KAUNAS UNIVERSITY OF TECHNOLOGY

FACULTY OF ELECTRICAL AND ELECTRONICS ENGINEERING

Aradhya Mallikarjunaiah Chetan

A METHOD TO FABRICATE SOLID FREE FORM SCAFFOLDS FOR LIVER TISSUE ENGINEERING BY USING 3D PRINTING

Master's thesis

Supervisor
Assoc. prof. dr. Vaidotas Marozas

Consultant
Assoc. prof. dr. Anders Wolff,
Technical University of Denmark

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Supervisor
(parašas) Assoc. prof. dr. Vaidotas Marozas
(data)

Reviewer
(parašas) Prof. habil. dr. Arūnas Lukoševičius
(data)

Author
(parašas) Aradhya Mallikarjunaiah Chetan
(data)

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Aradhya Mallikarjunaiah Chetan

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Final project „A Method to Fabricate Solid Free Form Scaffolds for Liver Tissue Engineering by Using 3D Printing“

ACADEMIC HONESTY DECLARATION

20 15 m. May 20 d.

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I confirm that my **Aradhya Mallikarjunaiah Chetan** final project on "A method to fabricate solid free from scaffolds for liver tissue engineering by using 3D printer" is written completely independently and all of the data or results are correct and received honestly. This work does not include of any plagiated part from printed or online sources, all other sources of direct and indirect quotes are provided in the list of references. I have not made any illegal payments of money for this work.

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SUMMARY

In recent years, there has been a great demand for the development of bio-artificial organs/tissues in the field of organ transplantation and in-vitro toxicological drug screening (L. G. Griffith & Swartz, 2006). Engineering of functional tissue/organs by controlling the cells microenvironment to resemble *in vivo situation* is of central importance. This has led to the evolution of various techniques for controlling the cellular microenvironment to facilitate cell proliferation, organization and differentiation. When engineering tissues in vitro, there is a requirement for structures or scaffolds that are able to support cell growth, and that closely mimic the physiological environment including the geometrical, topographical and physical features of the targeted tissue. Specifically for the generation of thick 3D tissues, the development of highly dense vascular networks that are able to meet the nutrient and oxygen requirements of large masses of living cells remains a tissue engineering challenge often limiting the size of engineered tissues to a few hundred microns (Khademhosseini, Vacanti, & Langer, 2009). The ideal tissue engineering scaffold supports the spatial distribution of cells in a three dimensional structure, provides mechanical stability to the cells and enables optimum nutrient transport and metabolic waste removal (Hoganson, Pryor, & Vacanti, 2008; Lu, Li, & Chen, 2013). There have been many approaches to create three-dimensional (3D) highly vascularized engineered tissue scaffolds to accommodate a high density of cells in high surface to volume ratio structures (Almeida & Bártolo, 2014; Lu et al., 2013). One strategy that has been employed is the use of highly porous structures with interconnected pores/micro channels that provide space for penetration and growth of cells and enable favourable mass transport characteristics (Langer, 2009)(Guillemette et al., 2010). The structural, mechanical and mass transport properties of such scaffolds are determined by parameters such as pore size, pore shape, porosity, pore interconnectivity, permeability, scaffold surface area, scaffold effective stiffness and scaffold material (Jeong & Hollister, 2010).

This work presents a novel approach for manufacturing structured pores/channels in a scalable 3D elastomeric scaffold. The method involves 3D printing (using a commercially available 3D printer) a sacrificial PVA mould whose geometrical features are designed according to the

required vascular channel network. In addition to its biocompatibility, PVA is an idea material for use as a sacrificial mould because it's water solubility in combination with its high melting temperature (190°C) which makes it robust for subsequent polymer casting and curing steps. The required polymer is cast around the PVA mould and following cross linking, the mould is dissolved leaving behind a structured porous elastomeric scaffold.

In brief, work carried out through these steps are, scaffold *design* is done through Solid Works and Make Ware software. 3D printing of mould by using PVA filament is done by Maker Bot printer. The two types of polymers are used, PDMS (synthetic polymer), Silk Protein (natural polymer) to fabricate tissue scaffolds. Fabrications steps are described below. Obtained scaffold was examined by using SEM and mechanical testing's.

Keywords (up to 8 words): PDMS scaffolds, Silk protein based scaffolds, Solid Freeform Fabrication, 3D printing for liver tissue engineering.

TABLE OF CONTENTS

ABBREVIATIONS AND GLOSSARY

SEM	Scanning electron microscope
PDMS	Polydimethylsiloxane - belongs to a group of polymeric organosilicon compounds that are commonly referred to as silicones
TE	Tissue Engineering
CATE	Computer Aided Tissue Engineering
CAD	Computer Aided Design
LiBr	Lithium bromide
PVA	Poly (vinyl alcohol) - a water soluble synthetic polymer.
ABS	Acrylonitrile butadiene styrene - is a common thermoplastic polymer. Its glass transition temperature is approximately 105 °C (221 °F) and used to clean the nozzles of the 3D printer
3D	Three Dimension
FDM	Fuse deposition modelling
SLS	Selective Laser Sintering

1 INTRODUCTION

The limited supply of donors and increasing morbidity have put new demands on tissue engineering as a treatment of organ failures, Tissue Engineering and 3d printing technology has an important role in the field of medicine. It is the combination of cells and Biomaterials to form a functional artificial organs with a goal of organ transplantation. Every year, millions of people suffer tissue loss or end-stage organ failure and the treatments include organ transplantation, surgical reconstruction, or implanting medical devices (Langer & Vacanti, n.d.). Though the organ transplantation has been a major scientific and clinical breakthrough in the 20th century, still it has associated with many problems, such as lack of organ donors and/or rejection of the transplanted organs and life-long heavy medication with side effects (Dolatshahi-Pirouz, Nikkhah, Kolind, Dokmeci, & Khademhosseini, 2011)(Saltzman & Olbricht, 2002). To address all these issues, the interdisciplinary field of tissue engineering has emerged with 3D culture models in the past few years to generate biological tissue constructs outside the body that maintain or enhance normal tissue function and also provides physiologically relevant models that suits for the study of normal cell/tissue functions and disease progression, as well as for the development of new predictive toxicological investigations(L. G. Griffith & Swartz, 2006)(Mazzoleni, Di Lorenzo, & Steimberg, 2009). Apart from serving for therapeutic purposes, 3D cultures are also being introduced into drug screening as promising platforms to avoid the high failure rate of models that miss or alter many tissue related functions and to analyse the effect of drug action, improving the effectiveness and reducing the investment of this process. They are able to recreate in a more realistic way the complexity of human tissues, while retaining the ability for high-throughput screening and cellular level imaging(Castells-sala et al., 2013).

1.1 3D Printing and culture models

The Tissue Engineering approach involves regenerating tissue within suitable scaffold with the goal of implanting the constructed tissue at the target site. The regeneration of functional tissue requires a suitable microenvironment that closely mimics the host site for desired cellular responses. Such an environment is typically provided by 3-D tissue engineering scaffold (Kundu, Rajkhowa, Kundu, & Wang, 2013).

Use of 3D tissue scaffolds as a template for regeneration is the basis of tissue engineering. These tools will enable a better understanding cell-scaffold interactions, including identification of

the relationships of cell response on 2D surfaces to that in 3D scaffolds, and will facilitate improved design of future scaffold-based medical products. These measurement tools and standards advance the ability of researchers to develop scaffolds that direct stem cell differentiation.

3D printing technology has been investigated for producing, biocompatible, scalable materials. Scaffold design previously focused on the incorporation of macro scale features such as interconnected pores for nutrient transport and tissue remodelling. One strategy to further augment the function of tissue-engineered constructs is to mimic the in vivo tissue microarchitecture and cellular microenvironment.

Three dimensional printing technology was developed in MIT (Ballarin & Cima, 2005) and was one of the first SFF techniques to be used in the fabrication of scaffolds and drug delivery applications. At present, the commercially available 3D printer (Maker Bot 3D printer) is used to for the 3d printing of tissue scaffolds. The working methods of Maker bot printer is discussed below.

The maker bot 3d Printer is a filament based printer, which uses the filaments like, PVA, PLA, and ABS. In this project the filament PVA is used to print the moulds which is fabricated later with different polymers. This PVA mould is used for both PDMS based scaffolds and Silk protein based scaffolds.

Advantages:

1. Computer guided fabrication process with direct linkage to CAD software packages. This allows for repeatable and accurate reproduction of scaffolds since the data is obtained from the same master file.
2. Bio-friendly manufacturing environment with no excessive use of heat or harsh chemicals.

The most powerful model for studying cellular functions such as cell-to-cell communication in a 3D environment is a living organism such as animal model. This model can display the integrated responses that result from complex interactions between tissues and organs. However, it fails to capture important facets of human responses, are very costly, time-consuming and ethically controversial(L. G. Griffith & Swartz, 2006). Hence, we need to develop model systems that mimic living tissue as accurately as possible, as the requirement for more physiologically relevant biological systems has become apparent resulting in a rapid increase in the use and development of model systems in recent years.

1.2 Scaffold based culture systems

Rather than simply introducing cells into a diseased area to repopulate a defect and/or restore function, in tissue engineering the cells are often seeded in or onto biomaterials before transplantation(Yarlagadda, Chandrasekharan, & Shyan, 2005). These materials serve as temporary scaffolds and promote the reorganization of the cells to form a functional tissue. Scaffold-based culture systems use synthetic (polyglycolic acid) or naturally derived (type I collagen) biomaterials that simulate the ECM and attempt to direct the growth of cells in a specific 3D spatial configuration while maintaining their differentiated function. A major goal of such system is to make the scaffolds capable of recreating the *in vivo* microenvironment, which is mainly provided by the ECM. Thereby, the important characteristics of engineered scaffolds are analogous to the functions of ECM in native tissues and are associated with their structural, biological, and mechanical features as shown in Table 1.

An essential function of the ECM is to give anchorage to cells. Indeed, the ECM highly porous nanostructure provides them a proper 3D microenvironment and imparts biochemical signalling through two mechanisms:

(i) the binding of a wide variety of soluble Growth Factors (GF), enzymes and other effector molecules, controlling their diffusion and local concentrations

(ii) The exposure of specific motifs that are recognized by cellular adhesion receptors. As a result, ECM is dynamically integrated with the intracellular signalling pathways that regulate gene expression and participate in cell phenotype determination(Frantz, Stewart, & Weaver, 2010)(Kim, Turnbull, & Guimond, 2011).

