SUPPORT OF PNEUMOCYTE SPECIFIC PROPERTIES BY DIFFERENT SCAFFOLDS UNDER DYNAMIC CONDITION

A DISSERTATION SUBMITTED

BY

ANISH MOHAN

IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

MASTER OF PHILOSOPHY

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY

TRIVANDRUM – 695 011

DECLARATION

I, **Anish Mohan**, hereby declare that I had personally carried out the work depicted in the dissertation entitled "**SUPPORT OF PNEUMOCYTE SPECIFIC PROPERTIES BY DIFFERENT SCAFFOLDS UNDER DYNAMIC CONDITION** " under the direct supervision of **Dr. A Maya Nandkumar**, Scientist in-charge, Division of Microbiology, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India. External help sought are acknowledged.

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CERTIFICATE

This is to certify that the dissertation entitled "**SUPPORT OF PNEUMOCYTE SPECIFIC PROPERTIES BY DIFFERENT SCAFFOLDS UNDER DYNAMIC CONDITION**" submitted by **Anish Mohan** in partial fulfilment for the Degree of Master of Philosophy in Biomedical Technology to be awarded by this Institute. The entire work was done by **him** under my supervision and guidance at Division of Microbiology, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Thiruvananthapuram-695012.

Thiruvananthapuram

Date 3/9/2011

Signature

Dr. A. Maya Nandkumar

The Dissertation

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Submitted

By

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For

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of

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Evaluated and approved

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Synopsis

Tissue engineering is a scientific field involving cells, engineered supports and their interaction in microenvironment. Lung even though an internal organ is at highest stake of injury due to constant exposure to environmental challenges forced upon it. Lung diseases are the fourth leading cause of death worldwide and yet lung tissue engineering lags behind organs like skin, cornea, and kidney. The lag in lung tissue engineering is due to the lack of understanding of unique engineering problems involving scaffold materials required, as lung is an organ which constantly contracts and expands in each respiratory cycle *in vivo*. Interactions between lung cell populations are entirely responsible for the lung specific properties like surfactant secretion, gaseous exchange etc. Among them Type1 pneumocytes help in gaseous exchange while Type II cells act as a reserve for Type1 pneumocytes and secrete surfactant proteins. Fibroblasts are the other major groups which are involved in overall maintenance of pneumocyte properties through inter cellular signalling. Maintaining these specific properties of the pneumocytes in culture, which include phenotype maintenance, is a daunting task.

Bioreactors are a befitting answer for the problem caused by the routine cell culture methods; they stimulate a microenvironment for proper cell growth *in vitro* by a combination of chemical and (or) mechanical stimuli.

Here our aim is to identify a suitable scaffold which supports pneumocyte attachment and proliferation in a bioreactor, to study their properties, specifically, surfactant protein secretion.

Surface characteristics of scaffolds will define cell-material interactions on them. AFM was use full in determining the distribution of cells on scaffolds. Lung epithelial cell-line, A549, which is an adenocarcinoma with characteristics of type II pneumocytes and Wistar rat primary lung epithelial cells were used for the study. The surfactant secretion properties of these cells were studied. The cells were seeded on to Polyvinylidene fluoride (PVDF), cellulose acetate (CA), mixed cellulose ester (MCE) membranes and static and dynamic culture conditions were adopted for the study. For dynamic system perfusion culture system was set up using the Minucell system.

The surfactant protein expression was used as functional marker; qRT-PCR was used to study surfactant protein mRNA modulation over the period of culture. Their expressions were normalized to GADPH, the house keeping gene.

m-RNA expression was high in dynamic system compared to static one, and expression of surfactant protein B (SP-B) m-RNA was maximal in dynamic followed by SPC and SPD. AFM microscopic analysis revealed that PVDF membrane promoted maximal cell adhesion and spreading. Surfactant protein expression was high in cells under dynamic system when compared to static, both cell types were positive for the above observation.

The study establishes the fact that the dynamic system is very important in maintenance of alveolar specific properties over the period of culture, while under static conditions these properties are lost rapidly. This may be due to the fact that in dynamic system metabolic wastes are continually removed and fresh nutrients supplied. This results in addressing removal of mass transport limitation in tissue engineered constructs.

INTRODUCTION

CHAPTER- 1

INTRODUCTION

1.1Background

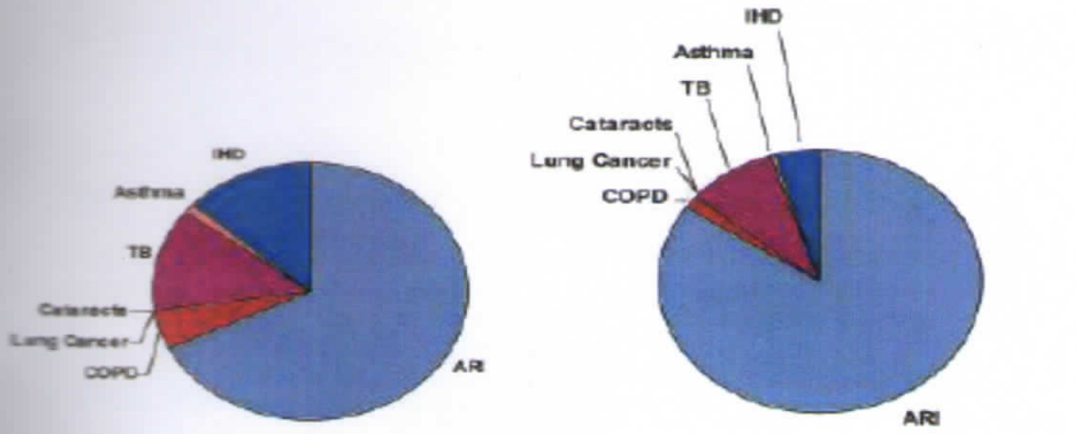
The great advancement in technology has found its expression in modern medicine leading to improved and complicated medical devices and equipments thereby has resulted in an increased life span for the general population. Organ assistance and substitution devices will play a larger role in future patient care. A great deal of research is ongoing on intravascular, arteriovenous, and thoracic gas exchangers for respiratory support, and several of these devices are entering their preclinical testing phase.

