

Development of an *in vitro* 3D Printed Liver Sinusoid Model

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In Partial Fulfillment of the Requirements for
The Degree of Master of Technology



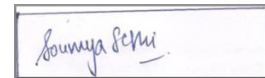
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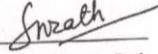
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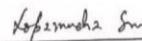
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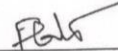
This thesis entitled "Development of a 3D Printed *in vitro* Liver Sinusoid Model" by Soumya Sethi is approved for the degree of Master of Technology from IIT Hyderabad.



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Abstract

Liver is one of the most remarkable organs of the human body as it performs various vital functions namely metabolism of fats and carbohydrates, drugs, detoxification, production of bile and has a capacity to regenerate. The intricate functional units of the liver called the sinusoids are of immense interest as they are early targets of many drugs and toxicants as well as have been reported to play a significant role in initiating liver regeneration and contribute to the pathophysiology of many liver diseases. Thus, understanding the functioning of these microvascular structures can be instrumental in providing us a multitude of information. Furthermore, as this area is targeted by many drugs and toxicants replicating the sinusoid in vitro can provide us with accurate drug testing devices as well as help in development of newer and more effective drugs. Here, we present a 3D printed liver sinusoidal model that contains a biomimetic liver decellularized extracellular matrix hydrogel to support the culture of hepatocytes. The prepared hydrogel has a potential to provide cells an environment similar to the native microenvironment of the sinusoids. The microarchitecture is mimicked by preparing a 3D printed scaffold emulating the native sinusoid to a certain extent. This is the first liver sinusoid model which has liver dECM gel to support the hepatocytes and thus has tremendous potential of becoming a device which can be used for drug testing as well as for studying the complex pathophysiology of life threatening liver diseases such as cirrhosis, fibrosis etc.

Nomenclature

%	Percentage
LSEC	Liver sinusoidal endothelial cells
HSC	Hepatic Stellate Cells
KC	Kupffer Cells
ECM	Extra cellular matrix
DLM	Decellularized Liver Matrix
dECM	Decellularized extracellular matrix
SLA	Stereolithography
3D	Three Dimensional
CAD	Computer Aided Design
STL	Standard Tessellation Language
PDMS	Polydimethylsiloxane
PEM	Polyelectrolyte Membranes
PET	Polyethylene terephthalate
SDS	Sodium Dodecyl Sulphate
DNA	Deoxy ribonucleic acid
RNA	Ribonucleic acid
GAG	Glycosaminoglycan
UV	Ultraviolet
DMMB	1,9 Dimethyl Methylene Blue
O.D.	Optical Density
BSA	Bovine Serum Albumin

DMEM

Dulbecco's Modified Eagle's Media

HNF

Hepatocyte Nuclear Factor

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Chapter 1

Introduction

1.1 Liver Anatomy

Liver is the largest internal organ of the human body and lies in the proximity to gall bladder and stomach, it weighs about 1.5 kg in a healthy adult. It contains 2 major lobes and performs the vital metabolic functions of the body, namely metabolism of fats and carbohydrates, synthesis of vital proteins, production and secretion of bile and detoxification of toxins etc. It has a remarkable capacity to regenerate unlike any other organ in the human body [1]

This fascinating organ receives only about 25 % of its blood supply from the heart via the left and right hepatic artery, in the form of oxygen rich blood. The rest 75% of its blood supply is through the portal vein. The portal vein directs blood from the gastrointestinal tract to the liver. Thus, the blood flow to the liver is unique as it receives both oxygenated and partially deoxygenated blood as shown in Figure 1-1

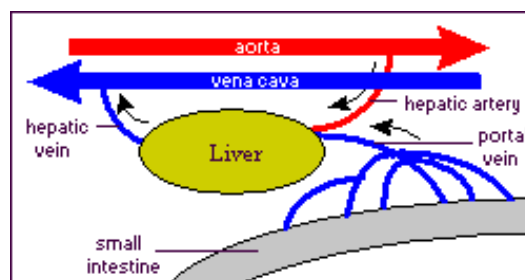


Figure 1-1: Blood flow to the liver, source: www.google.com

The microenvironment of the liver is very intriguing, it consists of millions hepatic lobules which are hexagonal in shape. Each of these lobules are encircled by branches of oxygen providing hepatic artery and nutrient providing, portal vein as shown in Figure

1-2. These then drain into the capillary like structure called sinusoids, which are highly fenestrated and distensible and are lined circumferentially by hepatocytes. Along with the branches of hepatic artery and portal vein, a bile ductile is also present which collects the bile produced by the hepatocytes. These three are together termed as the portal triad. The flow of bile is in the opposite direction with respect to the blood flow.

The oxygen rich blood from the terminal branches of hepatic artery and nutrient rich blood from the terminal branches of portal vein together drain into the sinusoids. Due to the presence of fenestrations in the endothelial cells and basement membrane, as blood flows through the sinusoid, a significant amount of plasma is filtered into the space between endothelium and hepatocytes. This space is of clinical importance and is termed as the 'Space of Disse'. Blood flows through the sinusoids and empties into the central vein of each lobule. The radial movement of blood through the sinusoid causes a gradual drop in the oxygen concentration, the blood reaching the central vein is deoxygenated, this deoxygenated blood drains into the hepatic vein, which then leaves the liver and is drained into the inferior vena cava.

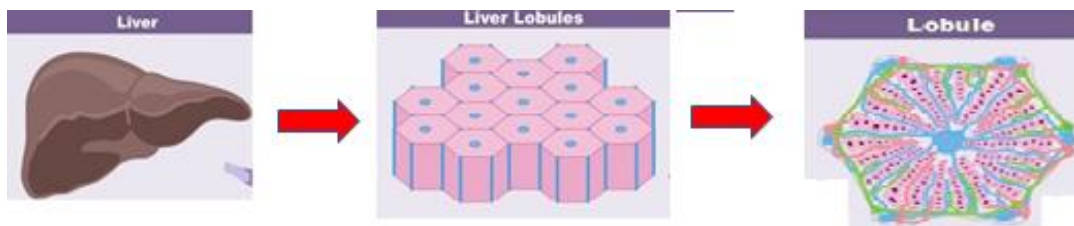


Figure 1-2: Structural Organization of Liver, source: www.google.com

1.2 Liver cells

Liver comprises of a vast variety of cells as shown in Figure 1-3 :

1.2.1 Hepatocytes

They are the most abundant cells in the liver and make up to 70-80% of the liver's cytoplasmic mass and are involved in a wide variety of functions like synthesizing proteins, bile salts, fibrinogen, cholesterol etc. These cells are instrumental in helping us to produce waste, transport materials, process toxins like insecticides and pollutants as well as drugs and steroids.

1.2.2 Liver sinusoidal endothelial cells (LSECs)

These are unique endothelial cells as they do not possess tight junctions as other existing endothelial cells and are known to be highly fenestrated and distensible.

They act as scavengers of nearby cells. When simulated they secrete cytokines.

1.2.3 Hepatic stellate cells (HSCs)

They are termed as the reserve army and comprise of only 5- 8% of the total liver cells. Their major function is storage of Vitamin A and some important receptors. Normally known to be in quiescent state and get activated and produce antibodies when there is some liver injury.

1.2.4 Kupffer cells

Mainly found in the sinusoidal lining of the liver and are popularly called as macrophages of the liver. They contain one quarter of the liver lysosomes. These cells work as immune surveillance in the liver.