1.3 Scaffold architecture design

The structure and architecture of scaffolds are crucial factors in scaffold-based tissue engineering as they affect the functionality of the tissue engineered constructs and the eventual application in health care. So, scaffolds should have suitable architecture and strength to serve their intended function(Yeong, Chua, Leong, & Chandrasekaran, 2004). It should be able to permit cell intrusion, nutrient and waste product permeation, and new capillary network formation(Walker & Ditor, n.d.). However, there are no hard and fast scaffold-design targets defined for a given tissue application. It is still unknown what mechanical properties scaffolds need to optimally function within specific tissue defects. Furthermore, despite significant advances in surface-modification methods and the development of new materials, there are still significant gaps in our knowledge as

to what surface chemistry, growth factor release, and mass-transport characteristics best accelerate a specific tissue formation(Lu et al., 2013). Thus, there are no guidelines specifying what material a scaffold should be made from, what linear or nonlinear elastic properties a scaffold should exhibit, what surface chemistry a scaffold should have, nor what permeability or diffusion properties a scaffold should exhibit. What is known are general targets of mechanical function, mass transport, and surface chemistry that may be broken down in categories based on hard versus soft tissue and vascular versus avascular tissues(Hollister, 2009).

Though it's not possible to define the definite structure and architecture parameters for scaffolds to assess the functionality of the scaffold, but still it's important to consider some of the parameters to achieve best results, which includes:

Pore size: One of the main parameters affecting the efficiency of initial cell seeding is the pore size. Pore size is critical in controlling both tissue in growth and the internal surface area available for cell attachment. If the pores are too big, the cells will not recognize the specific micro features of the scaffolds and if they are too small, then they become occluded by the cells, which will prevent cellular penetration, ECM production, and neovascularization into the inner regions of the scaffold(Loh, Choong, Oxon, Hons, & Mimmm, 2013). A further concern is the alteration of the effective pore structure *in vivo* because as the matrices biodegrade, the average pore size will increase and the interconnectivity of the pore structure will change overtime(Chua, 2001). Hence, the pore size is considered as a key factor in designing a substrate for 3D cell culture.

Scaffold morphology: Rapid prototyping fabricated scaffolds generally present surface roughness in form of edges and grooves. This surface roughness of the scaffold is important in cell–matrix interactions and might enhance cell adhesion. However, if the surface is too rough, the cells adhering to these materials might not be able to develop distinct focal adhesion plaques or bridge the irregularities. Moreover, the sharpness of the surface could damage the cell physically. The smooth surface of solidified materials cannot ensure firm cell adhesion and therefore require further surface modification or coating(Dolatshahi-Pirouz et al., 2011)(Yeong et al., 2004).

Pore characterization: Evaluating the pore characteristics of a fabricated scaffold is an important step in any tissue engineering approach(Loh et al., 2013). Porous materials including tissue engineering scaffolds may contain three types of pores (Figure 1.3.1).

(1) Closed pores that are isolated within the structure and are not accessible.

(2) Open pores that connect the internal structure to the outer surface and permit the flow of liquids and/or gases.

(3) Blind pores that connect with an exposed internal or external surface.

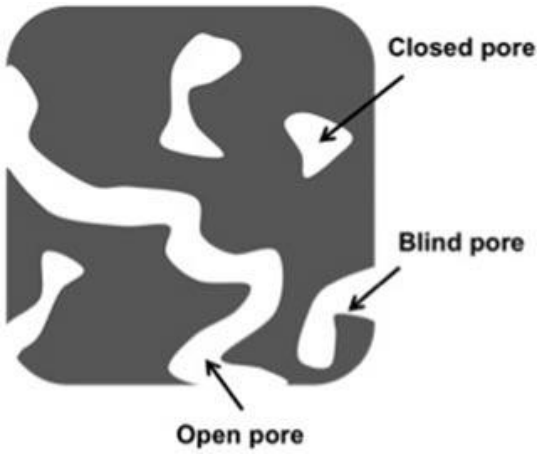


Figure 1.1 Different types of pores found in porous structures (Chung & King, 2008)

All of these pores have a role to play in tissue engineering; closed and blind pores can reduce the path length for diffusion of gases to the core of the scaffold, whereas open pores are key to ensure that the scaffold functions as a conduit for cell proliferation and nutrient diffusion. These different pore types increase the complexity and challenge of accurately characterizing the scaffold structure(Chung & King, 2008).

Porosity and interconnectivity: Scaffold porosity is a fundamental characteristic for providing available space for cells to migrate and for vascularization of the tissue. Furthermore, the larger the surface available, the more cell interactions will arise. Although high porosity is necessary for ensuring uniform cell delivery and tissue in growth, the mechanical strength of a scaffold tends to decrease rapidly as the total porosity increases (Yeong et al., 2004) (Papenburg et al., 2009). Thus, for polymeric scaffolds, there may be a conflict between optimizing the total porosity and maximizing the mechanical properties. So, the total porosity value should always be balanced with the immediate mechanical needs of the particular tissue that is going to be replaced. In addition to total porosity, cell transport within a scaffold, such as diffusion, attachment, and migration, is also controlled by pore interconnectivity, and the surface area of the scaffold. Scaffolds should be highly porous with an open, fully interconnected geometry, and a large surface area–volume ratio that will allow cell in growth, uniform cell distribution, and facilitate the vascularization of the construct(Chung & King, 2008).

Mechanical properties: In general, the mechanical properties of the scaffold should be consistent with the anatomical site into which it has to be implanted, also it should be strong enough to withstand typical hydrostatic or pulsatile pressures that can be found in the physiological

environment, as well as maintain the space or pore volume required for cell in growth and matrix production(O'Brien, 2011)(Chung & King, 2008).

In short we can say, a large surface to volume ratio would assist cellular adhesion. Moreover, pore sizes should be sufficiently large so as to encourage cellular in growth. Depending on the tissue type (soft or hard tissues), some of the tissue engineered constructs must possess sufficient mechanical strength so as to withstand loading, thus strut/wall thickness, anisotropy and the cross sectional area of the scaffold would also be of interest.

2 LITERATURE ANALYSIS

2.1 Scaffold Fabrication using SSF

To get a well interconnected fabricated scaffolds with suitable porosity, several traditional methods and SSF methods have been developed. The representative traditional methods are salt leaching, solvent casting, phase separation, gas foaming and freeze drying. Application of solid freeform fabrication (SFF) technologies in the design and fabrication of 3d scaffolds/constructs in tissue engineering constitutes the third component in CATE. SFF systems are directly linked to CAD software which facilitates the design of the scaffold in a virtual environment after which the CAD model is transferred to the systems for fabrication. Unlike, traditional machining, the SFF system builds parts by selectively adding material as specified by the CAD model. Since the 1990's, SFF technology has been primarily used in the rapid prototyping industry to make prototypes of parts that were designed in CAD. This technology allowed the rapid fabrication of conceptual designs, thereby allowing for design changes early on in the product development cycle. However, over the last 6yrs, researchers began to use these technologies to produce scaffolds for tissue engineering. The system's ability to achieve precise control over material distribution, high level of design capability in the model's internal structure and the ability to fabricate highly reproducible scaffolds with a variety of composite biomaterials makes it attractive for tissue engineering applications. These reasons have mainly contributed to making the SFF process the most favoured approach for the fabrication of engineered scaffolds/constructs.

Porous 3D scaffolds can be fabricated from various conventional and rapid prototyping techniques, depending on the type of materials used or type of pore structures needed(Loh et al., 2013). Several Conventional scaffold fabrication techniques have been developed to process synthetic and natural scaffold materials into porous structures. These techniques possess its own

advantages and limitations (Table 3) based on specific technology used out of various techniques available like Solvent-casting Particulate leaching, Gas foaming, Fibre meshes, Phase separation, melt moulding, emulsion freeze drying, solution casting and freeze drying (Sachlos & Czernuszka, 2003). These fabrication techniques are defined herein as processes that create scaffolds having a continuous, uninterrupted pore structure which lacks any long-range channelling microarchitecture(Visconti et al., 2010). However, there are inherent limitations in these processing methods, which offer little capability precisely to control pore size, pore geometry, pore interconnectivity, spatial distribution of pores and construction of internal channels within the scaffold(Leong, Cheah, & Chua, 2003; Yeong et al., 2004). So, these conventional techniques require good fabrication skills to maintain consistency in scaffold architecture.

Scaffolds produced by solvent-casting particulate-leaching cannot guarantee interconnection of pores because this is dependent on whether the adjacent salt particles are in contact. Furthermore, skin layers are formed during evaporation and agglomeration of salt particles makes controlling the pore size difficult. Moreover, only thin scaffold cross-sections can be produced due to difficulty in removing salt particles deep in the matrix. For gas foaming, it has been reported that only 10-30% of the pores were interconnected (Sachlos & Czernuszka, 2003). Non-woven fibre meshes have poor mechanical integrity. Excluding gas foaming and melt moulding, conventional scaffold fabrication techniques use organic solvents, like chloroform and methylene chloride, to dissolve synthetic polymers at some stage in the process. The presence of residual organic solvent is the most significant problem facing these techniques due to the risks of toxicity and carcinogenicity it poses to cells. In short we could say that these techniques are manual-based processes with characteristics that may not be suitable for achieving customized production. Further to this, the imperfections in scaffolds fabricated using these techniques due to the poor flexibility and control offered by the techniques, limit the application of the scaffolds(Choi et al., 2007).

In general, conventional fabrication techniques do not enable precise control of internal scaffold architecture or the fabrication of complex architectures that could be achieved by Solid freeform fabrication (SFF) techniques using computer-aided design (CAD) modelling (Loh et al., 2013). Computer-controlled fabrication via SFF technology may hold the key for a generic solution in automating scaffold production that can cater for variations in the shapes and requirements of different tissues and organs and also size variations between different individuals(Gauvin et al., 2012). The use of SFF techniques promises new cost-effective and rapid solutions to customized made-to-order TE scaffold production(Leong et al., 2003). The common SFF technology includes

3D printing, Stereo-lithography, Fused deposition modelling, selective laser sintering, 3D plotter, phase-change jet printing etc. Each SFF technique has some advantages or limitations over another (Table 1).