The lungs are internal organs; yet they are uniquely and constantly exposed to our external environment, a direct interface with the world outside. With each breath, a host of alien substances enters our bodies, leaving the lungs a ravaged battlefield. Lung disease is any disease or disorder where lung function is impaired. Lung diseases can be caused by long-term and immediate exposure to smoking (active and passive), air pollution (indoor and outdoor), carcinogens that trigger tumor growth, infectious agents, and over reactive immune system defences. According to WHO statistics estimated 210 million people have pulmonary disease worldwide. Lung Failure is the fourth leading cause of death worldwide. Almost 90% of deaths occur in low- and middle-income countries. Globalization in India has lead to emergence of occupational respiratory diseases. Latest WHO statistics show that India has an estimated 15-20 million asthmatics. After cardio-vascular diseases and cancers, chronic respiratory disease is the major cause for mortality in both the sexes in India (Smith KR 2000).

Lung has multiple roles in our body which includes: supplier of oxygen, remover of wastes and toxins, and defender against hostile intruders. They are actually far more complex than many other organs.

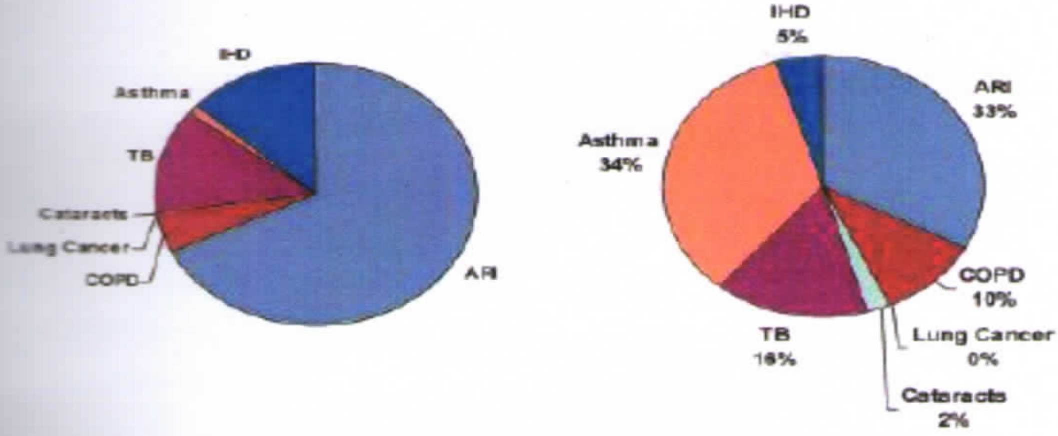
A single day, an astonishing 8,000 to 9,000 litres of breathed-in air meet 8,000 to 10,000 litres of blood pumped in by the heart through the pulmonary artery and its ability to retain its structure makes it unique. The pulmonary epithelium is uniquely situated so that it has a large surface area exposed to air facilitating efficient diffusion directly from air to blood. This site hence provides immense possibilities for understanding molecular pathology of alveolar diseases, development of novel treatment methodologies and as testing system for drugs, chemicals and pollutants.

Tissue engineering of the lung offers a way to develop an *in vitro* lung model where the *in vivo* situation can be duplicated *in vitro* for use as a testing system.



A. Deaths
400-550 thousand

B. Lost life years (YLLs)
11-15 million



C. DALYs
12-17 million

D. Sickdays
1.6 -2.0 billion

Fig 1.1: Annual health burden from indoor air pollution in India. (Smith K.R, 2000).

1.1.1 Lung Physiology and Development

Fig 1.2 illustrates the pulmonary system in an adult. Air is inhaled through the nose and mouth through the "airways," passing from the larynx and trachea and then into a rapidly dividing series of about 16 generations of conductive bronchi and bronchioles. Alveoli begin to appear in the walls of the 17th generation of bronchioles.

During early embryogenesis development of human lung is initiated at about fifth week of gestation and passes through four different phases.

1) The pseudo glandular stage (development of bronchial tree); 2) the canalicular stage (development of acini and vascularisation) 3) the saccular stage consisting of further differentiation of the acini into saccules, increase in saccules, and vascularisation, as well as differentiation of the epithelial cells into type 1 and 2 pneumocytes and 4) The alveolar stage (increase in number of alveoli and extensive increase in the surface area). Alveoli develop at the 28th week of gestation.

About 300 million alveoli are formed when a human attains 2-4 years. Type 1 and Type 2 alveolar cells constitute the epithelial component of the alveoli. Type 1 cells cover more than 90% of the alveolar surface area of peripheral lung and are highly attenuated, diameter of these cells can reach 100 μm.Type 2 cells are larger and round. They are found at the corner of the alveoli. They lay in close proximity to the underlying mesenchymal cells/Fibroblasts (Shannon MJ etal 1992).

Lungs are fundamentally invaginations therefore ventilation is "tidal" in mammals, and a certain proportion of the inhaled air never actually reaches the gas exchange regions of the lungs, making the process less efficient. Presumably because of the demand for high rates of metabolism during flying and reduced oxygen tensions at altitude, avian lungs are fundamentally more efficient than those of mammals: inhaled air passes through avian lungs, which are not inflated but conduct air to large sacs in the abdomen and bones. The inhaled air is subsequently routed from the body cavities by different portions of the airways, thereby minimizing rebreathing of exhaled air.

Oxygen needs in "endotherms" are much higher than those in their cold-blooded "exothermic" ancestors.