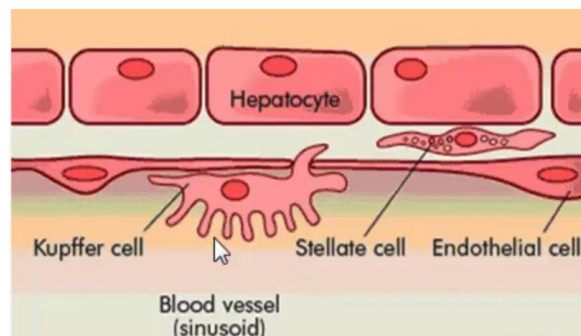


Figure 1-3: Cells of the Liver. Source: www.google.com

1.3 Space of Disse

The space of disse is a small compartment between the hepatocytes and a sinusoid, it encompasses the microenvironment that can facilitate the exchange between blood and hepatocytes. This perisinusoidal space comprises of plasma, collagen type III that forms a reticular framework. [2]

Moreover, the microvilli from the hepatocytes extend into the space of disse, thereby absorbing all the important plasma proteins and components from the sinusoid. The thickness of this protein rich space is 0.5-1 μm as depicted in Figure 1-4. In case of a liver pathology, there are reports stating that this perisinusoidal space may be damaged, thereby decreasing the uptake of nutrients and wastes by the hepatocytes. [3]

The hepatic stellate cells also known as the Ito cells are present in this perisinusoidal space, they are responsible for fat storage as well as vitamin A storage. When damage is inflicted upon the liver, there is an inflammatory response which can induce these Ito

cells to become myofibroblasts and thereby resulting in production of collagen production, fibrosis and cirrhosis.

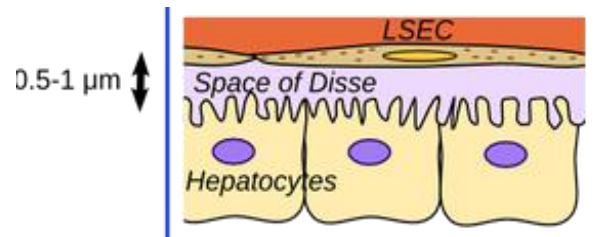


Figure 1-4: Space of Disse depiction, Source: www.google.com

1.4 Why liver sinusoid is important?

Liver sinusoids are the basic functional microvascular units of the liver. They contain liver endothelial cells, hepatocytes, Kupffer cells and Ito cells as shown in Figure 1-5. The liver sinusoidal endothelium is often referred to as a selective sieve between the blood and the hepatocytes [4]

Over the years researchers have understood the importance of the sinusoids and especially that of the LSECs. The endothelial cell barrier has been reported to be early targets for several drugs and toxicants [5] as well as allows rapid exchange of macromolecules, solutes and fluid between blood and the surrounding tissues. Thus, sinusoid is an important area of interest as many drugs target this area and thus it is much easier and rapid to understand the effect of a new drugs by fabricating in-vitro sinusoids. Furthermore, LSECs have been reported to have a major role in initiating liver regeneration and also contribute to many liver diseases like hepatitis, liver fibrosis and cirrhosis.[6]

Thus, understanding the mechanisms of action of LSECs can lead to development of newer and more effective drugs for liver diseases and provide us a wealth of information for clinical diagnosis.

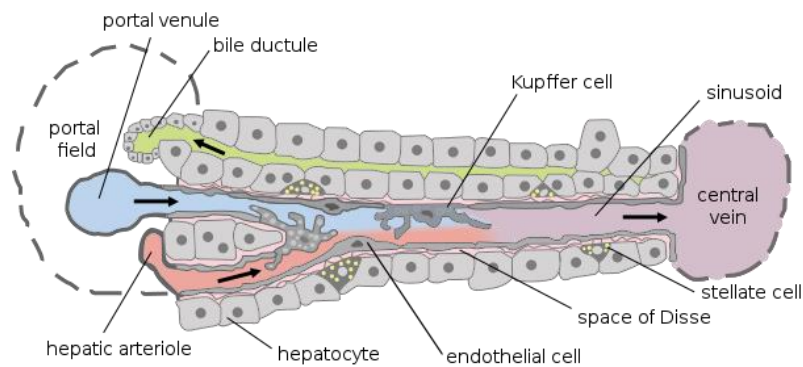


Figure 1-5 : Liver Sinusoid, source: www.wikipedia.com

1.5 Need for recapitulation of the liver microenvironment and sinusoids

Liver is a remarkable organ and has an intricate arrangement of cells and the microenvironment. The microarchitecture of the liver is of immense importance in understanding the pathophysiology of various liver diseases as well as in elucidating the normal working of this complex organ. The in-vitro mimicking of the liver sinusoids can give us a wealth of information about the functioning of the organ, disease progression in the organ and various pathways involved in the synthesis of proteins, production of bile etc.

Thus, there is a tremendous need to fabricate 3D bioprinted in-vitro liver sinusoidal model so as to:

- Better elucidate the pathophysiology of various diseases namely fibrosis, cirrhosis, hepatitis
- To better understand the pathological importance of the space of disse (perisinusoidal space)
- Understand the mechanisms of action of LSECs
- To understand the interactions between cells (hepatocyte-hepatocyte, hepatocyte- endothelial)
- To use as a drug screening device for checking the efficacy and toxicity of drugs[7]
- Development of new drugs

1.6 Drug Induced Liver Injury

Drug Induced Liver Injury or drug induced hepatotoxicity is a common cause of injury to the liver. It occurs due to adverse reactions of the drug on the liver, this can be either predictable or non- predictable as due to improper models of the liver, physicians are unable to understand the adverse effects of a particular drug.[22]

1.7 Motivation

Liver diseases affect over 600 million people worldwide and are known to cause about 1 million deaths worldwide per year. Understanding the pathophysiology of these diseases has still been a challenge as the only available models for disease study and drug development are the histologically challenged mice models which give incomplete information of the various pathways involved due to variations in the microenvironment of the organ.

Also, the pre-existing 2D cell culture models can hardly recapitulate any conditions *in-vitro* due to their spatial and temporal limitations.

Thus, a 3D bioprinted liver sinusoid model which can recapitulate the 3D microenvironment and microarchitecture of the liver sinusoids will be ideal to overcome the existing challenges and provide us with better insights of the functioning of the liver and help us understand the various liver diseases like fibrosis, cirrhosis etc. much better and thereby provide better and advanced platforms for drug testing.

1.8 Hypothesis

We propose a 3D printed functional biomimetic liver sinusoid model that contains liver dECM gel can mimic the *in vivo* sinusoid both structurally and functionally. Formation of the intricate structures like sinusoid, bile duct, space of disse is also predicted due to provision of the similar native ECM and architecture.

Chapter 2

Literature Review

Millions of people are suffering from liver diseases worldwide, namely hepatitis B, hepatitis C, cirrhosis, hemochromatosis, hepatitis A, fibrosis etc. Many of these diseases are still incurable and need urgent attention.

The liver is an organ which has been of immense interest in the field of tissue engineering due to its regenerative potential. Many researchers over the years have tried to develop in-vitro liver models with the hope to understand the organ better and elucidate pathways that lead to disease.[7]

2.1 Animal Models

For testing of new drugs and evaluation of pathways mice models have been used since a very long time but these animal models are histologically challenged and prediction of drug testing and toxicity testing is not accurate, thus there is a pressing need to recreate in-vitro models.

2.2 2D culture

Back in the 2000s, liver slices were used as in-vitro models and researchers used slices of liver to perform toxicity studies.[8][9]. Eventually, hepatocytes 2D culture was given prime importance but it was understood that culturing hepatocytes in monolayers was unfruitful as their phenotypic functions are lost. [10].

2.3 2D Co-culture

It was seen that the 2D co-cultures were successful in maintaining the phenotype over a period of time but these types of 2D co-cultures have a variety of limitations. These did not mimic the hepatic 3D architecture and have such co-cultures cannot exactly replicate the *in vivo* conditions and tend to give aberrant results when used for drug testing.[10].