Table 1 Capabilities and limitations of SFF fabrication techniques (Leong et al., 2003)

Technique	**Advantages**	**Disadvantages**
3D-P	Easy process High porosity High surface area to volume ratio Complete pore interconnectivity Macro shape control Independent control of porosity and pore size Wide range of materials	Use of toxic organic solvents Lack of mechanical strength Limited to small pore sizes
FDM	High porosity High surface area to volume ratio Complete pore interconnectivity Macro shape control Independent control of porosity and pore size Good compressive strength Solvent free	High processing temperatures Limited material range Inconsistent pore opening in x-y and z-directions Pore occlusion at boundaries Requires support structure for irregular shapes
SLS	High porosity High surface area to volume ratio Complete pore interconnectivity Macro shape control	High processing temperatures Limited to small pore sizes

	Independent control of porosity and pore size Good compressive strength Wide range of materials Solvent free	

SFF techniques involve building 3D objects using layered manufacturing strategies. Although there are several commercial variants of SFF technology, the general process involves producing a computer-generated model using computer-aided design (CAD) software(Leong et al., 2003). This CAD model is then expressed as a series of cross-sectional layers. The data is then implemented to the SFF machine, which produces the physical model. Starting from the bottom and building layers up, each newly formed layer adheres to the previous. Each layer corresponds to a cross sectional division. Post-processing may be required to remove temporary support structures (Sachlos & Czernuszka, 2003). SFF can improve current scaffold design by controlling scaffold parameters such as pore size, porosity and pore distribution, as well as incorporating an artificial vascular system, thereby increasing the mass transport of oxygen and nutrients into the interior of the scaffold and supporting cellular growth in that region. Optimizing the scaffold is essential if tissue engineering is going to be successful in replacing or repairing damaged human tissues. SFF has the potential to optimize these scaffolds and make them work(Hollister, 2005)(Karande, Ong, & Agrawal, 2004).

2.2 Silk Protein Fibroin for the 3D scaffold fabrication

Fibroin is a silk protein, which is insoluble protein made by the larvae of Bombyx mori, Spider, scorpions, mites, files and some other insects. Silk protein fibroin from Bombyx mori is can be effectively used as scaffolding material for tissue engineering and to produce a biomedical devices. It possesses good biocompatibility, suitable mechanical properties and is produced in bulk in the textile sector. The unique combination of elasticity and strength along with mammalian cell compatibility makes silk fibro- in an attractive material for tissue engineering.

Using synthetic polymer is having drawbacks such as hydrophobicity, degradability. To maintain stable cell culture conditions (such as, biocompatibility, hydrophobicity, small molecule absorption and degradability) Synthetic materials has a number of drawback issues such as biocompatibility, hydrophobicity, small molecule absorption and degradability. To maintain stable

cell culture conditions and to reduce the negative effects of synthetic materials, the use of natural biopolymers such as silk might be a sustainable materials of choice. Silk fibroin is a natural structural polymer protein purifying from domesticated silkworm (Bombyx mori) cocoons that has recently been demonstrated remarkable mechanical properties such as excellent biocompatibility, controlled biodegradability and low immunogenicity. Silk can be prepared in a range of material formats, including films, hydrogels and microspheres (Figure 3.3.1). Here, the silk fibers were used to prepare silk solution during fabrication process.

Silk in raw state consists of two proteins are sericin and fibroin, sericin is being a gum coating the fibers and allowing them to stick each other's. It has been linked to in vivo inflammation and therefore the silk is ‘degummed’ before further processing (Kundu et al., 2013). Later fibroin protein as silk fibers form used to prepare silk solution for silk based scaffold fabrication process.

Silk offers an attractive balance of modulus, breaking strength, and elongation, which contributes to its good toughness and ductility. Silk fibers are tougher than Kevlar, which is used as a bench mark in high performance fiber technology. The strength-to-density ratio of silk is up to ten times higher than that of steel. Considering the good strength and toughness of silk fibers, it is no surprise that silk has been exploited to develop scaffolds for load bearing tissue engineering.

Silk has several major advantages over other protein based biomaterials, which are derived from tissues of allogeneic or xenogeneic origins.

1. As such, the risk of infection is high for those materials.
2. Processing of such materials is also expensive due to the stringent protein isolation and purification protocols.
3. In contrast, silk is an established textile fiber and nearly 1000 metric tons of silk are produced and processed annually.
4. It is also economically advantageous to use silk for biomedical applications, because of available large scale processing infrastructure of traditional silk textile industries.

Biocompatibility of Silk: Silk has been used as suture material since centuries proves its biocompatibility. But, like any other non-autologous bio- materials causing foreign body response, some adverse immunological events associated with silk proteins cannot be ruled out, particularly due to its non-mammalian origin. Numerous in vitro studies have demonstrated that once sericin is extracted, fibroin supports cell attachment and proliferation for a variety of cell types. Silk-based biomaterials are at least as biocompatible in terms of inflammatory response and ability to support cell proliferation as many materials currently in use. The degradation behaviour can be

tailored, depending on the application, from a few days to many months. These materials are promising for use in wound healing and as tissue engineering scaffolds, particularly for the development of skeletal tissue. Other applications, such as the use of silk for nerve regeneration, are yet to be fully investigated.(Kundu et al., 2013)

2.3 Sacrificial moulds to fabricate scaffolds

This method usually involves casting of biocompatible material in a mould made by SFF systems and then removing or sacrificing the mould to obtain the final scaffold(Yeong et al., 2004). The use of sacrificial moulds in fabricating scaffolds avoids some of the processing issues caused by direct use of SFF systems in scaffold fabrication like difficulty in support powder removal, use of toxic organic binders, high temperature processing preventing the use of biological molecules and poor mechanical strength. The mould is designed to possess the negative shape of the envisioned scaffold and to retain the computer control and high resolution offered by SFF(Sachlos & Czernuszka, 2003). Moreover, such techniques enable the user to control both the external and the internal morphology of the final construct. In addition, indirect methods also require less raw scaffold material while increasing the range of materials that can be used and making it possible to use composite blends that might require conflicting processing parameters. The original properties of the biomaterial are well conserved because no heating process is imposed on the scaffold material(Loh et al., 2013)(Yeong et al., 2004)(Ahn, Lee, Cho, Chun, & Kim, 2011).

2.4 Aims and Objectives

The aim of this work - to develop biocompatible porous scaffolds with well interconnected micro-channels using solid free form fabrication process.

Objectives:

1. To design scaffold using computer aided design software
2. To print of poly vinyl alcohol mould using 3D printer
3. To design scaffold using polydimethylsiloxane polymer
4. To design scaffold using Silk Protein
5. To evaluate fabricated scaffolds morphology by porosity measurements and scanning electron microscopy

3 DEVELOPMENT OF 3D SCAFFOLD BY USING SOLID FREE FORM FABRICATION PROCESS

3.1 Materials and methods for PVA mould printing using Maker Bot 3d Printer

Tissue engineering and 3D printing have become vitally important to the future of medicine for many reasons. 3D printing can work in two patterns weather by combining things or by removing things. The Maker Bot 3d printer is the filament based printer which prints structure layer by layers and it is known as filament fuse deposition modelling or filament 3d printing. There are different types of filaments that can be used for 3D printing: PVA, ABS, or PLA. Here the material PVA (Polyvinyl alcohol) is used to design the 3D structure. PVA is a special plastic that is water-soluble. It is most commonly used as paper adhesive, as thickener, as packaging film etc. PVA is a water soluble synthetic polymer which helps as support material to fabricate tissue scaffolds using PDMS and Silk solutions. The finished object can be put into water until the PVA has completely dissolved, freeing the object of the support structure, without the need of any pesky manual post-printing curing, PVA is normally extruded a temperature of 190°C, but is not very easy to use, as it attracts water so much. Ambient air moisture will deteriorate the filament very quickly. PVA needs to be stored in a sealed box or container together with a desiccant and may need to be dried before use.

Figure 3.1 PVA filament

3.1.1 Maker Bot 3D Printer

Maker bot 3D printer is a filament based 3d printer, where PVA filament is used to print structures. The minimum resolution of this 3d printer is 100um thinness per layer. The printer has mainly 4 parts are, filament tubes where filament comes through, Extruders which can melt temperature up to 250˚C and moves X & Y directions, Built platform which holds the structure and

its moves in Z direction, and SD card slot where programing software file can load. The 3d design of scaffold was made in solid works software and required modification of scaffold is made in the software maker ware. Modification are made through adjusting the parameters like temperature, layer height, infill density, speed of nozzles etc. The Maker Bot printer is shown below.

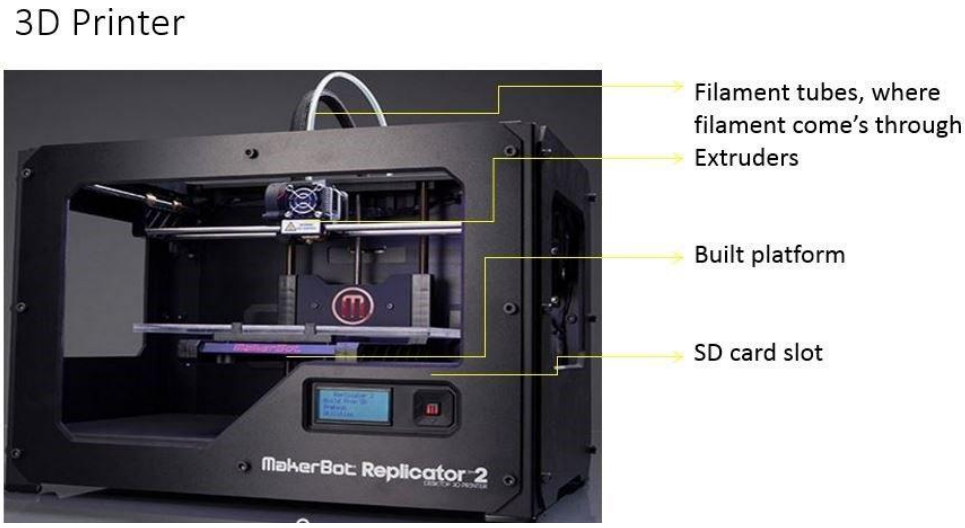


Figure 3.2 Maker Bot 3D Printer

3.1.2 Scaffold Designing

In the Beginning step, scaffold drawing is done by using 'SolidWorks' software (figure 3.1.3) and the G code file (DWG file format) which has created in 'SolidWorks' has to load into 'Maker ware' software, then parameters has to be set according to our requirements. All these process has shown below.