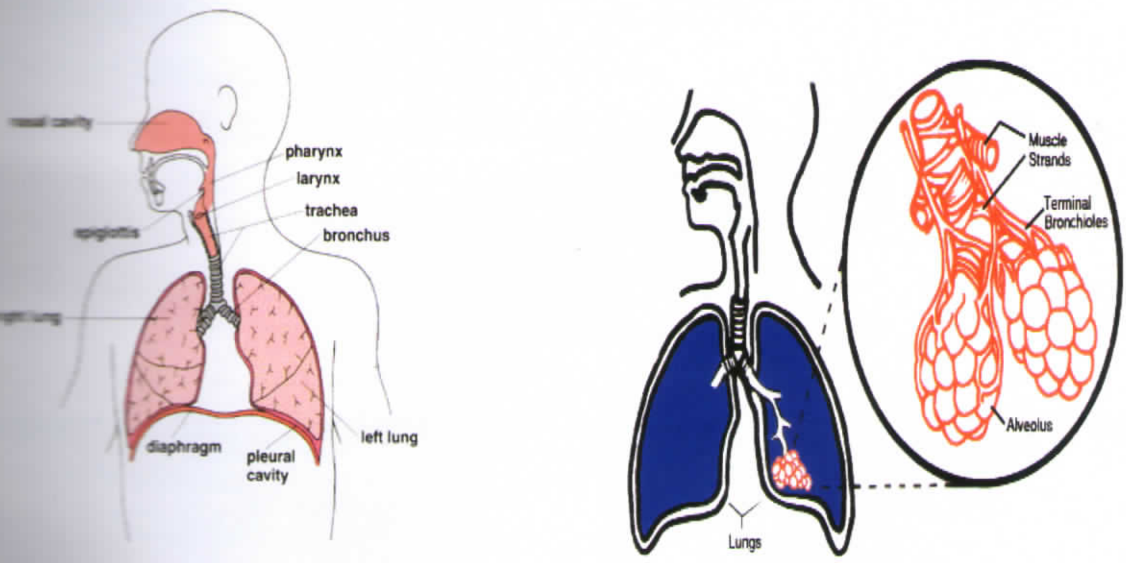


Fig1.2: The Pulmonary System.

1.2 REVIEW OF LITREATURE

Coupled with the elastic nature of the lung and Brown's discovery of dipalmitoylphosphatidylcholine (DPPC) was a major component of lung surfactant (Brown etal 1964) gave rise to the perplexing question of the functioning of lung, which has yet to be answered and is still lingering in its nascent stage.

1.2.1 Developmental aspects

The respiratory system arises from the ventral foregut endoderm. The process initiates with the establishment of respiratory cell fate in primitive foregut. This is followed by the development of a tree like system of epithelial tubules and vascular structures, which ultimately give rise to the mature airway and alveoli. The foregut endoderm differentiates into various epithelial cell types (Fig1.3), which line the inner surface of the developing lung and trachea. The lung mesenchyme originates from the lateral plate mesoderm. The lung vasculature forms, in part, by migration of blood vessels from the aortic arches and from the left atrium to the lung (angiogenesis). Blood vessels also develop by vasculogenesis in the lung mesenchyme near developing epithelial buds; a rudimentary capillary network initially forms and expands, and later connects to the larger vessels to give rise to the lung vasculature (Wood *et al.,* 1997; Demello *et al.*, 1997; Gebb and Shannon, 2000). There is evidence that, during organogenesis, blood vessels serve as a source of inductive signals to the epithelium (Lammert *et al.*, 2001; Matsumoto *et al.*, 2001). Recent studies have implicated fibroblast growth factor, sonic hedgehog bone morphogenetic protein, retinoic acid and Wnt signalling pathways, and various transcription factors in regulating the initial stages of lung development (Wellington V. Cardoso, 2006).

1.2.2 Alveoli

Lung is mostly blood (50%-60%) and wet surfaces tend to attach together due to surface tension. The walls of alveoli are coated with a thin film of water & this creates a potential problem. Water molecules, including those on the alveolar walls, are more attracted to each other than to air, and this attraction creates a force called surface tension. This surface tension increases as water molecules come closer together, which is what happens when we exhale & our alveoli become smaller (like air leaving a balloon). Potentially, surface tension could cause alveoli to collapse and, in addition, would make it more difficult to 're-expand' the alveoli during the next cycle of inhalation. Our alveoli do not collapse & inhalation is relatively easy because the lungs produce a substance called surfactant that reduces surface tension. Alveoli also have fibroblast cells in close proximity, which have functions which includes epithelial cell regulation, maintaining branching morphogenesis and cytodifferentiation in lung (Adamson *et al* 1988). Lung epithelial cells consist of both type I and type II cells.

Type 1 cells line the alveoli thereby providing a thin membrane for the gaseous exchange.

Type 2 cells are multifunctional, (Adamson et al 1988).

Their functions include:-

1) Surfactant secretion-to reduce surface tension in the alveoli.

2) Immune Response.

3) progenitor for Type 1 alveolar epithelial cells (Ian Y.R *et al*, 1987).