2.4 Importance of 3D culture

Over a period of time researchers have understood the importance of the sinusoids of the liver and are focusing on recapitulating it on microfluidic chips and in 3D culture.

2.4.1 Organotypic Models

Over the years, people have tried to emulate the liver microenvironment using three dimensional structures including multi-compartment co-cultures, cell culture substrates and thermally responsive polymers. [11]

In 2013, a unique organotypic model was formulated, which comprised of the three cell lines namely the hepatocytes, endothelial cells and the Kupffer cells. Furthermore, the model also comprises of a polymeric detachable interface made of polyelectrolyte multilayers (PEM) which is 400-800 nm thick that emulates the Space of Disse. It is said to have tuned to have the same young's modulus as that of in-vivo. This novel model opened new avenues in understanding the complexities of the liver sinusoid as it is the first one to understand the importance of the space of disse and include both hepatocytes and Kupffer cells along with the endothelial cells. Over the years, people have tried to emulate the liver microenvironment using three dimensional structures including multi-compartment co-cultures, cell culture substrates and thermally responsive polymers. [11]

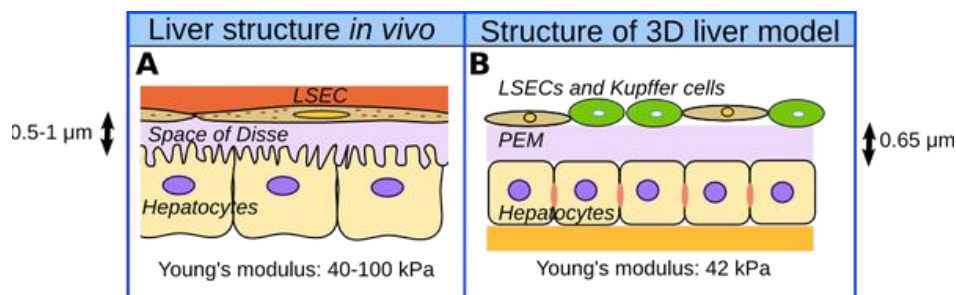


Figure 2-1 : Organotypic Model using PEM membrane, Source: [11]

2.4.2 Microfluidic Sinusoid Models

In July 2015, an organotypic model of liver on chip was devised, it was the similar recapitulation of the liver sinusoid but this time it was done on a miniature level. A microfluidic PDMS chip was fabricated with two inlets and two outlets, separating the two was a porous PET membrane, the length of the channels is 8mm and the height of

the top channel 100 micron comprise of liver endothelial cells and the bottom channel is 250 micron it houses the hepatocytes along with collagen as shown in Figure 2-2 The number of cells that can be seeded on this microfluidic device is less than 10,000 and the device was run on both static as well as dynamic conditions. The albumin synthesis as well as the urea excretion were analyzed.[12]The culture conditions were maintained for about 28 days. It was observed that the phenotype of the cells was maintained in both static and dynamic conditions but the synthesis of albumin and excretion of urea was higher in dynamic culture conditions.[12]Additionally, such in-vitro devices are formulated with an aim of its usage as a drug testing device but the material for synthesis of this chip is PDMS which is a highly hydrophobic material and can thus impede drug screening experiments due to non-specific adsorption of proteins and analytes.

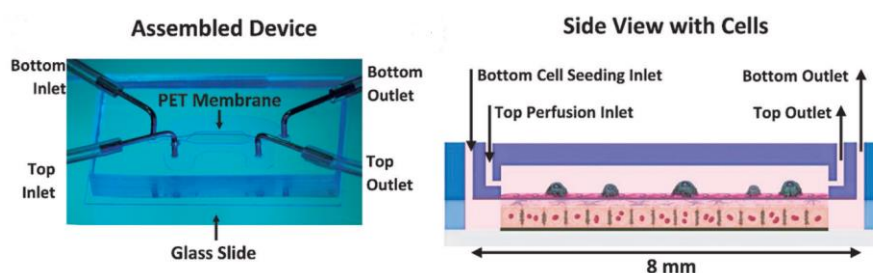


Figure 2-2 : Microfluidic Sinusoid, Source: [12]

Recently in 2018, researchers from China devised another liver sinusoid on chip device. In this device, the PET membrane employed before was eliminated as it has several disadvantages. PET does not mimic the in-vivo ECM conditions and is not biodegradable. This is prone to give results aberrant from the in-vivo conditions. Thus, a PDMS microfluidic device mimicking the liver sinusoid was fabricated, which consisted of hepatocytes on one side embedded in gel and endothelial on the other side embedded in collagen gel. The length of the channel was 20mm and dynamic media conditions was provided to the cells. The viability, albumin secretion and urea synthesis were assessed on days 3, 5 and 7. It was shown that biomimetic functions of the sinusoid was also achieved.[13]

Chapter 3

Aims and Objectives

3.1 Aim

We put forth an idea to construct a 3D printed in-vitro sinusoid liver model that reproduces **structural and functional** properties of the liver sinusoids and has **potential** of mimicking the microarchitecture and microenvironment of the liver sinusoids and the space of disse to study pathophysiology of various diseases like cirrhosis, fibrosis, hepatitis etc. and screen drugs.

3.2 Objectives

1. Fabrication of a 3D printed sinusoid which mimics the microarchitecture of the liver sinusoids
2. Preparation of decellularized extracellular matrix hydrogel from caprine liver which can a mimic *in vivo* like liver microenvironment
3. Cell Culture
4. Characterization

Chapter 4

Materials and Methods

4.1 Design Similarities

To mimic the in-vivo liver microstructure i.e. the sinusoid, an *in vitro* model was planned as shown in the Figure 4-1. It was then designed in a CAD software (AutoCAD 3D) and printed using SLA (stereolithography) printer.

The miniature bioreactor consists of 2 channels with separate inlets and outlets and in the middle contains a 100-micron mesh. The length of each channel is 20 mm, width is 1mm and height is 2.5 mm. This structure is similar to that of an in-vivo sinusoid. As we move from inlet to the outlet the oxygen gradient in the media reduces considerably mimicking the drop in the oxygen gradient as when moving towards the central vein from the portal triad. On both sides of the mesh we plan to seed the pre-formed decellularized liver pre-gel, next liver hepatocytes were incorporated in the pre-gel and added on both sides and gellated at 37°C for about 45 minutes. Media will be perfused in both inlets, both static and dynamic culture are planned.

Our model holds the potential to mimic all the structural and functional aspects of the liver sinusoid along with the very crucial space of disse.