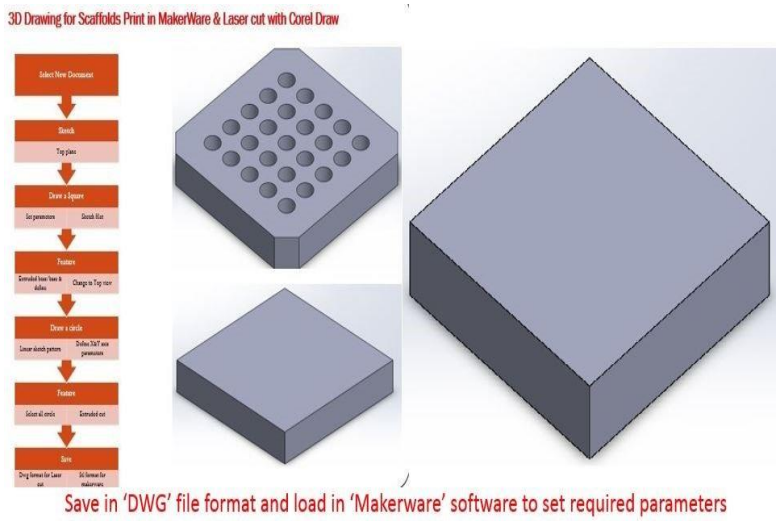


Figure 3.3 Scaffold design in Solid works software

Later the DWG files (Also X3g and STL files) was loaded into Maker ware software and parameters were set according to requirements and makerware software's description shown in figure 3.1.4.

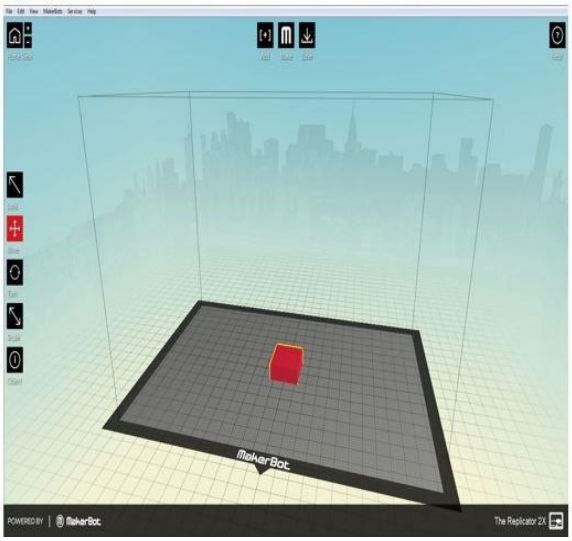


Figure 3.4 Makerware software

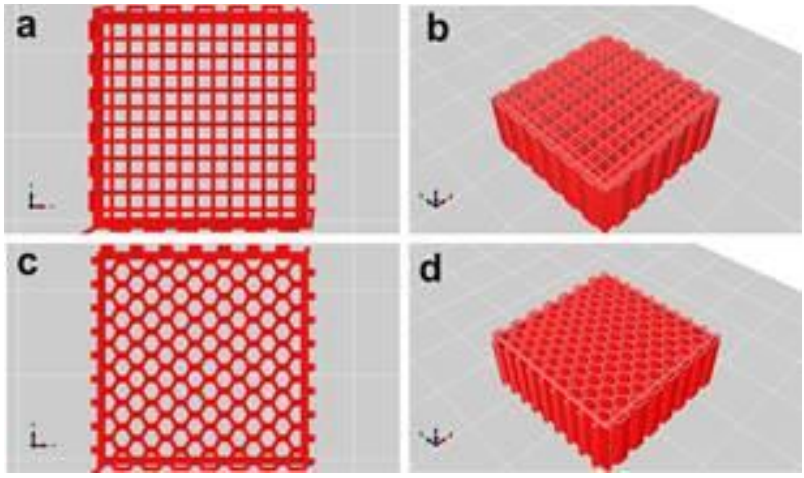


Figure 3.5 2D and 3D view visualizations of designed moulds varying in infill densities and pattern from Repetier-Host V1 Beta1 software

STL file was loaded into makerware software, then parameters was set according to our scaffold requirements. Those parameters are,

Parameters

- "Layer Height": It defines height of each layer
- "Extruder Temp": It defines the target temperature of extruders
- "Platform Temperature": It defines the target temperature of built plate
- "Number of shells": It defines the number of outline of scaffold

- “Roof thickness”: It defines the thickness of the roof of the scaffold
- “Floor thickness”: It defines the thickness of the floor of the scaffold
- ” sparselnfillPattern”: It defines the infill pattern
- “infillDensity”: It defines the infill density and must set between 0 & 1
- “feedrate”: Defines the speed of the extruder
- “firstlayer”: It defines the speed during first layer
- “infill”: It defines the speed during infill
- “outlines”: It defines the speed during outlines

Figure 3.5 shows the 2D & 3D view visualizations of designed moulds varying in infill densities and pattern from Repetier-Host V1 Beta1 software. The designed moulds were with 20p Infill density. Makerbot 3d printer can print two types of structures are linear (a) and Hexagonal (b) which is demonstrated in the figure 3.5.

After setting parameters we can go for printer settings, which are nozzle leaning, loading filament and level the base. After adjusting everything, we can able to start printing 3d scaffolds. The printed 3d scaffolds with different infill density are shown below.

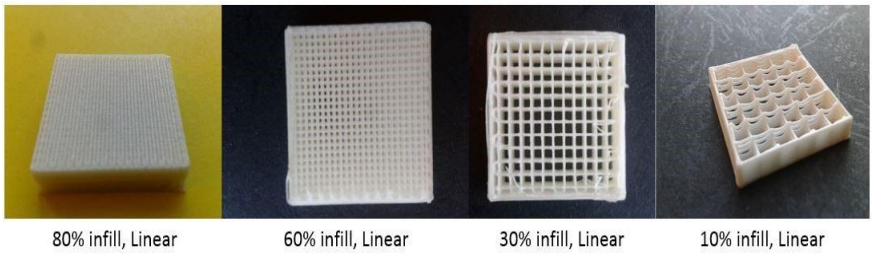


Figure 3.6 3D PVA moulds with different infildensity

The figure 3.1.5 shows the scaffolds with different infill density, as percentage increases, the length and width between filaments is decreasing. The filament gap is more in 10% infill than 80% infill scaffold. In MakerBot printer, we can print in two different structure are, Linear and Hexagonal.

3.2 Materials and methods for fabrication of PDMS scaffold

PDMS as a material is inexpensive, flexible, and optically transparent down to 230 nm (and therefore compatible with many optical methods for detection). It is compatible with biological studies because it is impermeable to water, nontoxic to cells, major advantage of PDMS it can be

fabricated and bonded to other surfaces. In this procedure this PDMS was bonded to 3D printed PVA mould to fabricate the PDMS scaffold.(Sia & Whitesides, 2003)

Polydimethylsiloxane called PDMS or dimethicone is a polymer widely used for the fabrication and prototyping of microfluidic chips. It is a mineral-organic polymer (a structure containing carbon and silicon) of the siloxane family (word derived from silicon, oxygen and alkane). Apart from 3d scaffold fabrication for tissue engineering, it is used as a food additive (E900), in shampoos, and as an anti-foaming agent in beverages or in lubricating oils. For the fabrication of 3d tissue scaffolds, PDMS (liquid) mixed with a cross-linking agent is poured into PVA mould and heated to obtain a elastomeric replica of the mould (PDMS cross-linked).

A little bit of chemistry will help us to better understand the advantages and drawbacks of PDMS for tissue engineering applications. The PDMS empirical formula is (C2H6OSi)n and its fragmented formula is CH3[Si(CH3)2O]nSi(CH3)3, n being the number of monomers repetitions. Depending on the size of monomers chain, the non-cross-linked PDMS may be almost liquid (low n) or semi-solid (high n). The siloxane bonds enable to obtain a flexible polymer chain with a high level of viscoelasticity. After cross-linking PDMS becomes a hydrophobic elastomer. Polar solvents such as water struggle to wet the PDMS (water beads and does not spread) and this leads to the adsorption of hydrophobic contaminants from water on PDMS surface.

Step A: PDMS preparation

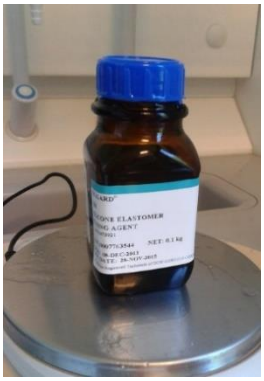
1: Then PDMS (polydimethylsilicones) mixture was prepared by mixing PDMS and its curing agent in the ratio of 10:1. This work carried out under fume hood for safety reasons.

2: This mixture was mixed for 3minutes and kept in desiccator to remove the bubbles.

3: PDMS mixture was removed from Desiccator after 20minutes.

a)

b)



c)

d)

Figure 3.7 Fume hood (a), PDMS (Silicon elastomer) (b), Curing agent (c), Desiccator (d)

Step B: Fabrication Process

PDMS mixture and 3D printed PVA mould were collected. The PDMS fabrication process produced three different scaffolds (PDMS sponge) are Structure, Random, and Combined structures.

Structured porous scaffold: 3D printing is a type of RP technique used to fabricate the moulds to prepare scaffold. This mould was printed using 3d printer was directly involved in casting process without salt particle or any other crystals. Prepared PDMS were directly poured into PVA scaffolds and kept in desiccator to remove bubbles and then kept in oven for overnight at 50°C. Later excess PDMS were removed and then dissolve in water of about 2 hours until PVA get completely dissolved. Then its SEM image was taken and shown in figure 3.2.2.

