Development of *Invitro* 3D Bronchi Model Using Novel Decellularized Smooth Muscle Matrix

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The Degree of Master of Technology

Department of Biomedical Engineering

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Declaration

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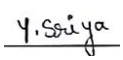

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Approval Sheet

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Abstract

Oxygen is the most important necessity for keeping us all alive. Lungs, in our body allows oxygen into our body. Any damage to this vital organ could be fatal. Incidences of tracheal and bronchial injuries are at a rise with increase in the number of road traffic accidents. Apart from traffic accidents, accidents that involve chest crashing, gun wounds, knife penetrations on chest area also damages the respiratory tract causing either blunt or penetrating injuries. Most of the injuries due to blunt trauma encompasses damage or rupture of trachea and mainstem bronchi. Not only injuries, several other conditions like tumors in the airway or congenital malformations like severe respiratory stenosis, bronchomalacia and bronchial atresia etc. also cause tracheobronchial damages. The theraupeutic approaches for all the cases are not well defined and may vary based on the site, extent of lesion and the severity of damage. There are problems associated with the traditional surgical approches and hence researchers are finding and developing new ways to overcome the complications and also to minimize the organ- demand and supply curve. In this thesis, we made an attempt to develop smooth muscle layer of the bronchi which is the part of the major project lab engineered bronchi which structurally and functionally mimics the native bronchi. Herein, we designed and fabricated a bioreactor using 3D printing technology and used this construct for development of smooth muscle layer of the bronchi model. To support the cells in the construct, we decellularized caprine smooth muscle and prepared its hydogel. Mesenchymal stem cells were embedded into the pre-gel and was incorporated into the printed bioreactor. In this process, we also tried to study the differentiation of MSC into smooth muscle cells in the decellularized smooth muscle matrix which stands as novelty in this project.

Nomenclature

TBI- Tracheobronchial Injury

DBE- Disk Bronchial Equivalents

ALI- Air-Liquid Interface

ECM- Extra Cellular Matrix

COPD- Chronic Obstructive Pulmonary Disease

IF- Immuno Fluorescence

UCMSC- Umbilical Cord Mesenchymal Stem Cells

α- MEM- α- Minimum Essential Medium

PBS- Phosphate Buffered Saline

SLA- Stereolithography Apparatus

SDS- Sodium Dodecyl Sulphate

dSMM- decellularized Smooth Muscle Matrix

GAG- Glycosamino-Glycans

BSA- Bovine Serum Albumin

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Chapter 1

Introduction

In order to develop new drugs, to understand the complex cellular pathways involved in many diseases or to study the basic underlying mechanisms inside a human body; two-dimensional culture and animal models are the most prevalent models available. However, these models do not completely mimic the in-vivo conditions, thus the drugs developed and tested on such models do not pass the human trials. Therefore, new concepts or models that can simulate the human in-vivo conditions are now gaining attention. Cell culture models that are developed in the 3D environment has a potential to mimic the microenvironment and microarchitecture similar to an in-vivo condition. Moreover, these models can also resemble the structural and functional features of the specific tissue or organ that is being developed.

3D printing technology has emerged as a rapid prototyping technique to develop models in 3-dimension easily and effectively. The concept of 3D printing was initially developed to manufacture complex and personalized machine parts, jewellery or to produce replicas of artifacts. This technology was later applied to create molds for the development of 3D structure from biocompatible materials. The concept of 3D printing when applied to biological research had transformed the organ/ tissue engineering methods and popped up as the finest solution in the field of medical research[1]. Bioprinting is a promising strategy to develop 3D models because of its advantages like high throughput and controlled delivery of scaffold and cells, growth factors etc. 3D bioprinting has emerged as a technology that ensures the development of in-vivo mimicking structures and is attaining immense attention in the recent past[2]. The artificially printed organs not only aid in research, training sessions but also solve the organ transplant shortage, which

eventually reduces treatment time. Multi-layered skin, heart valves, vascular grafts, bone marrow, thyroid glands are few noted structures that are developed using this technique[3]. Precise positioning, apt biomaterial, and cell source are the key factors for achieving the functional aspects of printed tissue.

Chapter 2

Aim and objective

This project aims at developing an *invitro* three-dimensional bronchi model that mimics the structural and functional features of in-vivo bronchi and also replicates the microenvironment found in the lower respiratory tract. The structure of the bronchi is achieved when cells are cultured inside a bioreactor which is designed and fabricated as part of the project. In order to accomplish this, an attempt is made to do the following.

1. Design and Fabrication of Bioreactor.
2. Decellularization of caprine smooth muscle
3. Preparation of smooth muscle gel
4. Cell culture
5. Characterization of the developed construct.

Chapter 3

Motivation

The Bronchi structure that was aimed to develop in this project serves as a solution for the ailments like tracheobronchial injuries, treating conditions like tracheobronchial malacia which is predominant in infants, bronchial anthracofibrosis- condition generally seen in elderly women.

3.1 Tracheobronchial Injury

Tracheobronchial injury is a rare yet serious condition that causes obstruction in airway resulting life-threatening respiratory distress. It is a condition that can result from high-speed traffic crashes, inhalation of harmful fumes or aspiration of objects or liquids[4]. The main stem bronchial injuries comprise the majority of tracheobronchial injuries[12]. Although, there might be instant death due to lack of oxygen at the time of injury, due to the advancements in the emergency care incidences of deaths before reaching hospitals are reduced, thereby increasing the diagnosis rate[13]. Rapid diagnosis and treatment are very important in case of TBI and any impairment in trachea or bronchus should be fixed as soon as possible, yet more often than not , diagnosis and treatment are delayed leading to further complications. Complications include pneumonia, bronchiectasis, stenosis, formation of scar tissue and infections in the injury site. Treatment options that are available to treat TBI are opening the obstructed airway by introducing an endotracheal tube, placing a stent or surgically performing end to end anastomosis. Although, surgery is not compulsory to treat TBI, surgical reparation is considered as the standard treatment in most of the TBI cases[5]. Surgical reparation includes end to end anastomosis and closing of flap using sutures. The damaged segment is resected and these separated parts are surgically connected using bio absorbable sutures in end to end anastomosis[15] (Figure 3.1). However, the maximum length of segment that can be resected should not be more that 4 cm[5] else there could be

shortening of the tract length and stress on the sutures thus complicating the issues further. After the surgery is performed stents are positioned in the surgical place so as to keep the airway open. Stents as a treatment option in TBI may loosen over time and there are higher chances that these stents relocate to smaller bronchioles and block the airway. Also, there are chances of granuloma formation around the stent and could lead to inflammation and infections. Not only these, there are several more problems when the above treatment options are considered. Few cases were reported that introduction of an endotracheal tube, deteriorated TBI instead of treating the condition. Multiple forced attempts, inexperience of health professionals, multiple repositioning of the cuff, inappropriate positioning of the tube are multiple factors that contribute for worsening the condition[14]