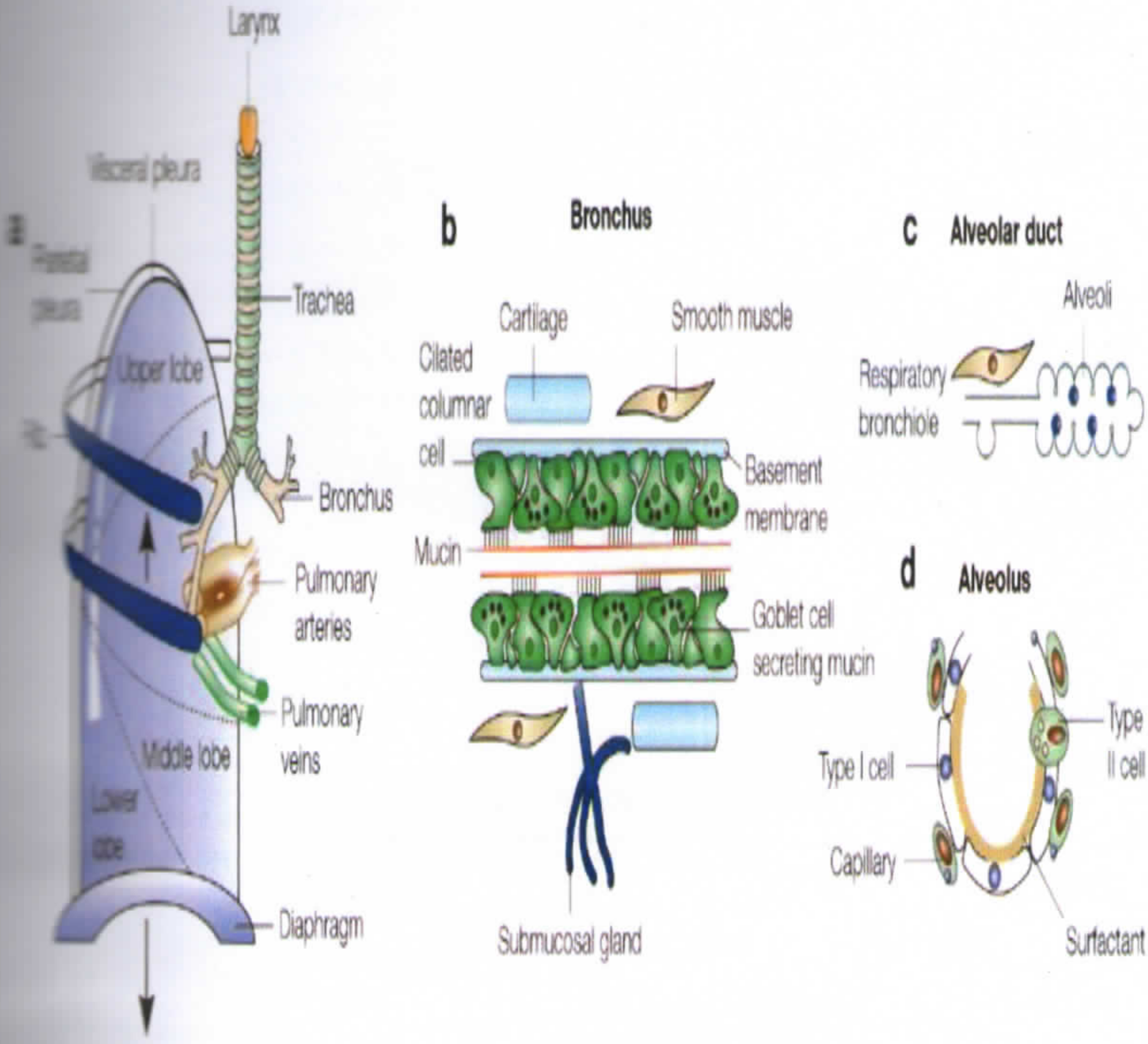


Fig (1:3): Cellular arrangement in lung (*Rooney SA et al*, 2001).

1.2.3 Surface tension

Surface tension is the property of liquids arising from unbalanced molecular cohesive forces at or near the surface, as a result of which the surface tends to contract and has properties resembling those of a stretched elastic membrane. In our lungs, despite the same pressure gradient at the alveolus, all of them will inflate with full inspiration. The variation in alveolar ventilation and alveolar size with respiration imply that the property of surface film do not remain constant.

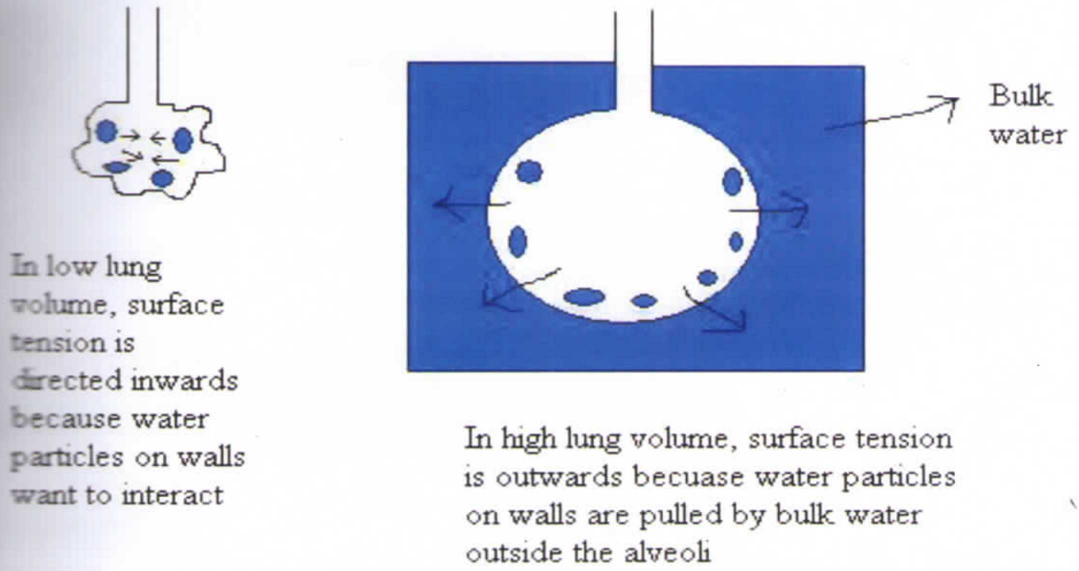


Fig (1:4): Effect of surface tension on alveoli (Richard M. Effros, Goyal and Shekhar)

Fig 1.4 shows the role of surface tension on the alveoli. Pulmonary Surfactants is a surface-active lipoprotein complex. They reduce the surface tension by adsorbing to the air-water interphase of the alveoli. This increases pulmonary compliance and prevents alveoli from collapsing. Dipalmitoylphosphatidylcholine (DPPC) is the main lipid component (Hildebran, 1979).

Lung surfactants are divided into two pools:

1) External pool-lines the alveoli.

2) Internal pool-stored in the type 2 alveolar cells.

Surfactants do contain 5-10% proteins which can be classified as SP-A, SP-B, SP-C, and SP-D. SP-B and SP-C speeds up the formation of surface film but during injury, when secretions of these proteins are impaired, SP-A takes on the function ,SP-A synthesis is carried out by type 2 cells (Cockshutt *et al*,1990). SP-B and SP-C are particularly hydrophobic, indicating their association with lipid layer. Their function is to improve flexibility of the layer and they contribute to lowering its surface energy on compression.

The heavier molecules, SP-A and SP-D are glycoproteins. SP-A binds phospholipids and to receptors on the type 2 cells. SP-D enhances phagocytosis of bacteria.

Surfactants have a high rate of turnover and are replaced with a half life of about 10 hours. Presence of SP-A reduces the secretion of new surfactant. This together with alveolar folding consumes functional surfactants. An increase in surfactant protein production is seen following hyperventilation.

Respiratory distress in premature infants of 28-32 week gestation is not due to the lack of pulmonary surfactants but the replacement by immature type 2 alveolar cells due to reduced removal, adsorption and recycling of these surfactants (Possmayer etal 1982). The use of corticosteroids can counter these problems to some extent. In adults with respiratory distress syndrome, due to poor cardiac output, fibrosis, atelectasis etc, the lung may lose its elastic property and surfactant function will not be normal.