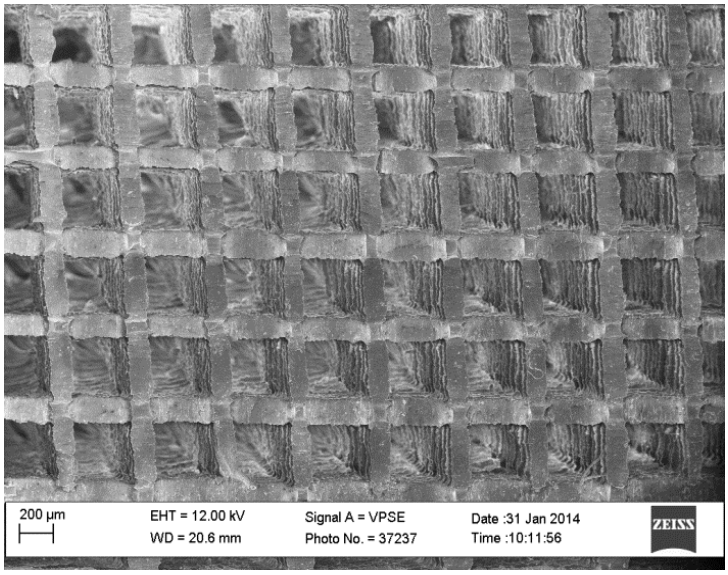


Figure 3.8 Structured structure

Random porous scaffold: In random porous scaffold fabrication method, the pore size of fabricated scaffold mainly depends on the size of particles used. In the present study, prepared

PDMS were directly poured into salt particles (size of 250um-300um) without any PVA mould and kept in desiccator to remove bubbles and then kept in oven for overnight at 50°C. Later excess PDMS were removed and then dissolve in water of about 2 hours. Then its SEM image was taken and shown in the figure 3.2.3.

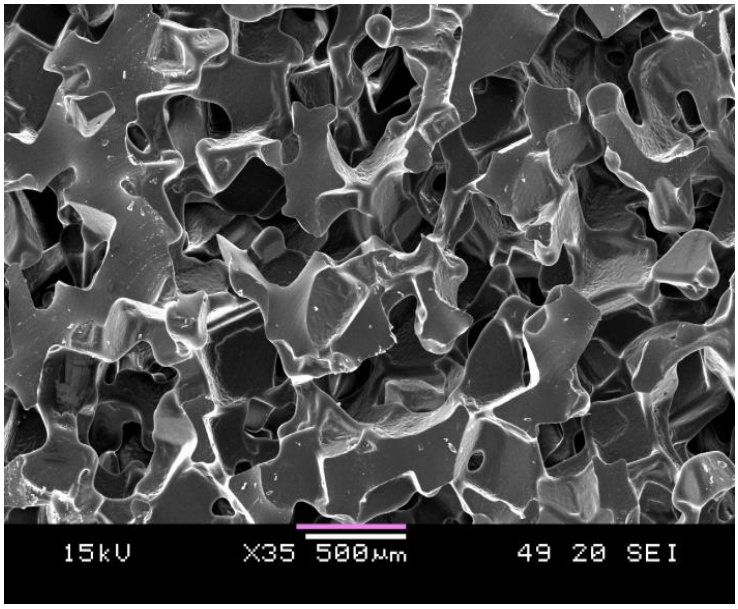


Figure 3.9 Random structure

Combined structures: In combined scaffolds, PVA scaffolds and salt particles (size of 250um-300um) both used. PVA scaffolds were filled with salt particles, and then Prepared PDMS were directly poured into PVA scaffolds and kept in desiccator to remove bubbles and then kept in oven for overnight at 50°C. Later excess PDMS were removed and then dissolve in water of about 2 hours until PVA get completely dissolved. Then its SEM image was taken and shown in the figure 3.2.4.

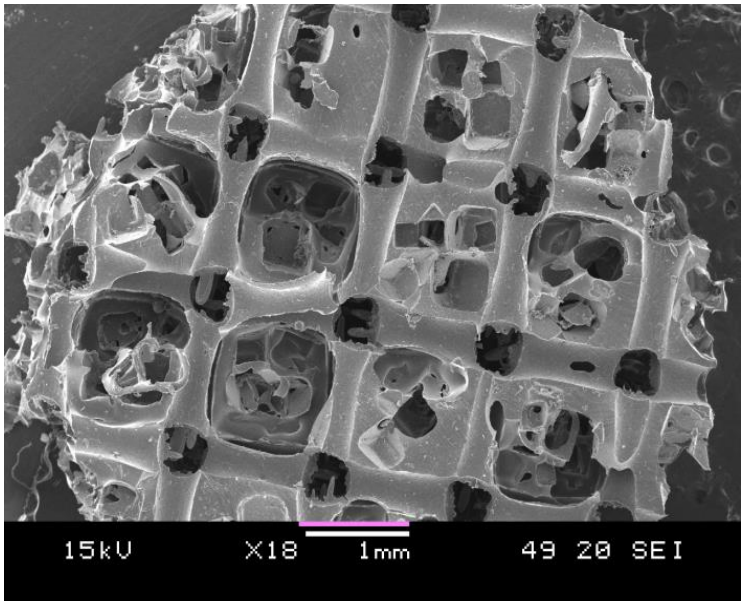


Figure 3.10 Combined structure

Later, Excess PDMS was removed using cutter and dissolved in water until PVA gets dissolved. Then obtained scaffold (figure 3.2.5) was dried in oven for about 10minutes.

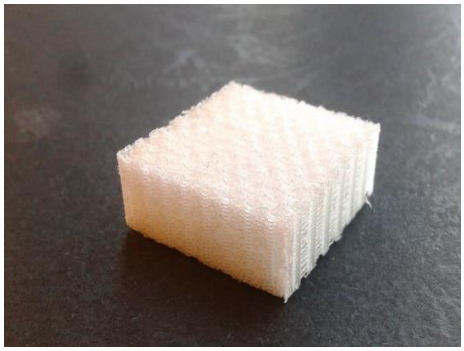


Figure 3.11 3D structured PDMS scaffold

But still this PDMS scaffold is hydrophobic, but for the cell research requires hydrophilic scaffolds. To get this, scaffolds was treated with oxygen plasma device for about one minutes.

The process of oxygen plasma treatment is described below.

PDMS oxidation using plasma changes the PDMS surface chemistry and produces silanol terminations (SiOH) on its surface. This helps to make the PDMS hydrophilic for thirty minutes or so. This process also makes the surface resistant to the adsorption of hydrophobic and negatively-charged molecules. In addition, PDMS plasma oxidation is used to functionalize the PDMS surface with trichlorosilane or to covalently bond PDMS (at the atomic scale) on an oxidized glass surface by the creation of a Si-O-Si bonds. Whether the PDMS surface is plasma oxidized or not, it does not allow water, glycerol, methanol or ethanol infiltration and consecutive deformation. Thus, it is

possible to use PDMS with these fluids without fear of micro-structure deformation. However, the PDMS deforms and swells in the presence of diisopropylamine, chloroform and ether, and also, to a lesser extent, in the presence of acetone, propanol and pyridine. It is transparent at optical frequencies (240 nM – 1100 nM), which facilitates the observation of contents in micro-channels visually or through a microscope. It has a low auto fluorescence. It is considered as bio-compatible (with some restrictions). The PDMS bonds tightly to glass or another PDMS layer with a simple plasma treatment. This allows the production of multilayers PDMS devices and enables to take advantage of technological possibilities offered by glass substrates, such as the use of metal deposition, oxide deposition or surface functionalization. PDMS, during cross-linking, can be coated with a controlled thickness on a substrate using a simple spin coat. This allows the fabrication of multilayer devices and the integration of micro valves. It is deformable, which allows the integration of microfluidic valves using the deformation of PDMS micro-channels, the easy connection of leak-proof fluidic connections and its use to detect very low forces like biomechanics interactions from cells. PDMS consists of repeating –Osi (CH 3) 2 - units; the CH 3 groups make its surface hydrophobic. This hydrophobicity results in poor wettability with aqueous solvents, renders micro channels susceptible to the trapping of air bubbles, and makes the surface prone to nonspecific adsorption to proteins and cells. The surface can be made hydrophilic by exposure to an air plasma (in a plasma cleaner for 1 min); the plasma oxidizes the surface to silanol (Si-OH). The PDMS scaffold has a channel inside for the proper flow of cell culture medium, but the PDMS surface is usually has hydrophobic nature, so we use oxygen plasma treatment to convert hydrophobic nature to hydrophilic nature. The treatment of oxygen plasma on PDMS introduces polar functional groups which is mainly the silanol group (SiOH). This group changes the surface properties of PDMS from being hydrophobic to hydrophilic. On the one hand, normal plasma treated surfaces undergo hydrophobic recovery within minutes after bonding and thermal treatment. On the other hand, extended plasma treatment induces undesirable surface cracks, which affect the bonding integrity of the device. The figure 3.2.6 shows oxygen plasma instrument setup which was used in lab. It is inexpensive compared to previously used materials (e.g. silicon). The PDMS is also easy to mould, because, even when mixed with the cross-linking agent, it remains liquid at room temperature for many hours. The PDMS can mould structures at high resolutions. With some optimization, it is possible to mould structures of a few nanometres. It is gas permeable. It enables so cell culture by controlling the amount of gas through PDMS or dead-end channels filling (residual air bubbles under liquid pressure may escape through PDMS to balance atmospheric pressure).