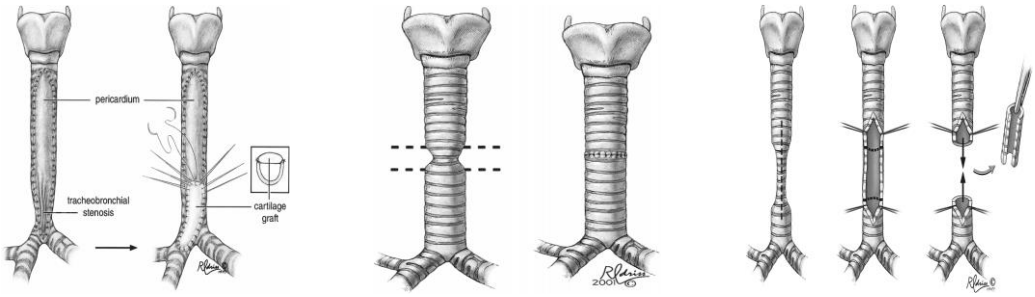

Figure 3.1: Surgical techniques to treat tracheal injuries [15]

3.2 Bronchomalacia

Bronchomalacia is a term for weak cartilage wherein either generalized or localized weakness of the bronchial wall collapses and obstructs the lumen (Figure 3.2). It is considered to be the congenital defect of the bronchus in the respiratory tract that mostly affects infants. In this disease, the main stem bronchi collapses, obstructs air passage causing respiratory distress. Because of the weak wall the bronchial lumen diameter reduces when patient exhales and increases on inhalation causing distress while breathing. This condition of bronchomalacia even worsens if the patient has abnormal vascular structure around the respiratory tract. The abnormal blood vessels compress the lumen of bronchi as the wall does not possess enough strength to maintain the airway open. Congenital defect in larger airways is one the causes of irreversible airway obstruction in children[6]. Bronchomalacia is treated by surgical

methods either by tracheotomy or inserting prosthesis so that airway remains open. Tracheotomy or tracheostomy is a medical procedure which involves insertion of a tube by creating an opening in the neck. The tube can be temporarily or permanently placed inside the trachea. This tube supports the weak wall of the respiratory tract and eases the passage of air. There are complications in this technique either immediately after the procedure or over time. The complications that develop over time are infections, dried secretions as the mucus is being blocked near the tube, rupture of lumen due to rubbing against tube. The next surgical procedure to overcome bronchomalacia is to insert stents or prosthesis into the airway. The stents are placed in the defective area and are sutured to the native trachea. These sutures are placed at the two extremities of the stent to minimize the movement of the stents inside the airway. The stents or prosthesis will support the wall till there is re-epithelialization typically 2-6 months after which they will be removed. As the age increases, size of respiratory tract modifies, there are very high chances of prosthesis relocation, granuloma formation and stent rejection, failure of the procedure due to inappropriate stent length, bacterial growth due to granulation tissue are complications that arise with the currently available stents[16]. Secondary bronchomalacia that is observed in adults due to recurring polychondritis[7] and chest crashing is also treated in the similar approach mentioned above with associated complications.

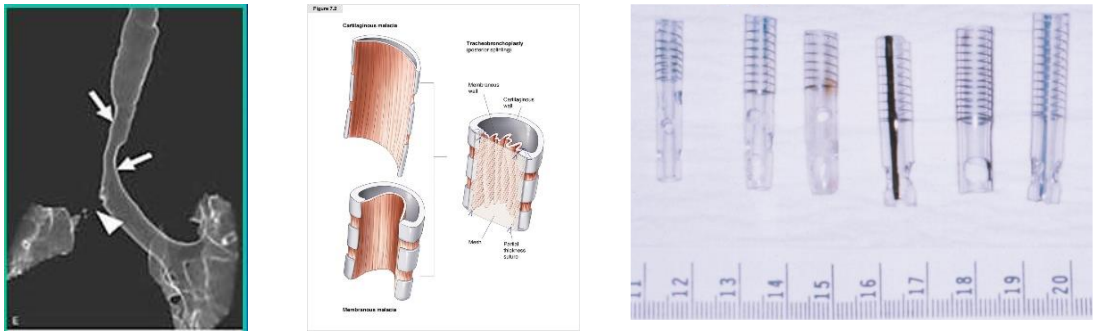

Figure 3.2: Bronchomalacia with current treatment options[16]

3.3 Bronchial Stenosis

Bronchial stenosis in adults is a condition that has been associated with several ailments in the lung which includes tuberculosis, sarcoidosis, trauma, prolonged

endotracheal tube intubation, bronchial sleeve resection, anthrocofibrosis and also after lung transplantation. Depending on site and severity of bronchial stenosis, it can be managed by various surgical techniques like cryotherapy, LASER therapy, surgical resection – re-anastomosis (Figure 3.3) and stent placement. Nevertheless, definitive treatment for stenosis is surgical resection and re-anastomosis. However, surgical procedures are not possible for patients whose health condition is very poor. For those patients, other alternative procedure is placing stents which compresses stenosis and opens the airway. Stents implantation often involves complications like stent rejection, relocation, granuloma formation. A study revealed that granulation tissue formation is the most frequent complication associated with stents. Due to several complications with the stents, recently bronchoscopic balloon dilatation procedure is being followed in the management of bronchial stenosis[17] (Figure 3.4). In this technique, under the guidance of bronchoscope a balloon is placed across the stenosis. This balloon is steadily inflated with saline solution and is held for some time. After deflation of the balloon if the airway is still narrow, the procedure is repeated with a balloon of larger diameter. The procedure is considered success if there is no narrowing of the airway after 6 months[16]. The potential complication of this technique is tearing of the bronchial wall due to excessive stretching of the balloon[24].

With the advancements in technology, there are treatment options available to treat above mentioned disease conditions. But, the treatments are followed with a wide number of complications for which the patient has to be treated repeatedly. Hence, there is a dire need for a tissue engineered construct that can provide appropriate mechanical strength, will not induce any stenosis and keeps the airway open once implanted or sutured in the site. Herein, we hypothesize that 3D printing technology has a potential to solve the primary disease condition without further complications.