Protein	Molecular weight range	Structure	Molecular weight of pp chain(kDa)	Polarity
SP-A	High	Octadecamer	26.0	Hydrophilic
SP-B	Low	Dimer	8.7	Hydrophilic
SP-C	Low	Monomer	4.0	Hydrophilic
SP-D	High	Dodecamer	39.0	Hydrophilic

Table1.1: Properties of surfactant proteins (Yu and Possmayer, 1988).

1.2.4 Properties of surfactant proteins

Table 1.1 enumerates surfactant protein characteristics. Immunological functions of surfactants: SP-A and SP-D can be called collectins and they enhance. phagocytosis of bacteria. They clear all the pollutants without creating any visible signs of inflammation in the lung. Type 2 cells can make proteins that can cause neutrophil chemotaxis (fig-1:5). Pulmonary surfactants are absorbed by macrophages and to some extend type 2 cells. Each alveolus contain an infinitely variable combination of immunoglobulins, cytokines, macrophage migration inhibiting factor and surface protein that may all affect the surfactant function.

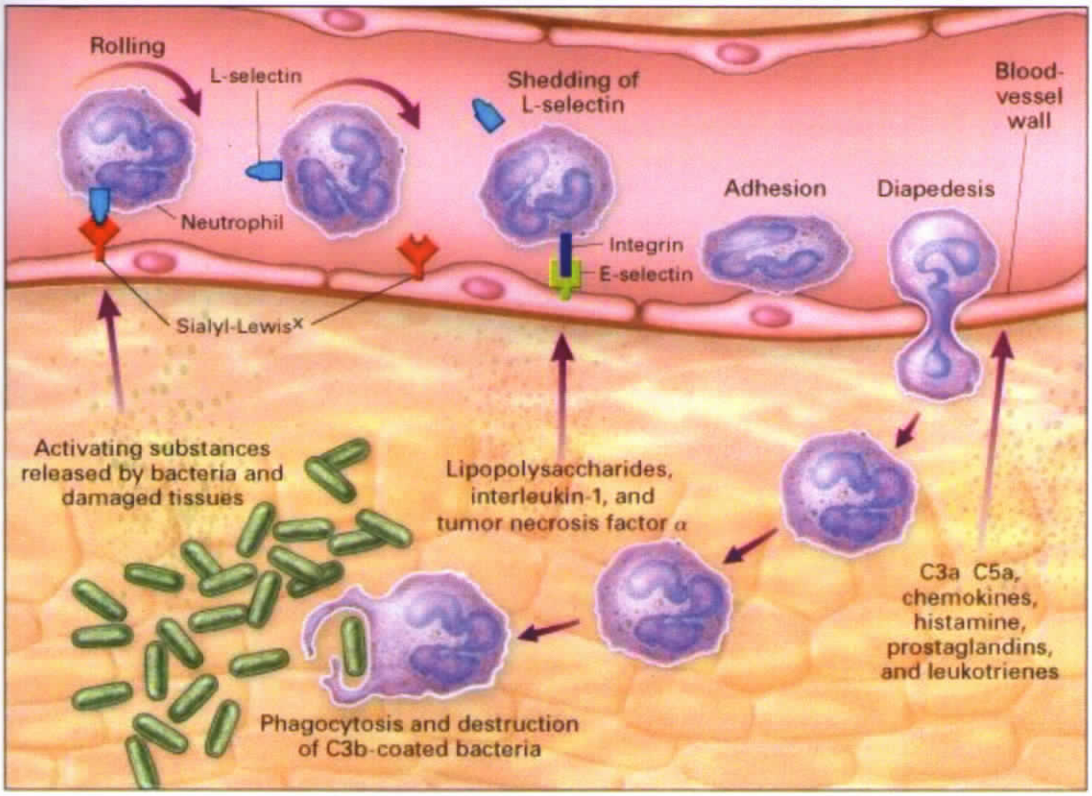


Fig (1:5): Chemotaxis mediated by collectins (Richerson et al, 1974).

1.2.5 Study models

In 1960's, physiological role of lung surfactants led to the recognition of the lung as an active metabolic organ. The advancement in pulmonary research has provided lot of information about lung function and about how the lung cells contribute towards the homeostasis. Over the years, a number of models have been used to study the pulmonary physiology and pathology of diseases. Both *in vivo* and *in vitro* models have been used to study pulmonary biology.

Initially before concept of tissue culture came up, animal models were used in respiratory physiology, pathology and toxicology studies. Major animal models included rodents like rats and rabbits along with canines like dogs. An inflammatory pneumonitis with mononuclear infiltrates histologically resembling those in delayed hypersensitivity cutaneous reaction has been produced experimentally in rabbit and guinea pig (Richerson, 1974). A number of animal models of aspergillosis have been developed for evaluation of antifungal agents and the analysis of fungal virulence factors (Tang et al, 1994). The major drawback in use of animal models is their maintenance. Moreover, in animal model many complex interactions come up as an outcome of their own immune response. Further variations also occur from animal to animal within an experimental batch. Thereby comes the need for *in vitro* models. Culture systems are the best *in vitro* models. They allow observations and manipulations of cells and tissues alone, where experimental variables can be controlled and minimized. With this advantage in mind, we set a goal to reproduce in culture, a system that mimics the *in vivo* physiological and pathological phenomenon of interest.

1.2.6 Culture systems

Recent advancements in tissue culture methodologies have enabled isolation of specific cells from tissues by mechanical or enzymatic dissociation. Tissue culture systems have helped in the propagation of cells as primary cultures or generation of cell lines. A primary culture is derived by enzymatic or mechanical dissociation of the tissue. Serial sub cultivation of a primary culture gives rise to a cell line. The cell line would be composed of the most rapidly growing cells of the original culture. A primary culture has its own advantage over cell lines, major advantage being the normal and original karyotype because cell lines tend to get karyotype modifications. Also conclusions draw from experiments using primary culture can be extrapolated to the *in vivo* situation with greater precision.