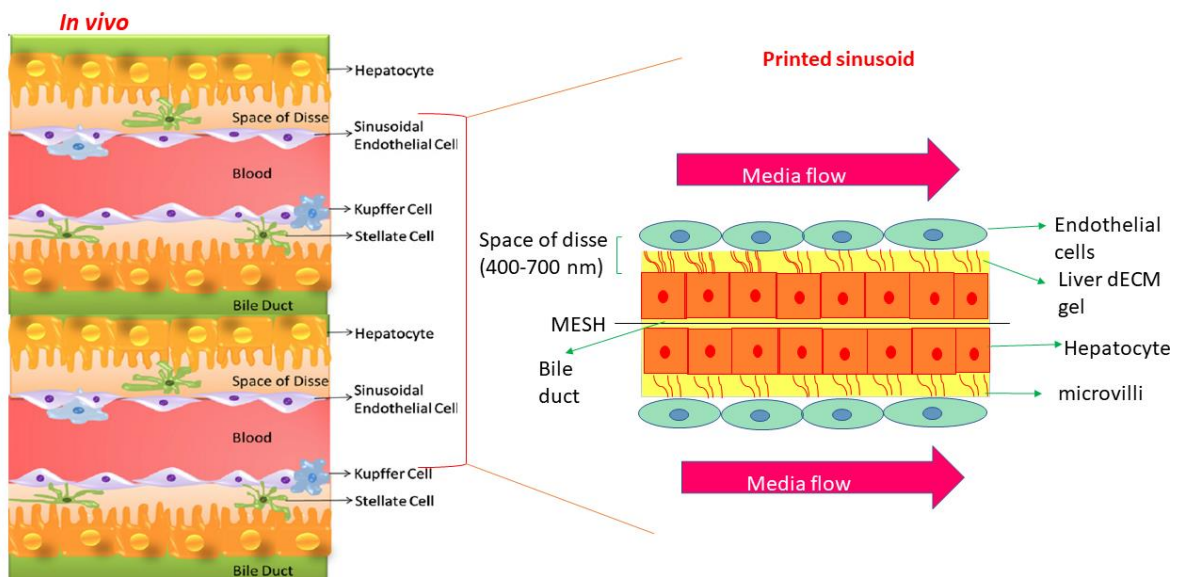


Figure 4-1: Design Similarity, Source (*in vivo* fig – www.google.com)

4.2 CAD Modelling

CAD designing

Various modelling software are used for preparing three dimensional models like AutoCAD 18, Solid edge V19. The size and dimensions are decided by considering several parameters like oxygen gradient, surface area etc.

The software employed for the designing of the miniature bioreactor is ‘**AUTOCAD 3D**’ version 2018 (student version). **AUTOCAD 3D** is a CAD i.e. computer-aided design and drafting software, developed by Autodesk. It is very popular among industries and institutes. It was widely used by us to design all the components of the miniature liver sinusoid model.

Designing

The design comprises of 2 parallel channels with inlets and outlets with a length: 20 mm, width: 1 mm, height: 2.5 mm. One 100-micron mesh with pore size: 50 x 100 micron is provided. 2 lids are designed to securely close the structure

Both channels will have media flowing from the inlet to the outlet which drains both the cell types. Hepatocytes also derive their nutrition from the liver dECM gel.

The **mesh** is designed to be 100 micron thick and pore size of the mesh 50X100 micron. The mesh will be sandwiched between the decellularized ECM formulated and will help in providing contact between hepatocytes through the gel as in the in-vivo conditions. To the best of our knowledge nobody has addressed this scenario in a 3D sinusoid model. The side of the hepatocytes facing the space of disse (i.e. towards the endothelial side) has multiple microvilli to increase the surface area whereas the other side facing the next layer of hepatocytes has very different transporter system and secretory systems. This face also has tight junctions or grooves between the cells called as the bile canaliculi.

4.3 3D printing

For prototype fabrication we used SLA, (Stereolithography) Form2 version 2, a laser-based technology that uses a UV-sensitive liquid resin for printing. STL files were loaded and printing has been done using **methacrylate** which has been layered one after another with UV crosslinking (405 nm) in each layer. Each layer gets shifted up after printing to facilitate next layer printing. The print temperature was 31 °C. After printing the constructs, the supports were removed and dipped in isopropyl alcohol for 30 min to remove the uncured resin.

About the printer

Figure 4-2 shows the SLA printer used in printing of the construct. SLA printer works on a laser-based printing process that has a photopolymer resin that is acted upon by a laser and then cured to form a solid. The laser beam is moved in the X-Y plane according to the 3D data supplied by the .STL file to the machine. The resin solidifies as the laser beam strikes the surface precisely. Once a particular layer is complete the Z axis movement comes into play and the upper layers are then traced out by the laser. This is how the entire object is formed and after completion of the print the platform is raised out and the print is removed.



Figure 4-2 : SLA Printer

Printer Specifications

Build volume: 145 x 145 x 175 mm

Layer thickness: 25-100 microns

Laser spot size: 140 microns

Operating Temperature: 35°C

Self- heating Resin Tank

Laser Specification: 405nm

Laser power: 250 mW

Printing

The .dwg file generated in the CAD modelling software was then converted into STL file and then given for printing.

4.4 Post processing

After printing the constructs, the supports were removed and dipped in isopropyl alcohol for 30 min to remove the uncured resin.

4.5 Flow test

After the post processing, the supports were cut and the construct was subjected to a flow test, whereby a blue colour dye was injected into the inlet 1 through a needle. The perfused dye travelled from inlet 1 to outlet 1. The same was repeated for inlet 2. This test was done to ensure that all the channels are open and the construct can be used for further experiments.

4.6 Diffusion test

The diffusion test was done to ensure there is transfer of nutrients from the top of the mesh to the bottom and that there is interaction between the top and bottom layers of the gel. We prepared 7.5% of gelatin and pipetted it onto the top side of the mesh, after letting it solidify, it was pipetted on the bottom of the mesh. After this the gelatin was allowed to crosslink using 1% glutaraldehyde for about 24 hours. It is important to note that the gelatin layers on the top and the bottom of the mesh is fairly continuous. We added trypan blue dye on the top mesh and allowed it to diffuse to the bottom.

4.7 Decellularization of Liver tissue

4.7.1 What is decellularization

What is decellularization

Decellularization is a process of complete removal of cells from a particular tissue, here liver, so as to obtain the non-cellular matrix of the tissue.[15] This decellularized tissue will have minimal immune reactions in-vivo as all the cellular components and debris have been removed. It can then be used to prepare a bioink for housing cells in a similar microenvironment. Such a liver bioink has all the necessary biochemical factors and proteins required for the survival of a liver cell.[15]

Decellularization Protocol

- 1) Caprine (Goat) liver was collected from a nearby slaughter house was used with the approval from the supplier.
- 2) The liver was then minced into small pieces of about 2-3 mm in size.
- 3) The chopped pieces were then subjected to decellularization by stirring in 0.5% SDS, in distilled water/PBS for 48 h.
- 4) It is then followed by treatment with 1% triton X-100 solution for 24 h.
- 5) For complete removal of cellular content degrading the leftover RNA and DNA in the ECM matrix RNase and DNase are used at 37°C.
- 6) The decellularized liver tissue is then washed thrice in Distilled water/PBS for about 30 min each to remove the detergent.
- 7) The obtained Decellularized Liver Matrix (DLM) is lyophilized and stored in -20 °C freezer till further use.

- 8) The lyophilized liver dECM is crushed into powder using a mortar and pestle with the help of liquid N₂. [16]

4.8 Digestion to form pre-gel

The digestion of the lyophilized liver ECM is done with the help of enzyme called pepsin. Pepsin, is obtained by the gastric juices of porcine and works by breaking down the collagen triple helix structure. Pepsin works best at a low pH (acidic pH) of around 2.8- 3. It is known to cleave the telopeptide regions of the collagen. [14]

Protocol for digestion

- 1) The required amount of DLM powder is weighed and digested in a solution of 0.5M acetic acid with 10 mg of pepsin for 100 mg LCM for 48 h.
- 2) After complete solubilization of DLM, the pH is checked (normally found in the range of 2.8–3).
- 3) The pH is adjusted with dropwise addition of cold 10M NaOH solution while maintaining the temperature below 4°C to avoid gelation of the DLM.

The pH-adjusted dECM pre-gel is stored in refrigerator

4.9 Bioink

To obtain bioink we should have done the following steps

- 1) Decellularization. 2) Pre-gel preparation. 3) pH adjustment

The following steps are then employed:

- 1 The required amount of 3% pre-gel is then taken, 10% of 10X of media added
- 2 Subsequently, cells are mixed with this pH adjusted pre-gel.
- 3 The formed bio-ink is subjected to gelation at 37 °C (optimal range: 34 °C – 38 °C) for about 30- 45 minutes.