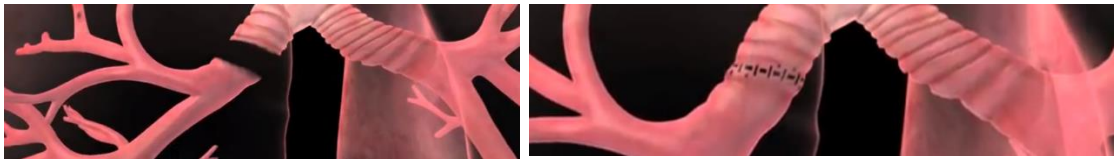

Figure 3.3: Resection of bronchi part and re-anastomosis

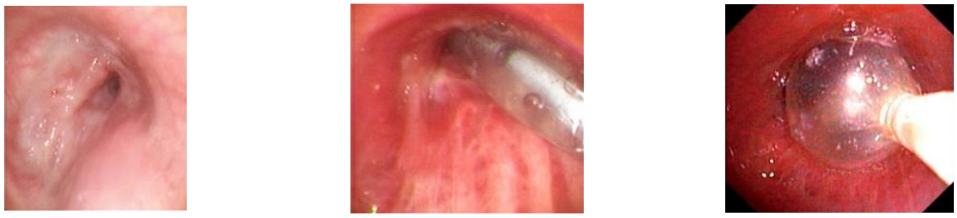

Figure 3.4: Constricted Bronchi lumen and management using balloon dilatation technique[24]

Chapter 4

Literature Review

4.1 Overview

The lower respiratory tract also called as a tracheobronchial tree to depict the branching assembly of the airway that provides a path for oxygen and carbon-di-oxide movement. This tract consists of trachea divided into bronchi which further divides into bronchioles and to the smallest unit of lungs i.e. alveoli. This complex system is partitioned into 23 generations where at each generation the airway branches into two smaller airways. The Path from Trachea (0^{th} generation) to terminal bronchioles (15^{th}-16^{th} generation) is purely conducting path where no gaseous exchange takes place. The later generations further divide into alveolar ducts that are completely lined up with alveoli which are gas exchange parts of the lung.

Bronchi is the part that is considered in this project. Similar to the trachea, bronchi are also bounded by C-shaped cartilaginous rings. Underneath cartilaginous rings lie sub-mucosa which consists of smooth muscle cell layer which controls the diameter of the lumen during inspiration and expiration, blood vessels to supply nutrients to cells present in sub-mucosa and sub mucosal glands. The innermost lining of the lumen is populated with a single layer of Pseudostratified ciliated epithelium and goblet cells that produce mucus to protect the mucus lining in the lumen. To connect bronchi and other tissues of the lung outer bronchial walls are covered with a band of adventitia that acts like a connective tissue.

In the recent past, researchers have started developing *invitro* bronchi models to study and understand the complex pathways involved in asthma. J.S.Paquaette et, al developed a three-dimensional human bronchial models using epithelial and fibroblast cells isolated from biopsies. They had produced two different bronchial equivalents Disk shaped (DBE) and tubular shaped. Initially, due to fibroblast

contractility the bronchial equivalents were not retaining their shape. But then the same group had shown that by using peripheral anchorages gel seeded with fibroblasts contractility can be reduced. Both these models were developed on such peripheral anchorages under four different culture conditions. It was observed that serum-free culture media with retinoic acid exposed to ALI culture is the best for maintaining bronchial cell properties[8]. A year later the same group used asthmatic/non-asthmatic epithelial cells and asthmatic/non-asthmatic fibroblast cells and produced asthma bronchial equivalents. A comparative study was done for four groups viz. asthma epithelium/asthma fibroblasts, asthma epithelium/non-asthma fibroblasts, non-asthma epithelium/ asthma fibroblasts, non-asthma epithelium /non-asthma fibroblasts. Results indicated that asthma epithelium cultured on non-asthma fibroblasts layers slowly reached confluency but with culture time epithelial layer started to detach from mesenchymal layer[9]. In another study Sato et.al proposed the use of a prosthesis that is developed for replacing the left main bronchus in canine. The main prosthesis is developed using polypropylene mesh which is reinforced with c monofilament strings (Figure 4.2). Using freeze-drying technique 5 mm thickness collagen was deposited on the mesh. This structure was implanted into canine whose left main bronchus was blocked due to stenosis. Severe stenosis developed in few models that has a lumen diameter of 12 mm. On the contrary, models with 15 mm diameter, prosthesis showed no stenosis development. Prosthesis coated with 2 % collagen showed complete epithelialization, no mesh exposure, and no stenosis. Researchers believe that this prosthesis is a promising alternative in the treatment of bronchial stenosis and obstruction[10]. In a recent study, Ishikawa et al. developed a 3D epithelial-mesenchymal co-culture model of human bronchial tissue to understand the effects of TGF-β1 on airway tissue remodelling. The gel containing lung fibroblasts that was applied to a cell culture insert and a layer of mucociliary differentiated bronchial epithelial cells were seeded on top of gel which enabled direct contact between epithelial and mesenchymal cells. ALI culture was also initiated to induce mucociliary differentiation. TGF-β1 was induced to stimulate airway remodelling in the model. Results showed that TGF-β1induced mesenchymal marker vimentin, increase in the number of elongated HBEC's, decrease in E-cadherin suggesting that there is Epithelial-mesenchymal

transition. Their findings indicate that TGF-β1 effects both epithelial and mesenchymal cells and induce gel contraction[11]. Vary recently Elliott et al, presented a case of using a decellularized cadaveric trachea, repopulating with cells and transplanting it in a patient who was suffering with long segment congenital tracheal stenosis. Because of recurrent stenosis the patient described here had undergone many surgeries like tracheoplasty, stent insertion, balloon dilatations, pericardial patch tracheoplasty, yet none were successful. Considering the condition of patient, success with autologous stem cell based tracheal transplant, with the consent of HTA, FDA, family, the clinical team proceeded with the procedure. A tracheal graft from cadaver was collected and it was decellularized. This decellularized trachea was repopulated with autologous cells ex-vivo and were cultured in bio-reactor[25]. Once the repopulated trachea was ready for implantation, the diseased segment was resected and replaced with the cadaveric trachea. The proximal and distal ends of the repopulated decellularized trachea was anastomosed with sutures. Immediately post operation, bronchoscopy revealed that the graft was intact but after 15 days, the patient developed ventilatory distress. Evaluation revealed that there was progressive narrowing of tracheal graft and the patient died after 24 h of evaluation. Even though the initial results were encouraging, the graft could not support or improve the condition of the patient. The reasons for the narrowing of the graft could not be confirmed as they could not retrieve the graft after post-mortem. Hence, there is necessity of improvising the techniques so that the failure rate reduces when implanted in the patient (Figure 4.3).

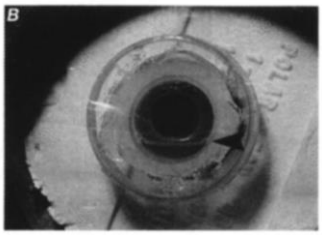

Figure 4.1: Disk bronchial equivalents[8]

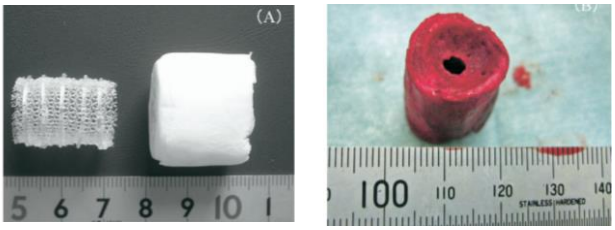

Figure 4.2:Polypropylene mesh prosthesis[10]