A cell culture system is greatly influenced by the cell culture medium used. For any cell type choice of medium is empirical. The growth rate, morphology and degree of differentiation of cells in culture depend on the chemical constituents and physical properties like pH, osmolarity, and temperature of the medium. Most cells grow in neutral pH and in media pH is maintained by a bicarbonate buffer, where CO_2 in the incubator maintains pH. The ideal osmolarity is the osmolarity of blood plasma and ideal temperature for cell culture is 37°C. Various growth factors and hormones form a part of the chemical constituents of the medium. A survey of literature available today gives a list of growth factors and hormones that modulate epithelial cell proliferation in general and airway epithelial cells in particular. These include Insulin, Cortisol, Cholera toxin (I,CT,F) etc. (Yang et al, 1986). Insulin stimulates cell growth and increases fatty acid and glycogen synthesis. It also stimulated amino acid transport by alveolar type II epithelial cells in primary culture isolated from rat lungs (Dobbs L.G *etal* 1997). Cholera toxin irreversibly increases adenylate cyclase activity and thereby favours epithelial cell proliferation *in vitro*. It also inhibits fibroblast cells (Yang et al, 1986). Cortisol elicits an array of physiological response in cultured cells via the modulation of gene expression and growth regulation. In our experiments, media used are Ham's F12K for A549, DMEM: F12 (1:1) I,CT,F as a growth media for lung primary cells.

Primary cultures of airway epithelium were initially used in several studies of ion transport. Data have been reported utilizing a variety of culture methods including rat and rabbit tracheal explants (Marchock et al, 1975). The first culture of dog tracheal epithelium was grown in a medium containing 5% FCS (Coleman et al, 1984). In our experiments Wistar rats (150g-200g) were used for establishing a primary culture and cell lines like A549 and WI 38 were also evaluated in a co-culture system.

Study done by Kimberly A. Foster et al, 1998, shows that A549 cell line exhibited metabolic and transport properties consistent with Type II pulmonary epithelial cells *in vivo*. Monolyers of Type II cells may not completely mimic the pulmonary epithelium where Type I and Type II cells coexist.

1.2.7 The Perfusion culture setup

Tissue engineering requires the interplay of scaffolds cells and the cellular niche. One of the limiting factors in such constructs when compared to ordinary static cell culture is access to nutrients and removal of metabolic waste. Perfusion system helps in this and helps in supplying constant nutrition to cells a condition as close to physiological *invivo* situation as possible. Stephen S *etal* in 2000, used PGA scaffolds to seed hepatocytes. He compared the seeded scaffolds in dynamic as well as in static state, from his study he concluded that the albumin synthesis by hepatocytes was higher in dynamic seeded cell compared to the static ones. The cell in dynamic state maintained a high metabolic activity and appeared healthy at both 2 and 7 days, the time frame used in the study. As perfusion culture resembles *in vivo* situation it can be used for drug metabolism studies, toxicant exposure studies like cigarette smoke, nano particles exposure etc. Paur HR (2008) used lung cells under dynamic condition to study the effect of varying concentrations of nanoparticles to validate the possibility of using such culture instead of animal studies. While Evangelia *etal* studied the effective biological dose of nano particle exposure for risk assessment in 2009, she came to a conclusion that a lower level of exposure throughout the day also leads to build up and majority accumulates at the pulmonary region. The clearance of particle takes place by macrophage activity with small amount of transport to the interstitium and less to the lymph nodes.

Petra Lynen Jansen *etal* 2008, used non-absorbable Polyvinylidene fluoride (PVDF) as scaffold to reconstitute the esophageal wall and to investigate the functional and histological consequences. PVDF even though nonbiodegradable supported the cell functions to a large extend. The PVDF stimulated complete mucosal regeneration and minimal inflammatory reaction with no anastomotic strictures even after 3 months of implantation. Rodrigues MT, 2008, used PVDF membrane to induce cell proliferation and differentiation in static and dynamic conditions which showed an increase in cell adherence and proliferation, it also showed to favor differentiation towards osteogenic phenotype. Muller FA etal 2006, used non-woven cellulose fabrics as scaffold for *in vitro* cartilage tissue engineering. He concluded that scaffolds treated with saturated calcium hydroxide solution were more favourable for chondrocyte proliferation and vitality.

The lag in lung tissue engineering is due to the lack of understanding of unique engineering problems involving scaffold materials required. In lung, Type1 pneumocytes help in gaseous exchange while TypeII cells act as a reserve for Type1 pneumocytes and secrete surfactant proteins. Maintaining these specific properties of the pneumocytes in culture, which include phenotype maintenance, is a daunting task. Bioreactors are a befitting answer for the above problem caused by the routine cell culture methods.

1.2.8 The Gap Junction

Gap junctions or connexons are hexameric transmembrane channels which play a part in cell to cell communication (Eugene Rannels, 2001). Alveolar epithelial cells express gap junction proteins connexins (Cx), which mediates the cellular communications. Data from Northern and Western blot analyses confirm that at least eight connexins are expressed in the gas-exchange region of the lung; these include connexin (Cx) 26, Cx30.3, Cx32, Cx37, Cx40, Cx43, Cx45, and Cx46. Type 2 cells express relatively high levels of Cx26 and Cx32 but little Cx43 and Cx46. Connexin 43 is ubiquitously expressed and found between Type 1 and Type 2. Sub culturing reduces connexins 32 but increases 43 and 46 (Valsamma Abraham *etal* 2001).

Connexin expression by type II cells *in vivo* may reflect the potential for assembly of gap junction channels between type I and type II alveolar epithelial cells and thus for physiologically relevant signalling networks within the alveolar microenvironment.

The interactions between alveolar epithelial cells and maintenance of morphological peculiarities of these cells in culture is a pre-requisite for maintaining the functional integrity of type II pneumocytes like surfactant synthesis for prolonged durations of culture. (Maya A. Nandkumar *et-al*, 2002).