4.10 Characterization

There are numerous characterization techniques for assessing the flow properties, mechanical strength and biochemical content of the prepared bioink. We employed DNA estimation, collagen estimation, GAG analysis, rheology.

4.10.1 DNA estimation

After completing the process of decellularization, it is imperative to check for the DNA content in the decellularized matrix. Ideally the decellularization matrix should have

- a) less than 50 ng of double stranded DNA per mg (dry weight) and
- b) during histological analysis with haematoxylin and eosin, there should be no visible nuclear material.[17] [18]

We performed DNA estimation using Nanodrop. It is known that the nucleic acids absorb light at a wavelength of 260nm, when a 260 nm light shines on the sample, the amount of light absorbed by the sample can be measured. For dsDNA at the Optical density of 1 at 260 nm correlates to 50 ng/ml concentration.

The measurements were taken with both native and decellularized samples and graph was plotted.

4.10.2 Collagen content estimation

Collagen is the major structural component of the ECM and provides the cells with adhesion sites, helps in their migration and also helps in the development of the tissue. [19]Collagen estimation is generally done by hydroxyproline assay.

Principle: The amount of hydroxyproline present is analysed in the matrix. It is a rapid protocol whereby hydroxyproline gets oxidized to form a reaction intermediate. This intermediate further reacts to form a coloured chromophore that can be visualized at 570 nm wavelength. The assay is reported to be very accurate.

Procedure:

- The lyophilized native and decellularized tissue is subjected to 0.5% collagenase digestion at 37°C overnight.
- 1 mg of tissue was dissolved in 500 µl collagenase.
- After the complete digestion, the assay buffer and chloramine T are added to the sample and the standard (hydroxyproline) and kept for incubation
- Next DMBA is added and the sample are given incubation at 60° C for 20 minutes
- After cooling the plate, the results are recorded at 570nm.

4.10.3 GAG estimation

GAGs are very important components of the ECM and are long unbranched polysaccharides. They are of many types: chondroitin sulphate, heparin sulphate, keratin sulphate etc. Spectrometric assays are valuable tools to measure GAG content in tissue extracts. DMMB Assay (dimethylmethylene blue assay) was performed to analyse the GAG content in the liver tissue. The dye DMMB is a thiazine chromotrope agent that brings about a change in the absorption spectrum by reacting with the GAG present in the sample and bringing about a visible colour change which can be analysed at 540 nm wavelength. Both native and decellularized samples were analysed for GAG content.

Procedure:

- 10 mg of native and decellularized lyophilized tissue was taken and digested in one ml of papain enzyme.
- The digestion was performed at 60°C for about 12 h
- After complete digestion, some amount of sample was taken from both and the experiment was performed in triplicates.
- 10mg/ml chondroitin sulphate was prepared in distilled water.
- Different concentrations of chondroitin sulphate were taken ranging from 5ug/ml to 50 ug/ml. This was also done in triplicates
- DMMB dye was prepared freshly and added in the standards and samples
- The readings were taken immediately after the addition of DMMB dye at 540nm.
- The O.D. was noted and graph between O.D. and concentration was plotted for the standards.
- The values for GAG in native liver tissue and decellularized tissue was then calculated from the graph and comparisons were made.

4.10.4 Rheology

Rheology, the study of flow properties of bioink under the influence of stress is a critical parameter for evaluating the flow behaviour. Viscosity and printability are the most critical rheological properties in case of bioinks [20]. We performed rheological analysis of the prepared liver dECM gel. Three tests were performed using a parallel

plate viscometer, a device used to determine viscosity based on rotation, temperature and time. We took liver dECM gel without pH adjusted and subjected it to:

a) viscosity v/s shear rate test b) strain v/s shear rate c) Amplitude sweep (storage and loss modulus plotted against strain%)

The temperature was set to 25°C during the entire duration of the tests and the plate was under continuous oscillatory motion. pH of the gel tested was around 3. The percentage of gel used for analysis was 3%

Graphs were plotted and the data was analyzed.

4.11 Cell culture

We used HepG2 cell line for our experiments. We obtained HepG2 cell line as a gift from CCMB, Hyderabad. We thawed cells from -196°C and cultured them.

Cell encapsulation in dECM gel

We cultured both cells and changed media over a period of 3 days. After the HepG2 cells achieved confluency, encapsulated them into the pre-prepared liver dECM (DLM) pH adjusted gel. We used 10^6 cells per ml of gel. Also, HepG2 cells were top seeded on the gel after the gelation process. This was then characterized by performing LIVE/ DEAD assay after about 72 hours and results were noted.

This was done to test the viability of the cells in the prepared gel.

The procedure for cell encapsulation and cell top seeding on gel was as follows:

- 1) The liver dECM gel was prepared and pH was set to around 7.4.
- 2) The gel was then transferred under cold conditions into the laminar hood.
- 3) About 100 μ L of gel of mixed with 10 μ L of cell suspension and 10 percent 10X DMEM media. This mixture was then plated into wells of a 24 well plate.
- 4) This plate was kept in the incubator to allow gelation of the gel for about 45 minutes.
- 5) After complete gelation, HepG2 cells were top seeded and the gel was observed under the microscope.
- 6) After about 30 minutes, DMEM low glucose media was added into the wells containing the gel.

After about 3 days we performed LIVE/DEAD assay using Calcein AM and ethidium homodimer.

Testing of the biocompatibility of the printed construct

After the post-processing the printed construct was dipped in 70% ethanol for about 30 minutes. The construct was then fully dried under the laminar hood and then cells were seeded onto the mesh of the construct and media was added. We could conclude that the construct is **bioinert** and not biocompatible.

Seeding gel and cells onto the construct

The construct was prepared for gel addition and cells seeding by dipping it in 70 percent ethanol for about 30 minutes and then air drying it completely in a laminar flow hood.

- The bioink was added on the top side of the mesh of the construct, it was then kept into the incubator for gelation for 45 minutes.
- After complete gelation, the same procedure was followed on the other side of the mesh.
- The construct was kept into a T-25 flask, this is the static culture
- Media was then added into the T-25 flask, touching the construct till the mesh.
- The construct was observed for some days and media was changed in a period of 3 days.

4.12 Cell viability and protein expression

LIVE/DEAD Assay:

Cell viability is very crucial in an in-vitro experiment. The trademark of live cells is an intact cell membrane and intracellular enzyme activity. These two characteristics form the basis of the LIVE/DEAD Assay. The Live cells exhibit intracellular esterase activity and appear green in colour and the dead cells have non-intact cell membrane and no esterase activity and take up the ethidium homodimer and appear red in under when observed under a fluorescent microscope.

The excitation wavelength for Calcein AM is 494 nm and emission are 517 nm, on the other hand excitation wavelength for Ethidium Homodimer is 517nm and emission is 617 nm.

Protocol:

- The gel in which cells were seeded is taken and transferred in an Eppendorf.

- To remove media completely, two PBS washes are given to the gel.
- Following which the dye is prepared in PBS by using 1:4 dilution of Calcein AM: ethidium homodimer.
- The dye is then added to the Eppendorf containing the gel and incubated at 37°C for about 45 minutes.
- The gel is then taken and observed under a fluorescence microscope at the required ranges.

Immunofluorescence:

Immunofluorescence is an assay to detect an antigen using specific antibody. It helps us to detect the presence of a specific antigen, used mainly for detection of expression of proteins, glycans and other small biological molecules. The protocol is quite elaborate starting from fixing of the sample using 4% formaldehyde, permeabilizing the cells if an intracellular marker is required using 0.1% triton X-100, next serum blocking using 1% bovine serum albumin (BSA) to avoid unwanted reaction. Primary antibody incubation and marked secondary antibody incubation is followed. For our experiments, we used

For nucleus staining: **Hoechst 33258**

It is a popular nucleus stain that emits blue fluorescence when bound to dsDNA, it is cell permeant and has an excitation at 355 nm and emission at 465nm.