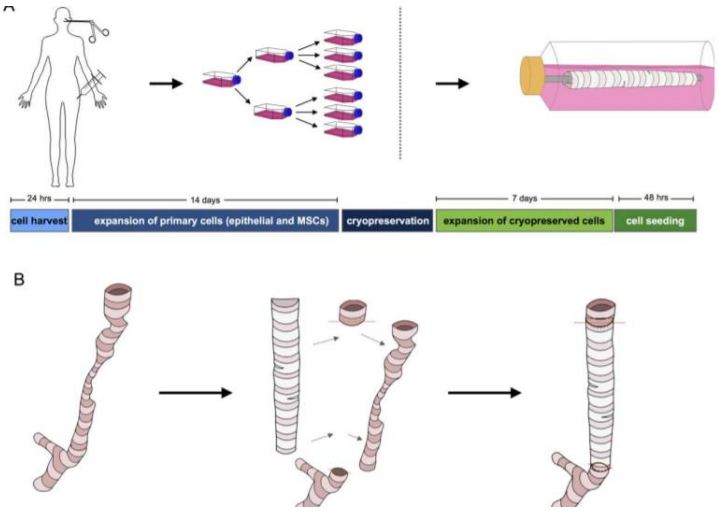

Figure 4.3: Recellularization of Cadaveric trachea with patient derived stem cells[25]

4.2 Decellularization

Decellularization is not a new term in tissue engineering and regenerative medicine. Decellularization principally means removal of cells from the tissue or organ leaving the structural and functional biological material in the organ itself. Most of the decellularization protocols involves chemical, physical and enzymatic methods to completely remove the cellular content from the tissue. Depending on the tissue source methods mentioned above are followed either individually or in combination. In the recent past researchers have started exploring decellularization of many different tissues and are using them to develop tissue constructs. Recently, Melo et al decellularized porcine trachea and used it for the development of bronchial wall. Porcine tracheal tissue was decellularized and is placed in a transwell. On the apical side epithelial cells were seeded and fibroblast, endothelial cells on the basolateral side of the transwell[18] (Figure 4.4). After performing all the necessary characterization techniques, the results demonstrated that this decellularized tracheal

tissue can be employed for developing bronchial equivalents. They also claim that as the co-culture of three different cell lines along with the native membrane mimics the in-vivo condition very closely. In another study decellularization of tissue was done to understand the role of bronchial epithelium ECM during epithelial cell differentiation in COPD patients. In this study Ulf Hedstrom et al obtained lungs from COPD stage 4 patients, the bronchial airways were dissected from the lungs and were subjected to decellularization (Figure 4.5). These decellularized bronchial tissues were repopulated with NHBE and are collected at different time points for histology, IHC and other characterization techniques. It was observed that primary human bronchial epithelial cells differentiate into airway epithelium on normal and COPD bronchial scaffolds[19]. Recent studies reveal that the cadaveric whole lungs[21] that are otherwise unsuitable can be decellularized and they might serve as source for whole organ engineering which is supposed to have potential to reduce the organ demand and supply[20].

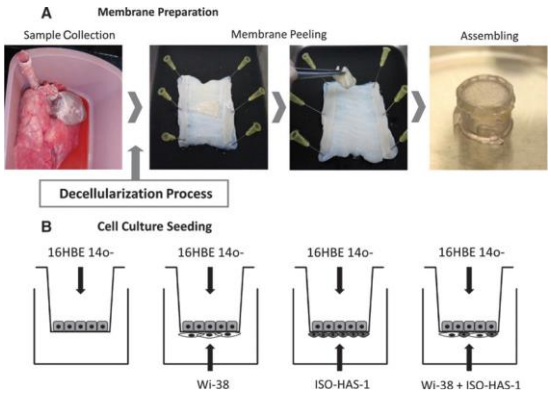

Figure 4.4: Development of bronchial wall in transwell[18]

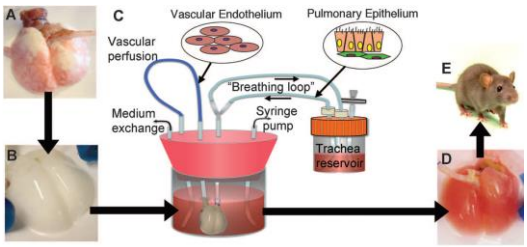

Figure 4.5: Whole lung decellularization[20]

4.3 Summary of the current state of bronchi tissue engineering

Not long-ago researchers have actively started to develop tissue engineered trachea either by using synthetic materials or collagen that is commercially available. After that came decellularizing of tissues to develop tissues that closely mimic the native environment. There are several studies that had shown decellularization of non-tracheal tissue to generate tracheal graft. Investigators then started decellularizing complete trachea as a whole, re-populate with autologous cells and replace with the diseased portion in the patients. As the number of bronchi related cases are emerging, researchers have started focusing on bronchi only in the recent past. To the best of my knowledge and available literature, a tissue engineered bronchi that can structurally and functionally mimic the native bronchi is not yet developed. Also, till date, there is no data on models that have bronchial epithelial cells co-cultured with bronchial smooth muscle cells in the decellularized smooth muscle matrix.

Chapter 5

Materials and Methods

5.1 Cell Culture

Umbilical cord mesenchymal stem cells UCMSC's that are isolated in our lab are used to develop the tissue engineered bronchi. Umbilical cord was collected from a nearby hospital and was transported to our lab in the solution containing PBS and antibiotics. The collected tissue was washed multiple times with PBS and antibiotic solution. Once the cord tissue is devoid of blood, it was then minced into small pieces of 2-3 mm and were kept in a sterile T-25 flask. After 14 days of explant culture we obtained MSC (Figure 5.1). These cells are used for the project. The cells whose passage number is 3 were used for the study. Cells were cultured in DMEM-alpha modification media with 10 % fetal bovine serum and 1 % Penicillin-streptomyocin. Once the cells are confluent in the flasks, they are detached using trypsin solution (Trypsin : PBS=1:1) centrifuged at 2000 rpm for 5 min and were resuspended in fresh media. 1×10^6 /ml of cells were mixed in the pH adjusted pre-gel and was injected into the temporary part and outermost cylinder of the bioreactor assembly (Figure 5.2).

Figure 5.1: Umbilical Cord MSC isolation using explant culture

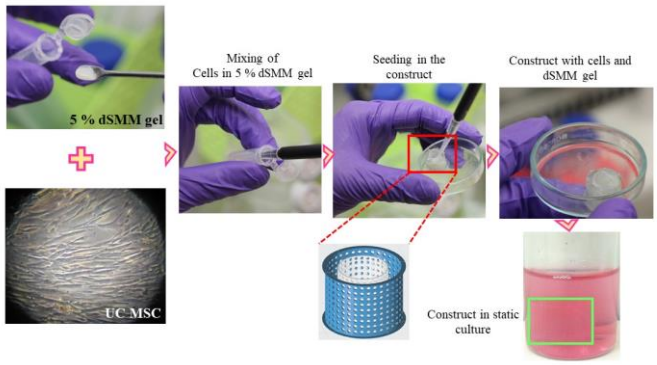

Figure 5.2: Cells + gel in the construct

5.2 Design of In-house Bio-Reactor

As discussed previously, to develop a tissue engineered bronchi, static culture conditions are being provided. In order to develop a tissue engineered bronchi that can structurally and functionally mimic the native bronchi, it is better to provide dynamic culture conditions for it to resemble the in-vivo bronchi. Herein, we developed a bioreactor model which is structurally similar to bronchi, hypothesizing that it would provide proper dynamic conditions to the cells that contribute to the development of engineered bronchi.