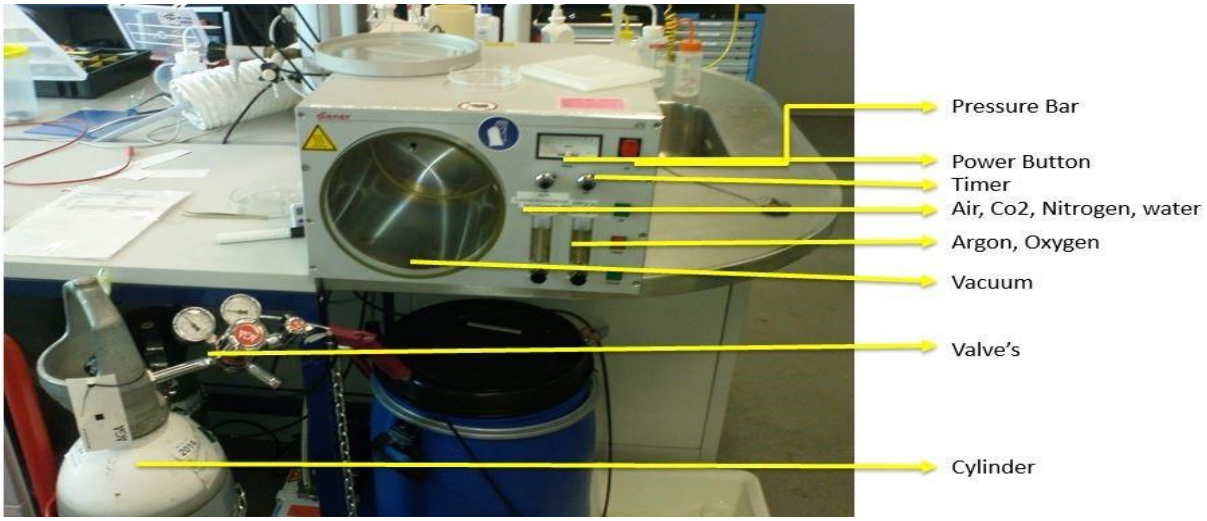


Figure 3.12 Oxygen plasma treatment

- Instrument settings:
    - Switch on O2 valves at back side
    - Switch on Cylinder and place the sample inside
    - Close the cap and generate vacuum
- Operation
    - Wash sample with acetone and isopropanol
    - Set time
    - Observe the pressure bar, when it goes below 0,2, apply plasma and wait for 1 or 2 min
    - After completion, switch of vacuum and take out sample and Switch off cylinder and machine

3.3 Materials and methods for silk protein based scaffold

In order to fabricate fibroin based tissue scaffold, mainly silk solution preparation is needed. First the silk protein 'fibroin' extracted from a naturally available silk cocoons i.e. Bombyx mori (silkworm). Bombyx mori (silkworm) silk is a unique material, biocompatible and has a slow degradation rate in in-vivo and its ability to be processed into multiple material formats such as film disks, gels, fibers. Physicians have used silk as a suture material for centuries. Because of large-scale cultivation of silkworms for the textile industry, there are abundant and reasonable cost sources for this natural polymer; however, for medical applications, adequate extraction and

preparation of the core protein is required. From the raw cocoons, the sericin component must be removed from the core fibroin fibers. Sericin is a group of soluble glycoproteins expressed in the middle silk gland of B. mori 7. These proteins cover the surface of fibroin, the silk filament core protein, in the cocoon filament. Once this adhesive protein is removed, the fibroin fibers are dissolved into an aqueous solution.(Rockwood et al., 2011)

a) b) c)

Figure 3.13 Silk cocoons (a), silk film disks (b), silk fibers (c)

Step A: Fibroin Extraction Process

1. 5L glass beaker was filled with 3 liter of ultrapure water, covered with aluminium foil and kept for heating at high temperature (500C) until it boils.
2. Meanwhile, cocoons were made into small pieces. Dust were thrown out and measured 7.5g of small cocoon pieces into a large weighing boat.
3. 6.36g of sodium carbonate was measured and added into water when it started boiling and mixed well until sodium carbonate dissolves.
4. After dissolving sodium carbonate, cocoon pieces were added and boiling continued for 30minutes. It stirred with spatula for each 10minutes to promote good dispersion of fibroin.
5. Silk fibroin were removed from boiling water exactly 30min and rinsed in ultrapure cold water.
6. Silk fibroin was rinsed in cold water and squeezed many times and water changed for 3 times for every 1hour.
7. After 3rd wash, silk fibroin was removed from water and spread it over clean aluminium foil to get dry for overnight.

(Rockwood et al., 2011)

Figure 3.14 Silk Processing Steps

A: Fume hood where the process took place
B: 5L Glass beaker with 3L of ultrapure water kept for boiling
C: 7.5g of Pieces of cocoons and 6.36g of sodium carbonate

D: Sodium carbonate and cocoons pieces were poured into boiling water (for 30min)
E: Plastic beaker with ultrapure cold water which contains Silk fibroin
F: Dried silk

Step B: Silk fibroin dissolving in LiBr

Material Required are: 1: Lithium bromide, weigh boat, scale

2: 50or 100ml graduated cylinder

3: 30 or 50ml glass beaker and stir bar

1: 9.3M LiBr solution was prepared for the 20% (wt/vol) solution based on the amount of dried fibroin. It means, 20% of the solution will be silk, and 80% will be LiBr. That is 1:4 ratio.

Multiply the amount of dried silk fibroin by 4 to obtain the total volume of 9.3M LiBr needed (X).

The total amount of LiBr is obtained by using (1):

$$\left(86.85\frac{g}{mol}\right)\left(9.3\frac{mol}{L}\right)\left(\frac{1L}{1{,}000ml}\right)(\mathrm{X}) = \_\_\_\_\_\ g\ of\ LiBr \qquad (1)$$

2: The silk fibroin was packed tightly into a 50ml glass beaker and required amount of LiBr solution were added on the top. Then mixed well.

3: silk fibroin was dissolved in oven at 60˚C for 4Hr. Once it is dissolved completely, it became transparent in colour. (Rockwood et al., 2011)

Step C: Dialysis (figure 3.3.3) and Centrifugation (figure 3.3.4)

Material required are: 1: Dialysis membrane 50-100um, Holding Sticks

2: 5L plastic beaker

1: Silk solution were transfer into dialysis membrane (50-100um)

2: Then it's both ends covered by holding sticks and dipped in 5l plastic beaker which contains 5l ultrapure water.

3: water was changed after 1Hr, 4Hr and after 12Hr. Six changes within 48Hr

(Rockwood et al., 2011)

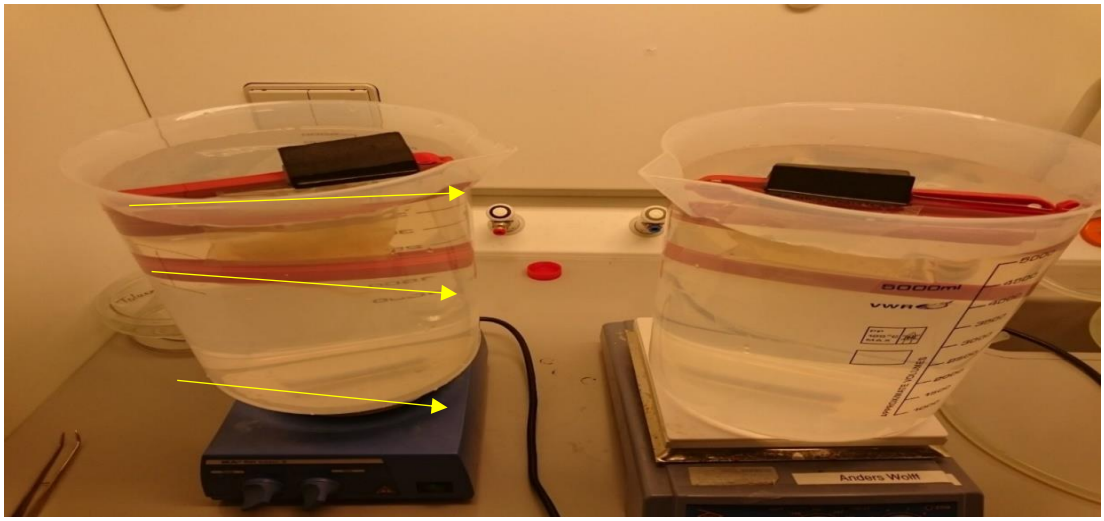


Figure 3.15 Dialysis, A-Holding Sticks, B-Silk solution in 50-100um Dialysis membrane, C-5L plastic beaker

Centrifugation:

1: There was little more impurities even after dialysis, Centrifugation process was used to remove those impurities.

2: Parameters were used are, 9000rpm, at 4˚C for 20min.

3: after centrifugation, solution were transferred to new falcon tubes and it was repeated for two times.

4: Silk solution were stored at 4˚C for a month.

(Rockwood et al., 2011)

:

Figure 3.16 Centrifugation device

After centrifugation, silk solution were stored at 4˚C.Then this solution was used for the fabrication of tissue scaffold. The process described below.

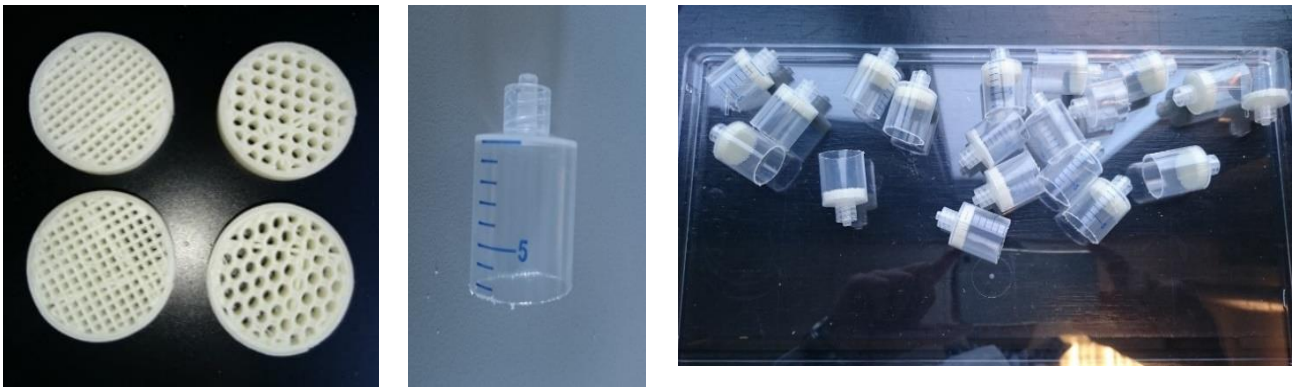
Fabrication Process

Step A: Syringe Mould Preparation with PVA

1: 3d printed PVA moulds and 20ml syringes were collected.

2: Syringe was cut into small piece as shown in figure.