HNF4 - α (hepatic nuclear factor 4 alpha) primary antibody was used to detect the presence of HNF4 - α antigen. HNF4 - α is the most abundant hepatic DNA binding protein that influences the expression of 40% of the actively transcribed hepatic genes including HNF 1A. It is produced by hepatocytes.

Alexa Flour 647: secondary antibody used. It is a marked antibody with
Excitation: 650 nm, Emission: 665 nm

It appears red under a fluorescence microscope.

Chapter 5

Results and Discussions

5.1 CAD Model

Figure 5-1 shows the designed CAD file in AUTOCAD software.

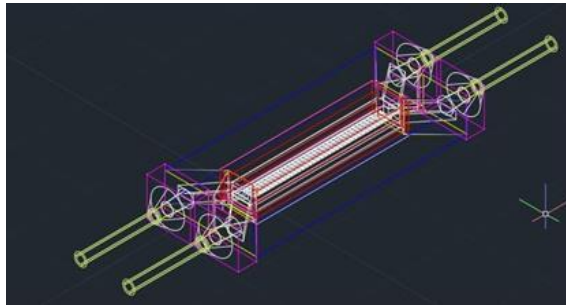


Figure 5-1: Designed CAD Model

5.2 STL File

The CAD file (.dwg) was converted to .(stl) format and sent to the printer for printing.

Figure 5-2 shows the isometric view of the designed construct

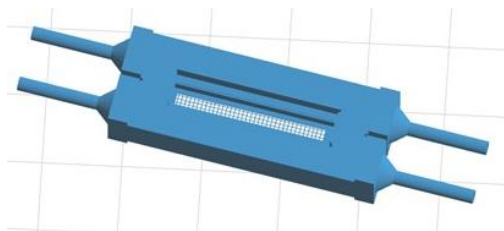


Figure 5-2: STL File

5.3 Design Outcome

It was found that the designed model had a

Length: 20 mm

Width: 1 mm

Height: 2.5 mm

Figure 5-3 shows the inlets and outlets as designed in the model.

Figure 5-5 displays the designed mesh 100-micron mesh has a pore size: 50 x 100 micron

Figure 5-4 shows the two lids are provided to securely close the structure.

Figure 5-7 shows the printed construct after post processing and removing the supports.

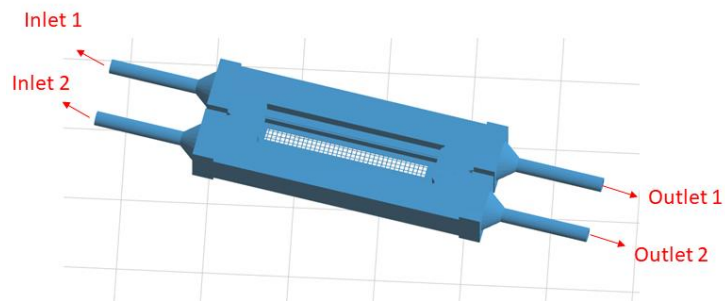


Figure 5-3 : Liver Sinusoidal Model STL



Figure 5-4: Top and Bottom Lids

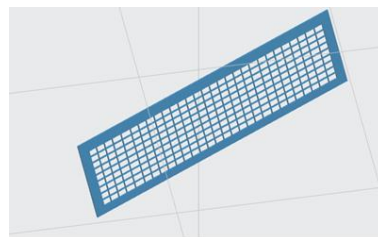


Figure 5-5: Mesh

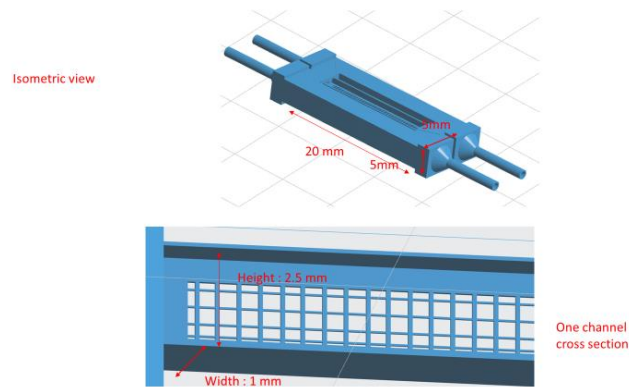


Figure 5-6: Measurements of the model and channel



Figure 5-7: Printed Model

5.4 Flow test

The flow test was performed successfully for both top and bottom channels. It was seen that the dye perfused effectively without any blockages. Thus, all the inlets and outlets are open without any obstruction. Figure 5-8 shows the dye perfusing through the channels.

It was interesting to note that there was no leakage of the dye from one side of the mesh to the other side due to strong capillary force. This ensures that during dynamic culture, the perfusion of media will be proper without any leakages.



Figure 5-8 : Flow test

5.5 Diffusion test

The diffusion test was performed. After addition of the dye the area was marked. We allowed the diffusion of the dye to occur. We observed the diffusion after 30 minutes and one hour. It was observed that after one hour the dye diffused almost completely to the other side of the mesh. This position was also marked and compared to the previous one. Figure 5-9 shows how the experiment was proceeded.

This experiment states that there is continuity between the seeded gelatin gel even though a mesh is present between the top and the bottom layers. Furthermore, it can be concluded that the porous gel allows diffusion of the dye from the top to the bottom, thus diffusion of nutrients in the gel must also occur.

Thus, the gel-mesh-gel system is a continuous system and allows effective diffusion.

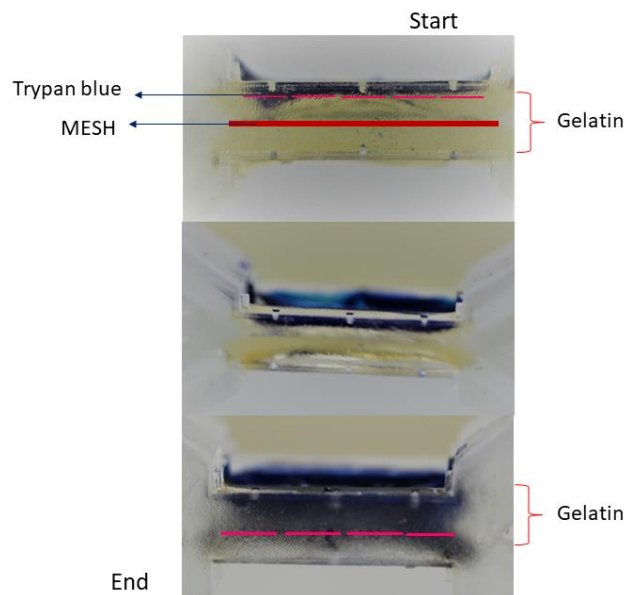


Figure 5-9: Diffusion Test

5.6 DNA estimation

DNA estimation was carried out using Nanodrop, it is usually done after lyophilization to check the efficiency of decellularization. DNA estimation is majorly required if we want to use our decellularized product/ hydrogel for *in vivo* applications. Minimal or no DNA content should be present in case of *in vivo* implantation or injection as it might lead to immune rejection of the hydrogel and cause many unwanted immune reactions.[18]

In our case, the dECM hydrogel formed will be used as a support for *in vitro* hepatocyte growth, the gel is added to provide a natural environment to liver cells and allow them to

grow in 3D. Additionally, in such a system the DNA removal effectiveness does not make a major difference. In our characterization using Nanodrop, we found the native DNA content to be 20,095 ng/ mg of dry tissue and decellularized DNA content to be 14,005 ng/ mg of dry tissue. Figure 5-10 shows that there is no major difference between the DNA content of native and decellularized liver samples.