5.2.1 CAD design of the bioreactor parts

The complete bioreactor has 6 different parts, each part having its own importance. All the 6 parts when assembled accordingly constitute the bioreactor setup mimicking the required microenvironment. The naming convention of the parts is as follows and the design specifications of each part are mentioned in the next section. For all the parts initially a 2D sketch is developed and its 3D modelling is done in AutoCAD v2017.

5.2.2 Part 1: Innermost cylinder

This part has a height of 15 mm and a diameter of 6 mm with a thickness of 250 µm. Two channels are connected on the either side of this cylinder, one to let in media and the other to let the media out. Thereby providing continuous media movement throughout. Cells are cultured on the outer surface of this cylinder. The media that is flowing inside the cylinder has to reach the cells cultured outside. For this nearly 160 holes of 600 µm in diameter are created on the curved surface of this cylinder.

The importance of this part is to provide nutrients from media to the cells that are on the outer surface of this cylinder through diffusion. The diameter of this part is chosen in such a way that it obeys the thickness to diameter ration as per the literature. The CAD modelled image of the innermost cylinder part is shown in the below (Figure 5.3).

Figure 5.3: Innermost cylinder

5.2.3 Part 2: Temporary cylinder

The next part after the innermost cylinder is the temporary part that surrounds the innermost cylinder. This has a height of 10 mm and diameter of 4 mm with a thickness of 250 µm. the main purpose of this part is to support the gel that is being filled in between the other parts of the bioreactor. This also has holes of 600 µm to diffuse media into the gel. The CAD modelled image of the innermost cylinder part is shown in the below (Figure 5.4).

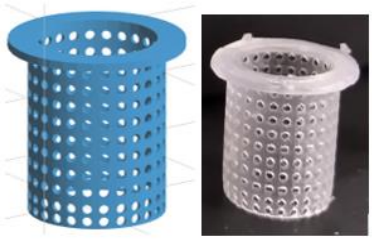

Figure 5.4: Temporary cylinder

5.2.4 Part 3: Capillaries

This part has nearly 20 hanging structures named capillaries with each of height 10 mm and each has a diameter of 1 mm. From literature is was found that nutrients from the media cannot diffuse more than 300 µm. The gap between the innermost cylinder part and the next part is nearly 2.75 mm and hence the cells in the inner layers might not get sufficient nutrients from the media and there are chances of necrotic core being formed at the center. To avoid this, we have these capillaries in

between the temporary cylinder and the next part, i.e. outermost cylinder (details in the next section). The CAD model is shown in (Figure 5.5).

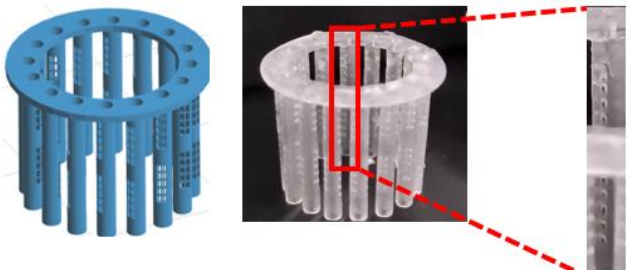

Figure 5.5: Capillaries structure

5.2.5 Part 4: Outermost cylinder

The outermost part is last part of the bioreactor assembly. This holds the gel and cells in the bioreactor. All the other above-mentioned parts are assembled inside this outermost cylinder. It has a height of 10 mm and a diameter of 12.5 mm with a wall thickness of 250 µm. Gel with cells will be present in between innermost cylinder and this part. This part also has holes of 600 µm throughout its circular surface. Equidistant spacing of 400 µm exists between the adjacent holes. The CAD model is shown in (Figure 5.6).

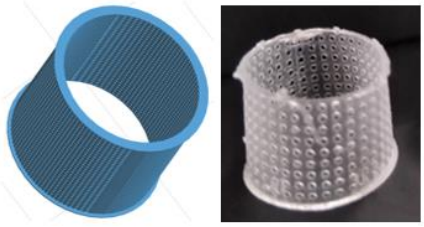

Figure 5.6: Outermost cylinder

5.2.6 Part 5: Outer Jacket

With a height of 16 mm and diameter of 23 mm this part holds all the above parts like a single unit. Inside this part there will be continuous flow of media and through diffusion from the small holes present on the circular surface of the outmost cylinder nutrients gets absorbed by the gel and cells. It has a conduit connected at the bottom to allow media to drain from it. Modelled image is shown below (Figure 5.7).

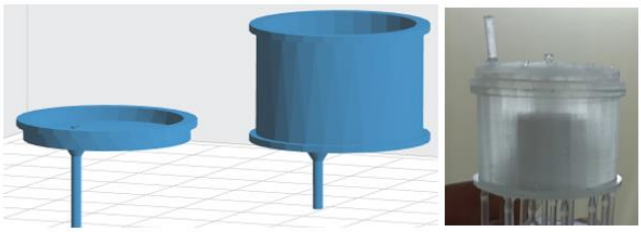

Figure 5.7: Outer-jacket

5.2.7 Part 6: Cap for outer jacket

Outer jacket has to be closed on top with a lid like structure for the bioreactor to maintain its sterility. This is the part which closes the outer jacket from top. Its diameter is same as the outer jacket diameter. A conduit is connected to the top of this part to allow media to flow in. Cad model is shown above (Figure 5.7).

5.3 Bronchi development strategy

The prepared dSMM gel in which cells are embedded is introduced into the space between temporary part and the outermost cylinder In between along with gel, capillaries part is also inserted to provide media in all directions. Once the MSCs are differentiated into myoblasts, a layer of epithelial cells will be coated towards the inner side i.e. lumen side. Then innermost cylinder is inserted into the bioreactor to provide epithelial media to epithelial cells. Once both the cell types are fully matured we planned to pass air through innermost cylinder and media outside the outermost cylinder. This concept of passing air and media at a time in the construct provides air liquid interface. Epithelial cells are exposed to air while the smooth muscle cells to media and nutrients. We hypothesize that this dynamic ALI culture plays a major role to maintain proper functionality of the developed bronchi.

5.4 Printing of the bioreactor parts

5.4.1 Modelling Software

Modelling software, a computer program that is used for developing any three-dimensional objects, also called 3D modelling. There are many commercial modelling softwares available, one of which is AutoCAD by Autodesk. The software that we used to develop the bioreactor in our project is AutoCAD. It consists of sketch-based features like geometric shapes and operation-based features

like extrude, fillets etc. There are few mathematical operations like subtract, union, intersection etc. The software has drag and drop option which facilitates easy modelling.