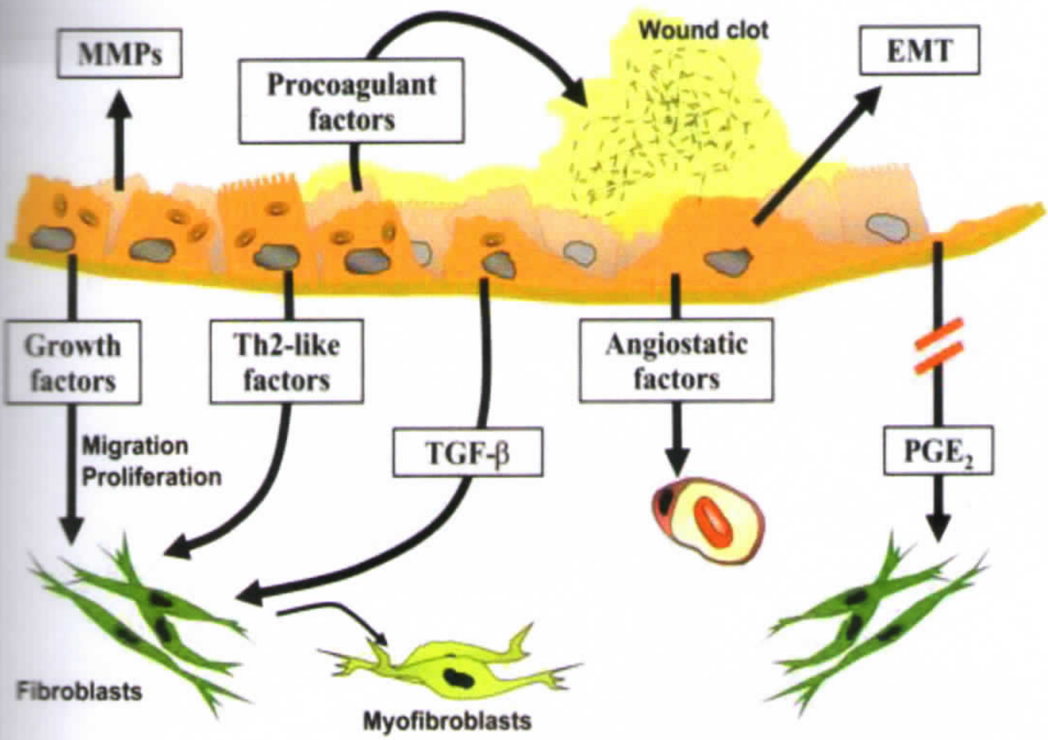


Fig (1:6): Interaction between fibroblast cells and lung epithelial cells. (Moise´s Selman and Annie Pardo, 2006).

Recent research in animal model suggests that when the communication between fibroblast and epithelial cells break, there develops a signalling cascade, which may lead to many abnormalities including idiopathic pulmonary fibrosis (IPF). Direct cell-cell interactions via gap junction (GJIC) channels may play a significant role in the regulation of alveolar epithelial cell phenotype and function. Several researches in pulmonary field suggest that cell-cell interactions via gap junctions are of both functional and physiological significance (Shannon J.M, 1987).

1.3 Hypo dissertation

The peculiar anatomical feature of the lung which is responsible for the respiratory function of the lung is also a hindrance in advancement of lung tissue engineering. For a successful tissue engineered construct a suitable scaffold is necessary. The scaffold provides a three dimensional architecture over which the concerned cell types can adhere and proliferate and assume structures suitable for maintenance of required functions. In standard cell culture of type II pneumocytes it is the property of surfactant synthesis that is lost first. This study hypothesizes that a suitable scaffold and dynamic culture conditions will help in the maintenance of alveolar epithelial cell specific properties *in vitro*.

1.4 Research objectives

The objectives of the study are:

- Determining which scaffold is suitable for setting up dynamic system among the chosen Polyvinylidene fluoride (PVDF), Mixed cellulose esters (MCE), and Cellulose acetate (CA).
- The functional evaluation of the scaffold and pneumocyte interactions by assaying pneumocyte specific property of surfactant synthesis.

MATERIALS AND METHODS

Chapter-2

MATERIALS AND METHODS

2.1 Materials

All media, EMEM medium, F12K medium, DMEM/F12 [1:1 v/v] medium. RPMI 1640 medium were purchased from Sigma. The powdered media is dissolved in deionised double distilled water and sodium bicarbonate is added. The pH is adjusted to 7.4 and is filter sterilized. Then media is supplemented with 10% FBS [sigma], Amphotericin B [sigma] [250 units/ml] and Gentamycin sulphate [Himedia] [50ug/ml].

2.1.1 Cell lines

A549 is a cell line established from a lung adenocarcinoma of a Caucasian male in 1972. It is a type II pulmonary alveolar epithelial cell line. A549 cells were obtained from ATCC [American Type Culture Collection] and maintained in Ham's F12-K medium (sigma), with 10% Fetal Bovine Serum and supplemented with gentamycin along with Amphotericin B. The cells were plated in cell culture flasks (T-25) and passaged on reaching 90% confluency using 0.25% trypsin-EDTA [Gibco].

Cell line was maintained in 5% CO_2, 37°C in a CO_2 incubator and 95% humidity.

2.1.2 Scaffolds used

Three scaffolds were used in the study, which were mixed cellulose esters (MCE), Cellulose acetate (CA), and Polyvinylidenedifluoride (PVDF). All the scaffolds were obtained from Genaxy.

2.2 METHODOLOGY

2.2.1 Contact Angle Measurement

The scaffolds, Polyvilydene fluoride (PVDF), Mixed Cellulose Esters (MCE) and Cellulose acetate (CA) were autoclaved at 121°C for 15 minutes. They were transferred onto the stage of the goniometer (Contact angle system OCA) using forceps and contact angle was measured.

2.2.2 Setting up Dynamic culture conditions using a perfusion system

Minucell system to maintain the perfusion culture was obtained from Minucells and Minutissue Vertriebs GmbH. The system was washed using mild detergent and rinsed thoroughly to remove all residual soap. The system was set up with all connecting tubules and 70% ethanol was pumped through it for two hours, followed by distilled water. The system was then autoclaved for 20 minutes at 105 degree centigrade and 5psi pressure. The sterile Minucell perfusion system was installed and readied for the perfusion culture. The various scaffolds chosen were seeded with A549 cells or primary alveolar cells isolated form Wistar rats. The cells were allowed to adhere to the scaffold under static condition overnight and then mounted in the perfusion system. Dynamic culture system was terminated at relevant time points for analysis. The flow rate for medium was maintained at 2ml/hour.

Fig 2.1: The perfusion system setup for dynamic culture.