3: then PVA moulds was inserted into syringe.



a) b) c)

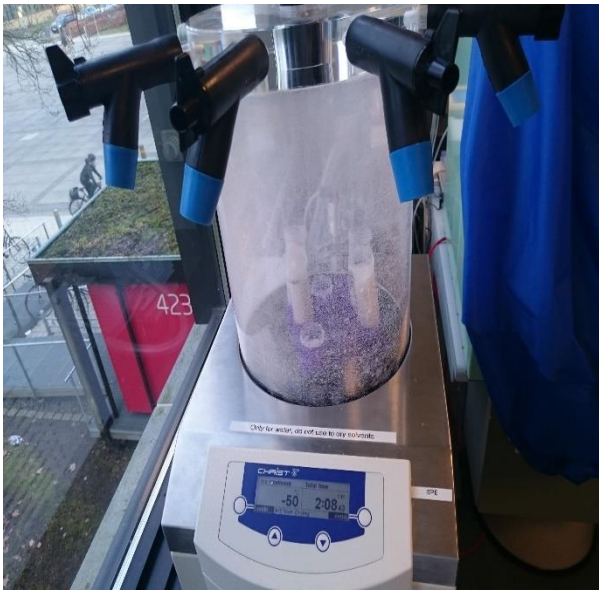
Figure 3.17 3D printed PVA mould (a), 20ml Syringe was cut into small piece (b), PVA moulds (c)

Step B: Silk solution injection into moulds

1: Silk solution was injected into syringe moulds, bubbles were removed carefully during injection process.

2: Then immediately mould was kept in -80˚C for 4 hrs.

3: Moulds from deep freezer was having water content, freeze drying process was helped to remove the water content from moulds. The sample moulds were kept in Freeze dryer at -50˚C for 48hrs.

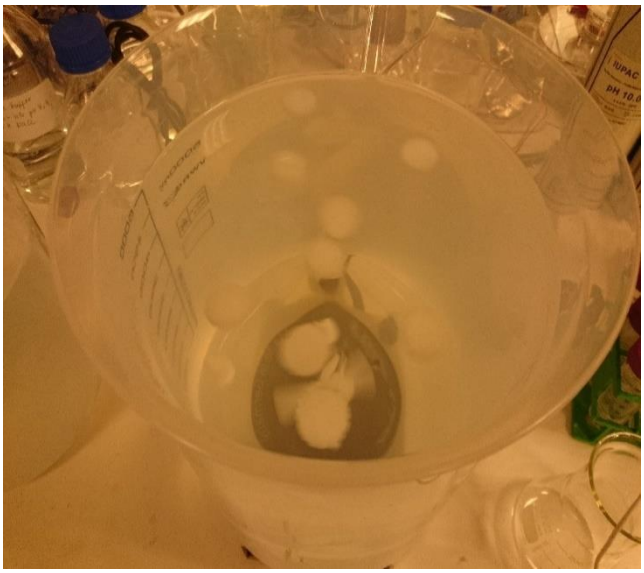


a) b)

Figure 3.18 Silk solution injection process (a), Deep freezer (-80˚C) (b)

Step C: Dissolving Process (to dissolve PVA) shown in figure 3.3.7(A)

1: After 48hrs from freeze dryer, the sample moulds were dissolved in 5L water in plastic beaker for overnight to remove PVA from the sample mould. Then the silk scaffolds were obtained as shown in the figure below.



a) b)

Figure 3.19 Dissolving Process in 5l water in plastic beaker (a), Obtained silk scaffold (b).

3.4 Scaffold characterization

3.4.1 Surface Structure analysis Using SEM equipment

To confirm the morphology of the scaffolds, SEM equipment was used. SEM is used study the characteristics of PDMS & Silk Protein based fabricated scaffold. The scanning electron microscopy (SEM) is a powerful instrument which used to study the surface characteristics of scaffolds, and composition, crystallography, chemical composition of the material and other properties. A Scanning Electron Microscope provides details surface information by tracing a sample in a raster pattern with an electron beam. The process begins with an electron gun generating a beam of energetic electrons down the column and onto a series of electromagnetic lenses. These lenses are tubes, wrapped in coil and referred to as solenoids. The coils are adjusted to focus the incident electron beam onto the sample; these adjustments cause fluctuations in the voltage, increasing/decreasing the speed in which the electrons come in contact with the specimen surface. Controlled via computer, the SEM operator can adjust the beam to control magnification as well as determine the surface area to be scanned. The beam is focused onto the stage, where a solid sample is placed. Most samples require some preparation before being placed in the vacuum chamber. Of the variety of different preparation processes, the two most commonly used prior to SEM analysis are sputter coating for non-conductive samples and dehydration of most biological specimens.

In addition, all samples need to be able to handle the low pressure inside the vacuum chamber. The interaction between the incident electrons and the surface of the sample is determined by the acceleration rate of incident electrons, which carry significant amounts of kinetic energy before focused onto the sample. When the incident electrons come in contact with the sample, energetic electrons are released from the surface of the sample. The scatter patterns made by the interaction yields information on size, shape, texture and composition of the sample. A variety of detectors are used to attract different types of scattered electrons, including secondary and backscattered electrons as well as x-rays. Backscatter electrons are incidental electrons reflected backwards; images provide composition data related to element and compound detection. Although topographic information can be obtained using a backscatter detector, it is not as accurate as an SED. Diffracted backscatter electrons determine crystalline structures as well as the orientation of minerals and micro-fabrics. X-rays, emitted from beneath the sample surface, can provide element and mineral information.(Carrassi & Abati, 2007)

SEM produces black and white, three-dimensional images. Image magnification can be up to 10 nanometers and, although it is not as powerful as its TEM counterpart, the intense interactions that take place on the surface of the specimen provide a greater depth of view, higher-resolution and, ultimately, a more detailed surface picture.

The PDMS sponge & Fibroin sponge (Prepared scaffolds) which need to be analysed in SEM imaging, has to coat with gold in Gold spotter machine (figure 3.4.1) to absorb the electron beam from SEM, the process of gold spotting and operating SEM imaging is described below.

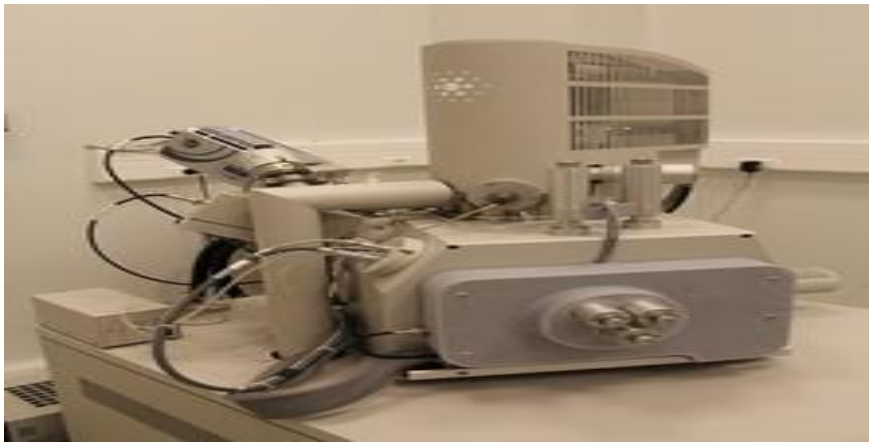


Figure 3.20 Gold Spotter

Using Gold Spotter:

- Instrument settings:
    - Switch on pump & valves to be tight
    - Load sample and close upper valves
    - switch on Machine
- Instrument Operation:
    - 3time rinsing
    - Hv on → Open the shutter → Adjust current and time
    - After completion, take out sample & release gas at side valve
    - Switch off pump and machine

SEM:

Figure 3.21 SEM setup (http://www.wdyl.com/#sem)

The figure 3.4.2 shows the Scanning electron microscopy's instrument setup which is connected to computer display. The description of method of using SEM is mentioned below.

Using SEM:

- Instrument setup:
  - Access Lab Manager
  - Load sample into SEM with alignment
- JSM Software
  - Menu→ Spc Vent
  - Load sample → Spc Evac → Push valve inside → Acc-Vott
- Operation
  - Find Spot and magnify the sample
  - Scan 2 & adjust magnification → wobble & adjust x,y axis
  - Stig → Adjust Contrast & Brightness
  - Scan 4 & save image

3.4.2 Porosity Calculation

In cell scaffolds, porosity is an important parameter because the porosity indicates the total space proportion in the scaffold for cell proliferation. In this study, the porosity of the fabricated scaffold was calculated using eq. (2). For each type of scaffold, 10 scaffolds were used for the following calculation:

$$Porosity\ (\%) = \frac{Vo}{Vo+\left(\frac{A}{\rho}\right)} \times 100\% \qquad (2)$$

Where V0 is the apparent volume of the scaffold, which is calculated using the outer dimension of the scaffold, m is the dry mass of the scaffold.

Example 1: Porosity calculation of PDMS scaffolds was done by using equation 1. Two samples from scaffolds with infill density 20p, 40p, 60p and 80p were used. The calculation is shown in the table 2.

Table 2 Porosity calculation of PDMS scaffold

Infill (%)	**(A) Dry Wt. (gm)**	**(B) saturated wet weight (gm)**	**density of PDMS (gm/cm3) ρ**	**Porosity (%)**	**Average of Two samples: Porosity (%)**
20-(1)	3.6033	4.3969	0.965	17.52809	18.45677
20-(2)	3.4676	4.3317	0.965	19.38545	
40-(1)	2.4132	3.8408	0.965	36.34119	35.61858
40-(2)	2.3853	3.7102	0.965	34.89597	
60-(1)	1.7852	3.763	0.965	51.67007	53.51269
60-(2)	1.6421	3.752	0.965	55.35532	
80-(1)	0.801	3.4405	0.965	76.07611	77.22019
80-(2)	0.686	3.2608	0.965	78.36428	

A= Dry weight of PDMS sponge

B= Wet Weight of PDMS sponge

ρ= Density of PDMS sponge (0.965)