3D parts of the bioreactor developed in this project are modelled in this software. All parts are individually designed and modelled. Later after printing all the parts were assembled

5.4.2 Printing technology

Stereolithography is the 3D printing technology that is used to print the parts of the bioreactor. Stereolithography, a layer-by-layer printing technology that uses stereolithography apparatus (SLA) (Figure 5.9) to convert liquid parts to solid objects. The main components in the machine includes LASER source, photosensitive resin, XY scanning mirror, lenses, elevator, vat to hold the resin, wiper to maintain uniform surface of resin while printing (Figure 5.8).

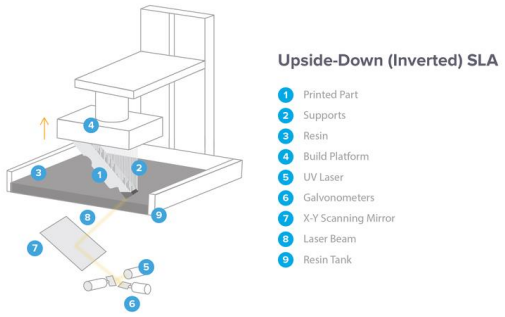

Figure 5.8: Parts of SLA (Source: Formlabs webpage)

Figure 5.9: SLA desktop 3D printer by Formlabs

5.4.2.1. Principle of Operation

The model that has to be printed is sent to the printer. A vat holds the photo-sensitive resin; a thin layer of photopolymer is exposed to the light source. As per the model, the LASER light hits the polymer. The photo-sensitive liquid hardens when the LASER light touches it, forming the first layer of the 3D object. Once this first layer is printed, the elevator rises in Z-direction exposing a new layer of uncured resin to print the next layer. This process is repeated until the printing of entire object is done. Once the print is done, post processing of the object removes any uncured resin from the print (Figure 5.10).

Figure 5.10: SLA operation principle (Source: gcreate- gmax 3D printer forum)

5.4.3 Printing software

Printing software is necessary to convert the files from one format to the format which a machine understands. The output format from the modelling software is quite different from the machine language. In our project, parts that are modelled in AutoCAD has an extension ".dwg". These CAD files has to be converted into printing software understandable language. Standard Tessellation Language (.stl) is one such type of language that is commonly used for stereolithography and other additive manufacturing processes. ".stl" is the format that is understood by the printing software Preform. The software preform is provided by Formlabs which takes .stl as input and gives output as .preform job file which is proprietary format of Formlabs. From the software itself when we give the print command, the file is exported to the printer for printing.

5.5 Decellularization of smooth muscle

Caprine smooth muscle was collected from a nearby slaughter house and was used for the research with the approval from the trader. The decellularization of smooth muscle was performed following the protocol that was published for decellularizing

skeletal muscle with modifications[22]. Briefly, the tissue was washed in distilled water to remove any biological wastes and chopped into small pieces of around 2-3 mm. These chopped pieces were stirred in 1 % SDS in distilled water for 24 h. This is followed by treating the chopped pieces with 1 % Triton X-100 for 24 h. Then they are thoroughly washed in distilled water to remove any left-over detergents. After washing the decellularized tissue is lyophilized and stored at -20 ^{0}C for further use (Figure 5.11).

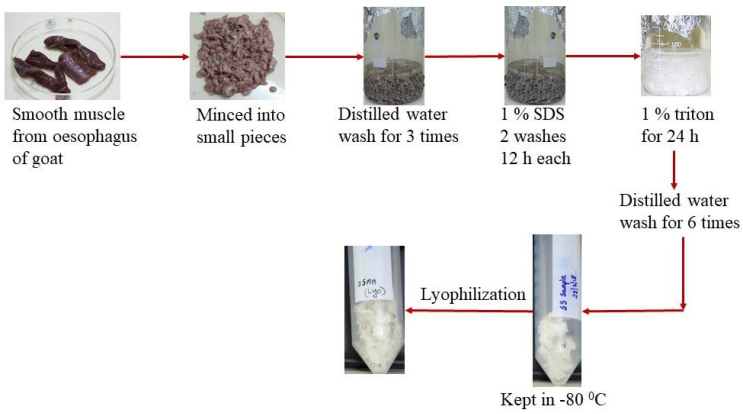

Figure 5.11: Decellularization process

5.6 Preparation of decellularized smooth muscle matrix hydrogel

Lyophilized decellularized Smooth Muscle Matrix (dSMM) is crushed in the presence of liquid nitrogen. Required amount of crushed tissue is weighed and digested in 0.01 M HCl in the presence of pepsin (10% of the total weight of tissue) for 24 h.[22] After complete solubilization, pH was checked and was in the range of 2-2.5. pH was then adjusted to nearly 7.4 by adding 10 M NaOH solution in cold condition preferably less than 4 ^{0}C. After adjusting pH the pre-gel was stored at 4 ^{0}C until further use.

5.7 Characterization of prepared dSMM gel

5.7.1 GAG estimation

To analyze the amount of GAG present in the native tissue and decellularized tissue GAG estimation was done. This analysis will help us understand the extent of ECM components that are being preserved during decellularization. The native and decellularized tissue were digested in papain solution for 16 h at 60 ^{0}C. the GAG

content was estimated using 1,9-dimethylmethylene blue solution. The absorbance was measured at a wavelength of 492 nm. For comparison, a standard curve was generated in advance using chondroitin sulphate solution

5.7.2 DNA estimation

To quantify the amount of DNA present in the native tissue and decellularized tissue, we performed DNA estimation using Biodrop instrument. For this estimation, the native muscle and decellularized muscle were digested in papain solution at 60 ^{0}C for 16 h. after initial calibration of the instrument, 1 µl of digested native muscle solution was loaded to the orifice and the DNA content was noted. Then, 1 µl of water was added to clean up the orifice, so that there is are no previous sample particles. Then 1 µl of digested decellularized muscle solution was added to the orifice and the DNA content was noted.

5.7.3 Collagen estimation

To quantitatively measure that collagen content present in the decellularized tissue and the native tissue, hydroxyproline assay is performed. In this assay, both native and decellularized tissue samples are digested in 0.5 % collagenase A solution and were incubated at 37 ^{0}C overnight. Then freshly prepared assay buffer and chloramine-T is added to the sample. After 15 min at RT, DMBA solution is added and kept at 60 ^{0}C for 20 min. Later absorbance reading was taken at 570 nm. The standard that was used for comparison is hydroxyproline.

5.7.4 Rheology

The mechanical properties like viscosity and modulus of the pre-gel that was prepared was measured using rheometer. The decellularized Smooth muscle matrix was digested in pepsin and the pre-gel was used for measurements. Sample was run on a parallel plate rheometer (Anton Par MCR301, Software: Rheoplus V3). The three measurements that were done on the sample are viscosity, stress and storage-loss modulus. For viscosity measurement, sample was loaded onto the fixed parallel plate and the strain rate was varied from 10 s^{-1} to 1000 s^{-1} and the graph viscosity vs. strain rate was plotted. In the same measurement we could also plot a graph between Shear stress and strain rate. Amplitude sweep is another graph that was plotted. In the measurement, angular frequency is kept constant at 10 rad/s and strain is

increased from 1 % to 100 % with a slope of 5 pt./Dec. In the amplitude sweep test, we get the values of storage and loss modulus with respect to change in strain percentage. The temperature at which the tests were performed was 25 ^{0}C.

