight fraction differ significantly.

<table>
<thead>
<tr>
<th>SV</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>Fs</th>
<th>Fs</th>
</tr>
</thead>
<tbody>
<tr>
<td>AG</td>
<td>12</td>
<td>0.432956</td>
<td>0.03608</td>
<td>0.083514</td>
<td>29.62547</td>
</tr>
<tr>
<td>LG</td>
<td>1</td>
<td>0.432017</td>
<td>0.432017</td>
<td>5.061.497</td>
<td></td>
</tr>
<tr>
<td>DLG</td>
<td>11</td>
<td>0.000939</td>
<td>8.54E-05</td>
<td>0.005853</td>
<td></td>
</tr>
<tr>
<td>WG</td>
<td>23</td>
<td>0.3354</td>
<td>0.014583</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>35</td>
<td>0.768356</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
$F_s > F_{(0.05,[1,2])}$, therefore we reject null hypothesis IV that there is no regression. Hence there is linear regression between salt weight fraction and porosity of polyurethane scaffold.

<table>
<thead>
<tr>
<th>Regression C</th>
<th>1.341667</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y intercept</td>
<td>-0.32222</td>
</tr>
<tr>
<td>Regression equation</td>
<td>$-0.322 + 1.3417X$</td>
</tr>
</tbody>
</table>

2) Statistical mean pore diameter studies using MINITAB version 15 and Microsoft Excel.

2.1) Effect of salt weight fraction (0.7, 0.8 and 0.9) on mean pore diameter of scaffold

$H_{II0}: \mu_{0.7} = \mu_{0.8} = \mu_{0.9}$ (Null Hypothesis)

$H_{IIa}: \mu_{0.7} \neq \mu_{0.8} \neq \mu_{0.9}$ (Alternate Hypothesis)

2.2) Effect of salt particles size (250 – 176 $\mu$m and 177 – 140 $\mu$m ) on mean pore diameter of scaffold

$H_{III0}: \mu_{250-176} = \mu_{177-140}$ (Null Hypothesis) $H_{IIia}: \text{At least one}$ (Alternate Hypothesis)

<table>
<thead>
<tr>
<th>SV</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>Fs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subgroups</td>
<td>5</td>
<td>35943.3</td>
<td>7188.66</td>
<td></td>
</tr>
<tr>
<td>A(col)/SWF</td>
<td>2</td>
<td>678.1625</td>
<td>339.0812</td>
<td>0.009992304</td>
</tr>
<tr>
<td>B(rows)/SPZ</td>
<td>1</td>
<td>33934.24</td>
<td>33934.24</td>
<td>50.99458241</td>
</tr>
<tr>
<td>A * B(int)</td>
<td>2</td>
<td>1330.896</td>
<td>665.4479</td>
<td>0.001506238</td>
</tr>
<tr>
<td>Within</td>
<td>96</td>
<td>42412300</td>
<td>441794.8</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>101</td>
<td>42448243</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

|             |       |           |           |      |
| Row/SPZ     | $F_{0.05,[2,96]}$ | 3.07       |           |      |
| Col/SWF     | $F_{0.05,[1,96]}$ | 3.92       |           |      |
| Int         | $F_{0.0012,96}$  | 11.4       |           |      |
* SP = Salt particles size

* SPF = Salt weight fraction

As $F_{SWF} (50.995) > F_{0.05,[1,96]}$, therefore we reject null hypothesis II and therefore, mean pore diameter of scaffold with salt weight fraction 0.7, 0.8 and 0.9 does not differ significantly.

As $F_{SWZ} (0.009) < F_{0.05,[1,96]}$, therefore we fail to reject null hypothesis III and therefore, mean pore diameter of scaffold with salt particle size 250 – 176 μm and 177 – 140 μm differ significantly.

Also, $F_{int} (0.001) < F_{0.001,[2,96]}$, therefore it is proved that salt particles size range (250 – 176 μm and 177 – 140 μm) affects the mean pore diameter of scaffold with 3 different weight fraction (0.7, 0.8 and 0.9)

1.4) Reliability test for 2-D image analysis

<table>
<thead>
<tr>
<th></th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>Fs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among grp</td>
<td>3</td>
<td>19.18616</td>
<td>6.395386</td>
<td>0.005133</td>
</tr>
<tr>
<td>Within grp</td>
<td>6</td>
<td>7475.048</td>
<td>1245.841</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>9</td>
<td>7494.234</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$Ft=4.76 > 0.00513$, Groups do not differ significantly. The mean pore diameter measured on day 1, day 3 and day 5 didn't differ significantly.