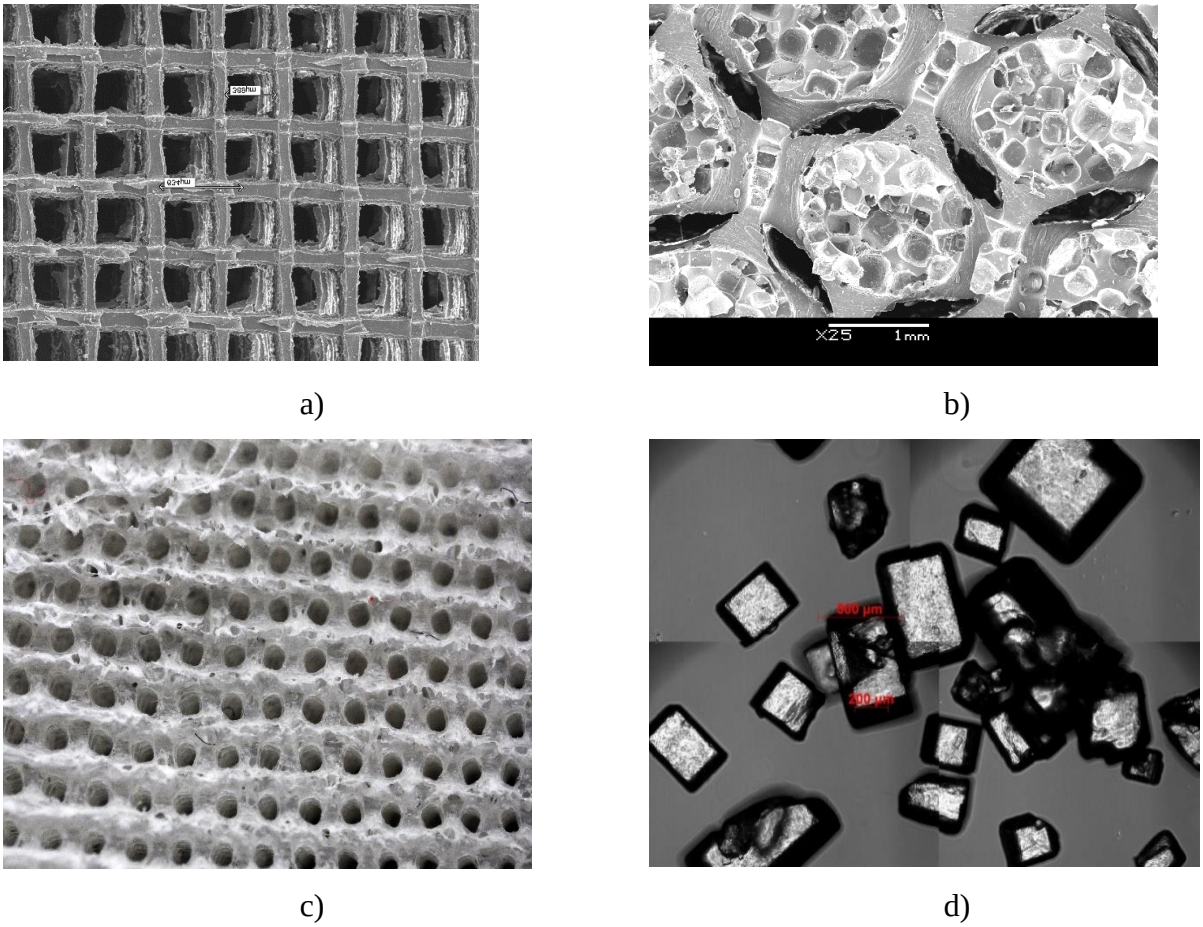
4 RESULTS

4.1 Surface Structure analysis of PDMS and Silk Protein scaffolds

This 3D scaffold fabrication techniques that involves synthetic polymer (PDMS) and natural polymer (Fibroin) microfabrication of 3d scaffolds surface images is shown in the Figure 4.1. 3d scaffold of PDMS, with precise micro-architecture (pore size and geometry) and surface micro-textures (surface topography) that could potentially be used as scaffolds for tissue engineering applications. PDMS is biocompatible and has been used in some implantable applications.

Figure 4.1 shows scanning electron micrographs of the cross section of the porous scaffolds. The morphology is uniform, and the pore size distribution is homogeneous in all the scaffolds.

Using SEM (JSM-5410, JEOL,) equipment, surface morphology and interconnectivity of fabricated scaffolds are observed.



a) b)

c) d)

Figure 4.1 SEM images of surface of the 3D scaffolds which shows interconnected network of pores: PDMS Scaffold (a) and (b), Fibroin scaffold (c), Cross sectional image of the 3d scaffold for the measurement of pore size (d)

Figure 4.1 (a), (b) and (c) are surface images of PDMS scaffold and Silk protein scaffold which were taken by using SEM. As shown in the above figure, uniform, well interconnected network pores were observed in the 3d scaffolds with pore size of 389um. These interconnected pores can provide high quality space for cell migration and proliferation.

Pore size were measured by using imageJ (software) from the SEM images of the scaffolds from different regions. Dimensions of more than 20pores were measured and averaged to obtain a mean pore size of 367.69um. The obtained graph shown below.

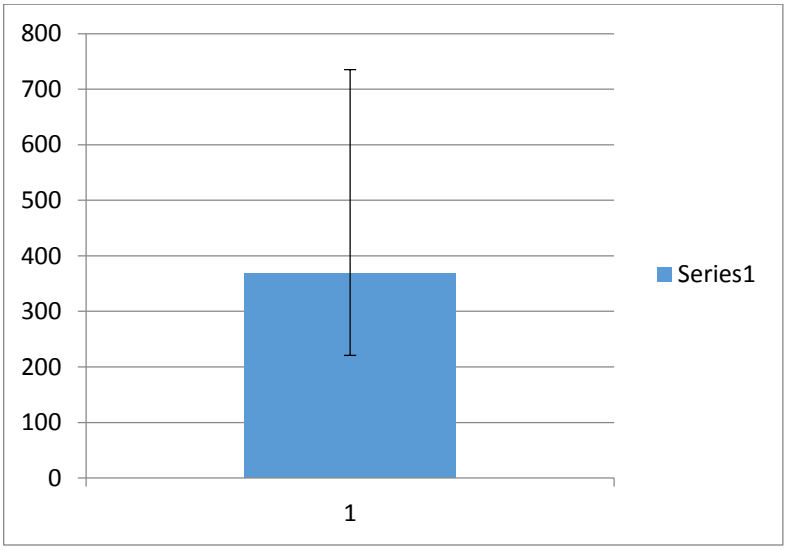


Figure 4.2 Pore size calculation graph

4.2 Porosity Measurement of PDMS based scaffolds

As depicted in figure 4.3, the porosities of the 3D scaffolds of graphs was plotted versus Infill density and calculated porosity of the scaffolds. The microstructure of the samples was tested with SEM for each group of different concentration. The cylinder shape of fibroin net was prepared in a test tube. The nets were composed of a mesh of randomly oriented fibers that ranged between 10 μ m and 30 μ m in diameter. Branch points and three-dimensional open spaces were distributed throughout the structure with an average pore size of about 177.9 ± 40.0 μ m [8]. The individual fibers generally exhibited a smooth surface as revealed in Figure 2A. Three-dimensional scaffolds are required in tissue engineering to support for the formation of tissue-relevant mimics as well as to promote cellular adherence, migration, formation of new extracellular matrix, tissue ingrowth and to foster the transport of nutrients and metabolic wastes.

The pore shape and size were strongly influenced by the size of the salt particles used. When the size of the salt particles are less the pore size is also a less, here salt particles size used is 260um-360um, this is good agreement with the 250um-300um range of salt used to fabricate the scaffolds, although pores could also be found somewhere under SEM test (figure 4.1.1 D). The silk protein based scaffolds was able to form the pores with a complete sponge structure and the most homogenous distribution (figure 4.1.1 C). The pore size ranged from 250μ m to 380μ m with an average size of 367.6μ m, as shown in Figures 4.2. It is generally considered that pore sizes larger than 200 micron in diameter with an interconnected structure is a minimum requirement for such systems based on cell sizes and migration. The pore size of the silk fibroin net seems to be able to

meet the needs for the potential use in tissue engineering. These PDMS scaffolds and fibroin scaffolds had highly homogenous and interconnected pores with pore sizes ranging from 250 µ m to 380 µ m, depending on the mode of preparation. The final structure prepared in the present experiment was more like a hierarchical structure. Although its pore was almost the ideal size for tissue engineering, this structure may be able to be used for drug deliver, bio membrane, cell studies or other purposes.

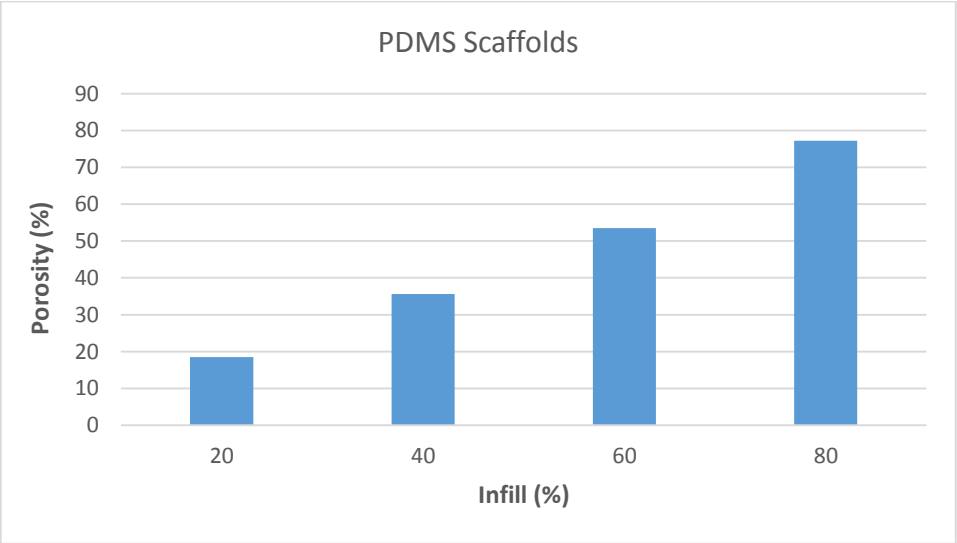


Figure 4.3Porosity Measurement of PDMS scaffold

Two samples from each PDMS scaffolds with infill density of 20p, 40p, 60p & 80p were tested for the porosity measurement. The graphs explains that, as infill density increases the porosity of the scaffolds also increases.

5 CONCLUSIONS

Fabrication of 3D engineered scaffold was done by using a method of solid free form method with the two different materials - PDMS (Synthetic polymer) and Fibroin Silk protein (Natural polymer). Surface analysis was done by using SEM where well interconnected pores were observed, average pore size obtained is 367.69 µm and this is good agreement with the 250 µm-300 µm range of salt used to fabricate the scaffolds. Silk protein based scaffolds also showed well interconnected pores, but it still needs more characterization to perform in vitro studies. Porosity calculation of scaffolds was done and the results were more comparable with the standard which proves that as infill density increases porosity of the scaffolds also increases.

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