5.8 Cells viability and gene expression

5.8.1 Live dead assay

Live dead assay was performed to check the viability of cells when embedded in the dSMM gel. UCMSC cells were embedded into the gel and was kept in the incubator for 45 min. After gelation media was added and was kept in incubator at 37 ^{0}C for 48 h. Post 48 h, media was removed and gels were washed with PBS to completely remove the media. Calcein, ethidium homodimer was mixed in the ratio of 1:4 in 1 ml of PBS. The washed samples were then immersed in the assay solution prepared and was incubated for 45 min. After 45 min, the samples were taken on a glass slide and was observed under fluorescence microscope at the excitation/emission wavelength of 494/517 nm for calcein and 528/614 nm for ethidium homodimer respectively.

5.8.2 Immunofluorescence

Immunofluorescence is a technique that is used to locate the distribution of biomolecules in the sample primarily making use of fluorophores to visualize the location of antibodies. In this project, Umbilical cord Mesenchymal stem cells are embedded in the decellularized smooth muscle gel. We hypothesize that these UCMSC will differentiate into Smooth muscle cell due to the presence of similar cues in their surroundings. In order to check the differentiation of MSC to SMC we used alpha smooth muscle actin (an early stage marker for SMC differentiation) primary antibody and Alexa fluor 647 anti-mouse as secondary antibody. We used Hoechst stain for staining the nucleus. The samples under study were initially fixed in 10 % formalin and are washed multiple times with PBS. Then, they are washed with 0.1 % triton which breaks the cell membrane. Samples were then added to 1 % BSA solution which acts as blocking buffer that blocks the unwanted sites in the sample for 30 min at 37 ^{0}C. After BSA, primary antibody is added and is kept in 4 ^{0}C for overnight. Next day, after thorough washing with PBS, secondary antibody i.e. Alexa fluor 647 (Bright red fluorescence dye) that binds to primary antibody is

added and was incubated at 37 ^{0}C for 2 h. Later Hoechst stain was added and was taken for confocal imaging. The excitation/ emission wavelengths that are used for secondary antibody and Hoechst stain are in the range of 650/665 nm and 355/465 nm respectively.

Chapter 6

Results and Discussion

6.1 Development of In-house bioreactor

In order to develop a three-dimensional lab engineered bronchi that mimics the structural and functional aspects as the native bronchi, the main part was development of the bioreactor which resembles the native tissue. So, to mimic this the first thing was to create a cylindrical structure, then to support the main structure and to make sure that media perfuses into all the directions and into all the layers we designed all the above-mentioned parts. Holes were also created on the cylindrical surface of the cylinder so that media enters into the construct with ease. After the shape, the next thing was the size of the construct. From literature, the thickness to diameter ratio should be in between 0.2 to 0.3. So, as per this we decided upon our diameter and thickness and the T/D ratio of our construct is 0.23 which is well within the range. Once all the parameters are decided, we started modelling in AutoCAD. The modelled constructs were exported to preform printing software and was printed using Formlabs 3D SLA printer. After printing, the printing accuracy i.e. measurement of the holes was measured using Zeta 3D optical Profiler. The average size of holes that were measured was found out to be 510 µm while the designed diameter was 600 µm (Figure 6.1). There is a decrease of almost 15 % in diameter after printing. As the size decreases the accuracy also goes down because as the area to solidify is less nearby layers are also exposed to light and might get polymerize to some extent. This could be the reason for decrease in print dimensions when compared to design.

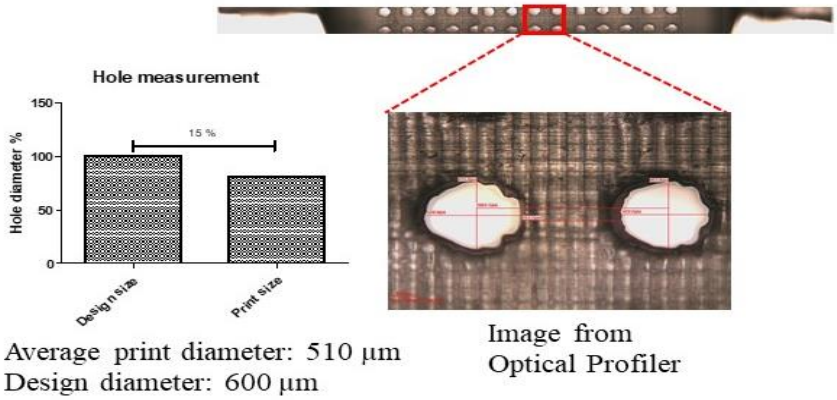

Figure 6.1: Hole measurement using 3D optical profiler

6.2 Preparation and Characterization of decellularized smooth muscle matrix (dSMM)

The main aim of decellularization is to remove the maximum cellular content while retaining the majority of ECM components. We had made an attempt to decellularize the native smooth muscle tissue using chemical detergents like SDS and Triton X-100. SDS is a detergent that disrupts the cell membrane. Triton X-100 disrupts the hydrogen bonding in the lipid bilayer and enters the interiors of the cell releasing the cellular contents into the matrix. To analyze the extent of protein retention in the decellularized matrix we performed GAG and Collagen estimation with DMMB assay and Hydroxyproline assay respectively. The GAG content in the decellularized matrix was found out to be 9 % more than the native tissue (Figure 6.2). Also, the collagen content in the decellularized smooth muscle matrix was almost 200 % more when compared to the native smooth muscle tissue (Figure 6.2). When we decellularize a tissue, the cellular contents get washed off compressing the matrix. So, if we take same amount of native and decellularized tissue it is hypothesized that the protein content in decellularized is more as there are no cells and protein content is less in native tissue as there are cells along with the matrix. This probably could be the reason for increase in GAG and collagen content in decellularized ECM. We could observe only slight decrease of about 13 % in the DNA content in decellularized tissue when compared to native tissue (Figure 6.3). The probable reason could be due to insufficient decellularization. When we

compare DNA content with ECM components retention in the decellularized tissue, it can be also concluded that due to inadequate decellularization we could observe more ECM retention.