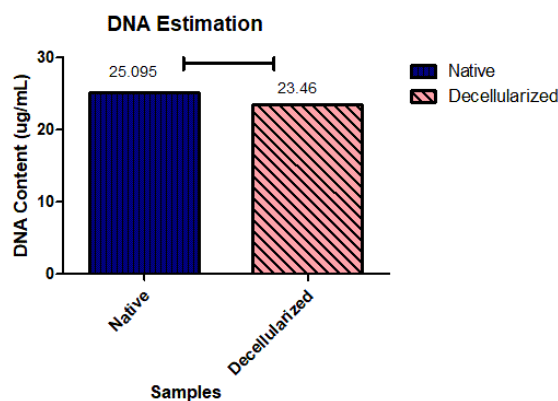


Figure 5-10 : DNA Estimation

5.7 GAG estimation

GAG estimation is performed by using DMMB Assay. The papain digested native and decellularized samples were subjected to DMMB dye and absorbance at 540 nm was noted. Figure 5-11 shows that the decellularized GAG content was 76.9% less as compared to the native liver tissue. 0.12 mg of GAG per mg of dry weight of liver tissue was obtained in decellularized sample. It is interesting to note that a considerable amount of GAG content is retained after the process of decellularization although some leaching of GAG was observed. Thus, the process is decellularization followed is effective in retaining the ECM components and therefore the dECM obtained can be used for *in vitro* biomimetic applications.

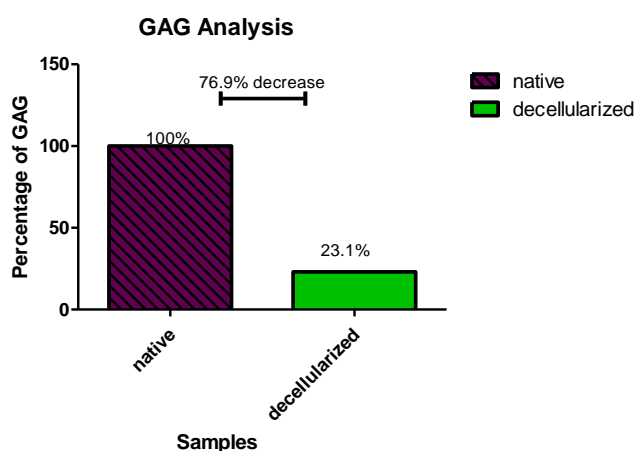


Figure 5-11 : GAG Content Analysis

5.8 Collagen estimation

Collagen estimation is done using hydroxyproline assay. It was found that there was no significant difference between the collagen content of native and decellularized liver. Figure 5-12 shows that only 7.7% decrease in collagen content was found in the decellularized samples. 14.2 ug of collagen content was found in per mg of tissue (dry weight).

We can conclude that almost negligible collagen leaching took place and the process employed for decellularized is apt for retaining the major ECM component collagen. Our results are in accordance to many other research groups who have tried to decellularize tissues like heart, adipose, cartilage, skin etc. as they also found that the decrease in collagen content is minimal or very little. [21][16]

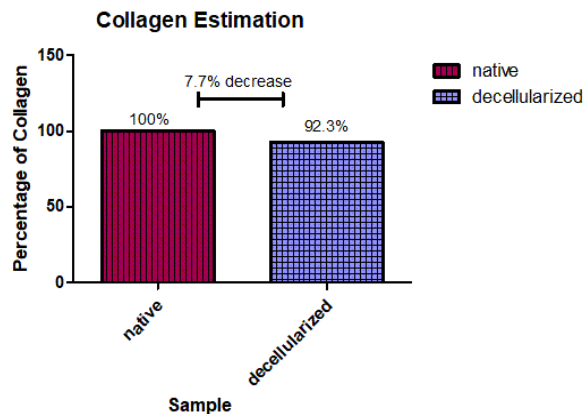


Figure 5-12: Collagen Estimation

5.9 Rheology

The data was analyzed in the equipment RHEOPLUS/32 V3.61 2 and graphs were plotted. Figure 5-13 explains the rheological profile of the samples.

From the viscosity v/s shear rate graph it was observed that the pregel possesses shear thinning property as there is a gradual decrease in the viscosity with increasing shear rate. Thus, our gel is ideal for the process of 3D printing. The graphs for strain v/s shear rate confirmed the shear thinning property of our 3% liver dECM gel.

The graphs plotted for storage modulus and loss modulus v/s strain percentage show that the storage modulus is greater than the loss modulus at all strain percentages, thus, confirming that the hydrogel has viscoelastic property and has shape fidelity i.e. can retain shape after printing process.[16]

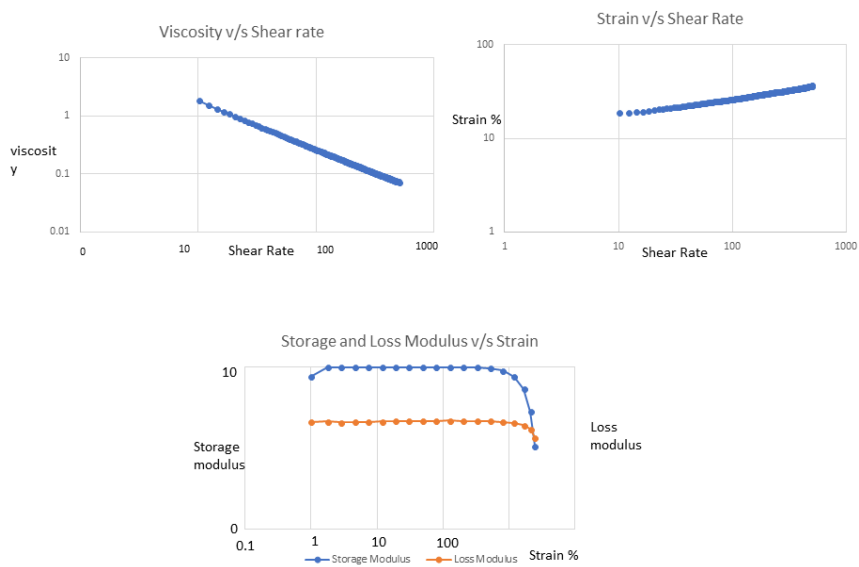


Figure 5-13: Rheology Curves

5.10 Cell seeding in gel

Figure 5-14 shows the cells used for the experiment i.e. HepG2 cell line. 10^6 cells were used per ml of gel. Cells were cultured in DMEM low glucose media and Figure 5-15 shows that the cells were incorporated in the gel for preliminary studies and was finally seeded onto the construct. Figure 5-15

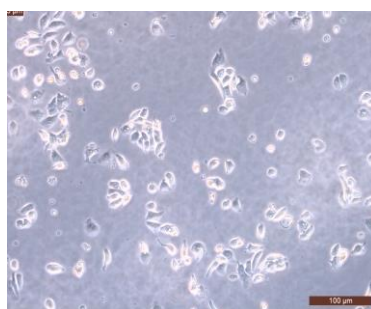


Figure 5-14: HepG2 cells

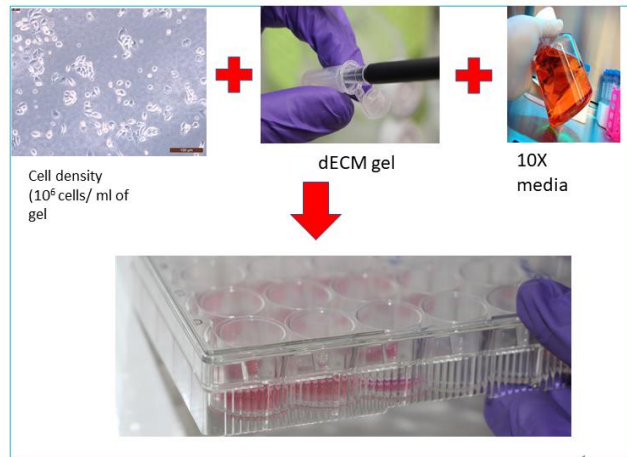


Figure 5-15: Cell seeding in gel

5.11 Cells seeding in construct

Figure 5-16 shows that the cells and pre-gel (bioink) were added in the construct. Figure 5-16 Figure 5-17 shows that the construct was cultured in T-25 and kept in culture for 7 days in incubator at 37°C. Figure 5-17 The construct was then fixed using formaldehyde and then taken for confocal imaging after staining.