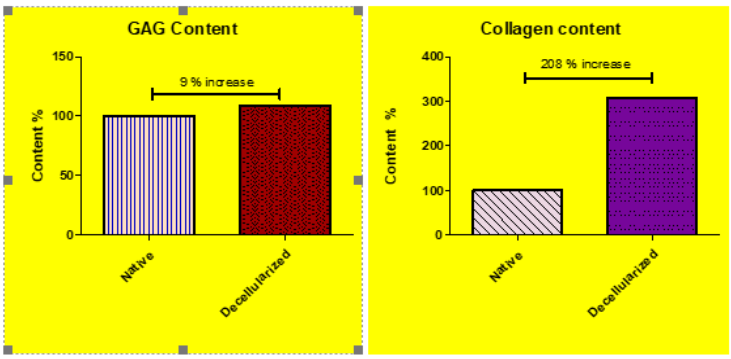

Figure 6.2: GAG, Collagen Content in %

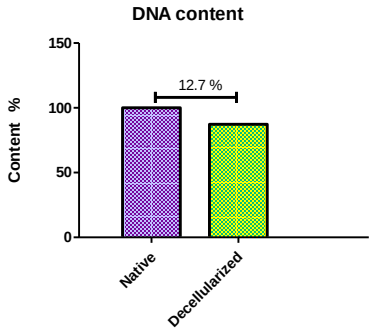

Figure 6.3: DNA Content expressed as percentage

6.3 Rheological Characterization of dSMM hydrogel

The percentage of gel to be prepared was decided based on viscosity. We want our gel to provide support for the cells we embed in the gel and hence 5 % gel was prepared which had high viscosity when compared to 1 % and 3 % gels. The digested dSMM was acidic in nature and was adjusted to neutral pH by adding 10 M NaOH. After adjusting pH, when allowed to incubate at a temperature 37 ^{0}C it formed a stable gel. The 5 % gel (pH ~1) was subjected to rheology tests to evaluate its behavior when it is subjected to a shear rate in the range if 10 – 1000 s^{-1} at 25 ^{0}C. The gel showed a shear thinning behavior which is the behavior of gels that is required for printing (Figure 6.4). Amplitude sweep test was performed to measure the dependence of loss and storage moduli with change in strain %, in which

frequency was constant at 10 rad/s, while strain % increased from 1 % - 100 %. At strain % between 10 % - 100 % the elastic property was more when compared to viscous property indicating the hydrogel's ability to store deformation energy in elastic manner (Figure 6.4). So higher the storage modulus the hydrogel can retain its shape in this strain conditions. Higher G' implies higher strength and mechanical rigidity.

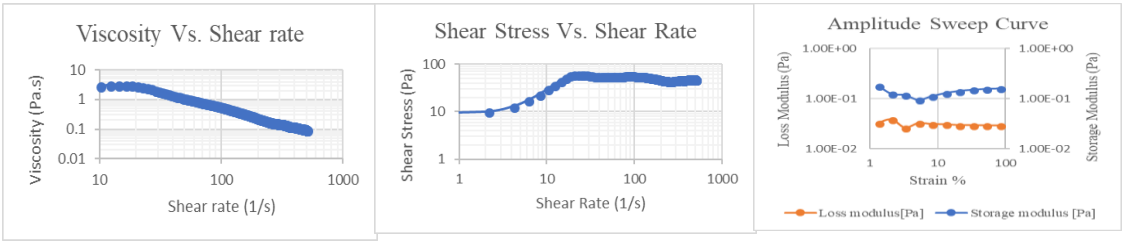

Figure 6.4: Graphs indicating shear thinning property and higher G' implying high rigidity and mechanical strength

6.4 Cell Viability

Cells were embedded in two different percentages of gels, 3 % and 5 %. Both these gels were considered for checking the viability of cells. It was observed that the cells that are embedded in 3 % gel were almost dead, no viable cells observed (Figure 6.5). While 80 % viability could be observed in 5 % gel on Day 3 of culture (Figure 6.6). Stiffness of the gel could be factor that is affecting the viability of cells in the two different percentage gels.

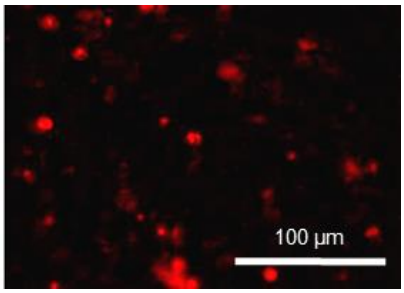

Figure 6.5: Ethidium homodimer staining dead cells in 3 % gel

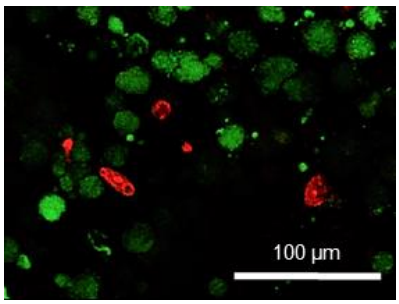

Figure 6.6: Green Fluorescence by Calcein Stain indicating live cells in 5 % gel

6.5 Immunofluorescence

After culturing UCMSC in the gel for about 7 days, we fixed the sample and stained it with alpha SMA, an early marker for SMC differentiation, Hoechst stain to stain nucleus and observed under confocal microscope. We could observe nucleus stained blue in colour and the secondary antibody showed florescence surrounding the nucleus (Figure 6.7). This implies the potential of decellularized smooth muscıe matrix to support UCMSC to differentiate into SMC in the dSMM gel. This indicates the importance of ECM microenvironment for directing the cells to specific lineage.

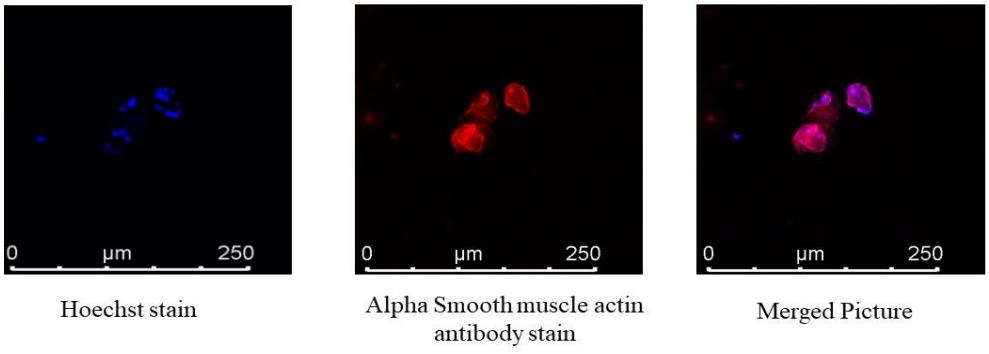

Figure 6.7: Nucleus stained with Hoechst blue fluorescent dye; alpha SMA stained red; merged picture indicating actin filaments surrounding nucleus

Chapter 7

Conclusion and Future Scope

In conclusion this study shows the feasibility of using the fabricated bioreactor for bronchi tissue engineering. This study revealed that decellularized smooth muscle hydrogel can be a potential candidate for the reconstruction of bronchial muscle as evidenced from the differentiation of UCMSC to SMA expressing cells, though it is a preliminary data. We also supported by the practicality of preparing decellularized smooth muscle hydrogel successfully as evidenced from the high GAG and Collagen content, though the DNA content demands modification in the decellularization protocol. Rheological behavior of the gel revealed the suitability of the prepared gel for 3D bioprinting applications.

As part of future scope, we would like to include epithelial cells layer once the smooth muscle layer is developed. This will be followed by ALI culture in dynamic conditions and further validating the construct. These outcomes in this preliminary study encourages us to take the project forward in development of complete 3D in vitro bronchi, which has a potential to overcome the complications that are encountered during standard treatment procedures.

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