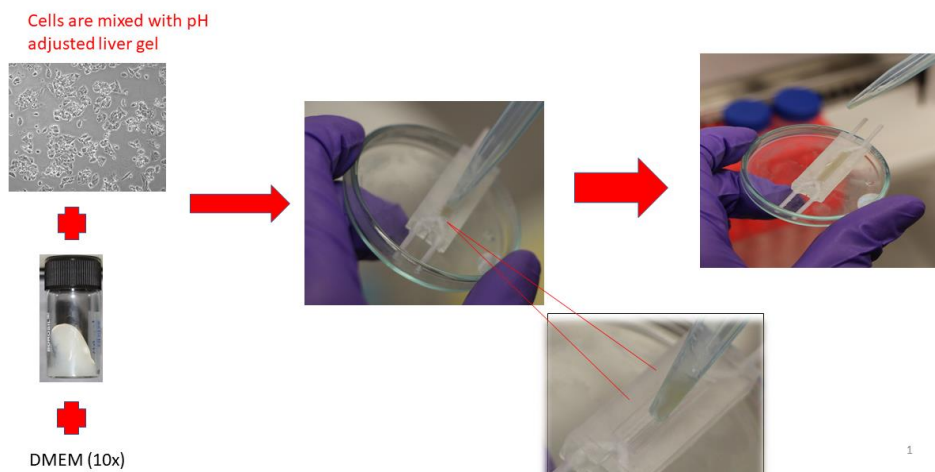


Figure 5-16: Cell Seeding in the Construct

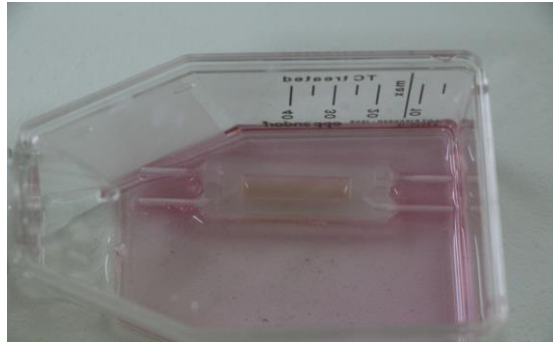


Figure 5-17: Static Culture

5.12 Live/Dead Assay

The cells embedded in the gel stained on Day 3 with Calcein AM and ethidium homodimer were viewed under a fluorescent microscope. At 494 nm wavelength many green fluorescence cells were observed indicating the live cells and at 517 nm wavelength few red colour cells were observed indicating the dead cells.

Figure 5-18 shows almost 80% cell viability, thus stating that the cells are viable in the liver dECM gel. Hence, the liver dECM gel is supporting the HepG2 cell growth and so we concluded that this gel can be used for further studies as it provides a biomimetic environment for the HepG2 cells.

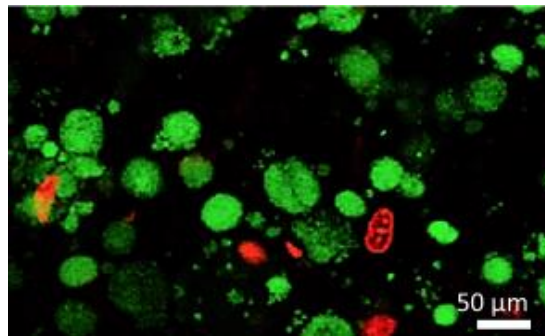


Figure 5-18: LIVE/DEAD Assay

5.13 Immunofluorescence

After observing the stained samples under confocal microscope, we could observe the blue coloured nucleus of the cell stained by Hoechst 33258 dye at excitation 355 nm and emission 465 nm. Furthermore, red colour was seen co-localized with the nucleus indicating the presence of HNF-4 alpha antigen at excitation 650 nm and emission 665 nm. Figure 5-19 depicts the expression of HNF 4 alpha in red colour and the nucleus was seen in blue colour.

This shows that the HepG2 cells are functional in the liver dECM gel as they are synthesizing the transcription factor HNF 4- alpha. This HNF 4- alpha is crucial for the up regulation of other liver specific genes of hepatocytes.

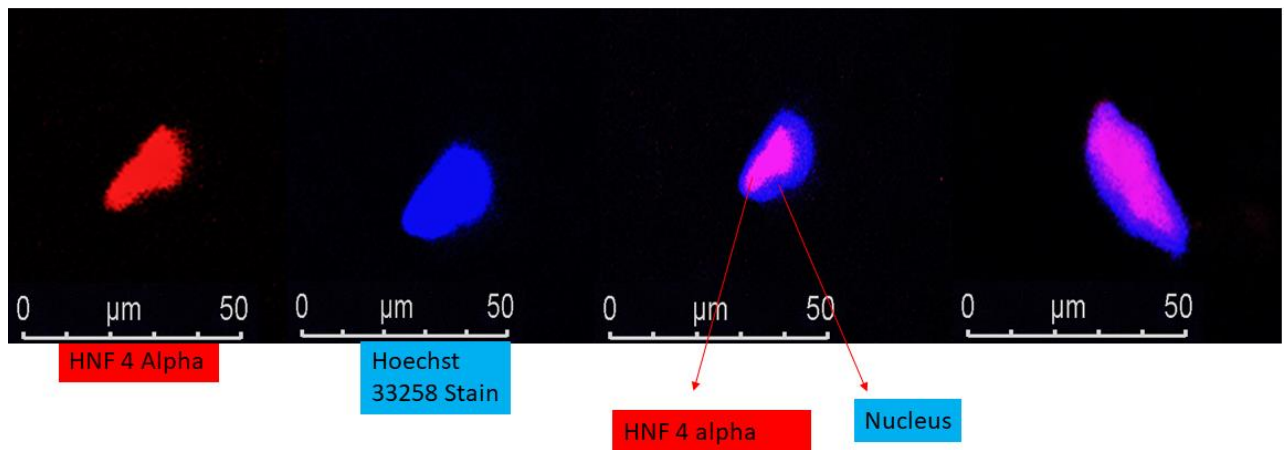


Figure 5-19: Immunofluorescence Results

Chapter 6

Conclusion

It was observed that the 3D printed sinusoid model has architectural similarities with the native liver sinusoid and can be exploited to completely recapitulate the sinusoid with all its physical and functional peculiarities. Also, the prepared decellularized liver extracellular matrix hydrogel retained the major extracellular matrix compounds and found to be supportive for functionalization of the hepatocytes in the *in vitro* sinusoid model. Further long-term studies and characterizations are required to establish the complete *in vivo* like functional liver sinusoid unit.

Future Work

In the present work we have fabricated a 3D sinusoidal liver model as well as assessed the functionality of the HepG2 cells in the liver dECM gel. This study will be taken forward and we plan to do the following in the future:

1. We plan to seed HUVEC (endothelial cells) in the construct.
2. Assessing of protein expressions
3. Dynamic Culture
4. Characterization for bile duct formation, space of disse and oxygen gradient.

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