2.2.3 Isolation of primary cells from lung tissue

A primary heterotypic culture of alveolar cell types was set up. The cell source was Wistar rats having body weight in the range of 150-200g. Institutional Animal ethics Comm. clearance was obtained for these studies and CPCASEA rules were followed in the care of experimental animals. Wistar rats used in the experiment were withdrawn from feed 16-18hrs prior the experiment, with water *ad libdum*. The rats were sacrificed by overdose of intra-peritoneal injection of thiopentone. The lungs were excised and cut free of bronchioles. It was finely minced and subjected to dissociation with 0.5% collagenase type I [Himedia] and Elastase pancreatic solution Type I [sigma] in RPMI medium at 37°C in an incubator with gentle shaking at a speed of 80 rpm. The cells were monitored under a phase contrast microscope for optimal stromal dissociation. A few drops of DNase [sigma] were added to prevent cell clumping. The cells were washed off collagenase and alveolar epithelial cells were enriched by density gradient separation on preformed percoll gradient. Gradient was prepared by adjusting percoll to physiological salt and pH condition by addition of RPMI medium and spinning at 12,000 rpm for 60 min in a fixed angle rotor. The epithelial band was collected, washed off percoll and plated on scaffolds. Cultures were maintained at 37°C and 5% CO_2 initially for adhesion. The cells were allowed to adhere overnight and then transferred to Dynamic culture in the Minucell system. Culture medium used was DMEM/F12 (1:1v/v) containing 10% FBS, Insulin [50 µg/10 µl], CT [10ng/ µl], F [5 µg/µl] along with antibiotics.

Controls cultures were maintained at 37 ^{0}C and 5% CO_2 in CO_2 incubator under static conditions for duration of culture.

2.2.4 Functional analysis

m-RNA expression of various surfactant proteins were looked at, to understand whether the dynamic culture system would support and maintain functionality of the cells in culture.

2.2.4.1 RNA extraction

Total RNA was extracted from the cells using the TRIzol® Reagent [Invitrogen]. Protocol of the kit was followed strictly. In brief, cells were collected from the dish using trizol reagent and kept on ice. For every 1ml of trizol 200µl of chloroform was added and mixed for 15 seconds. It was then incubated at room temperature for 5-10minutes followed by a spin of 15minutes at 12000 rpm at 4°C. Three layers were formed - Upper aqueous phase [colourless], interphase and lower phase [pink]. The upper aqueous phase was transferred into a new tube and 500µl of isopropanol was added. It was incubated at room temperature for 5-10 minutes and then centrifuged at 12000 g for 8 minutes, 4°C. RNA got precipitated as a white pellet at the bottom of the tube. The pellet was then washed with 1ml of 75% ethanol by centrifuging it at 7500 g for 5 minutes, 4°C. Pellet was air dried for 3 minutes and resuspended in 10-15µl of RNase free autoclaved water and stored at -20°C. It was quantified using nanovue plus spectrophotometer (GE Health care, Amesrsham).

2.2.4.2 Real – time reverse transcription with polymerase chain reaction

RT-PCR was done using SYBR Green chemistry and Eurogentec two step kit. Kit comprised of a Reverse Transcription core kit and a MESA GREEN qPCR Mastermix plus for SYBR assay – dTTP. The protocol mentioned in the kit was followed. In brief, mRNA extract was primed with random nonamer and reverse transcribed with Euro script RT to produce cDNA. A 10µl reaction was made. Constituents of the reaction mixture are listed below in table 2.1.

Constituents	Concentration
10X reaction buffer	1µl
2.5mM dNTP	2µl
25mM $MgCl_2$	2µl
Random nanomer	0.5µl
Euro script RT	0.25µl
RNAase inhibitor	0.20µl
mRNA extract	1-2µg/µl concentration
Nuclease free water	To make up the total volume to 10µl

Table 2.1: Constituents of reaction mixture for cDNA synthesis.

cDNA amplification was done using Chromo4™ system and the conditions of the experimental setup is listed in table 2.2.

Temp	Time
25^0C	10 min.
48°C	30 min.
95°C	5 min.

Table 2.2: PCR conditions for cDNA synthesis

Gene	**Annealing temperature (°C)**	**cycles**	**Primer sequence**
SPC	53°C	40	5'ACC CTG TGT GGA GAG CTA CCA 3' 5' TTT GCG GAG GGT CTT TCC T 3'
SPB	53° C	40	5'GAA CAC CAG TGA ACA GGC TAT G 3' 5' AAA CTG TTC ACA CTT TTG CCT GTC TA 3'
SPD	53°C	40	5' CAA GAA GGA AGC AAT CTG ACA T 3' 5' AAG ACA AGC ATG GAG AGA AAG G 3'
GAPDH	53°C	40	5' TGT GTC CGT GGT GGA TCT GA 3' 5' CCT GCT TCA CCA CCT TCT GA 3'

Table 2.3: PCR Primer Sequences

Specific primers were used, optimized to amplify fragments from the various genes of interest, as listed in Table 2.3. A 25µl reaction was set. The constituents of reaction mixture and the PCR conditions are given in table 2.4 and 2.5 respectively.

Constituents	**Amount**
2x reaction buffer with SYBR Green	12.5µl
Forward primer [100nm]	0.025µl
Reverse primer [100nm]	0.025µl
Nuclease free water	11.45µl
cDNA	1µl

Table 2.4: Constituents of reaction mixture for cDNA amplification.

<table>
<tr><th>Temperature</th><th>Time</th></tr>
<tr><td>95°C</td><td>10 min.</td></tr>
<tr><td>95°C</td><td>15 sec.</td></tr>
<tr><td>53°C</td><td>20 sec.</td></tr>
<tr><td>72°C</td><td>40 sec.</td></tr>
<tr><td colspan="2">Plate read</td></tr>
<tr><td colspan="2">Go to step two for 39 more times</td></tr>
<tr><td colspan="2">Melting curve analysis</td></tr>
<tr><td>72°C</td><td>5 min.</td></tr>
<tr><td>10°C</td><td>Forever</td></tr>
<tr><td colspan="2">end</td></tr>
</table>

Table 2.5: PCR conditions for cDNA amplification

It was programmed for 40 cycles. Melting curves were used to establish the purity of the amplified band. The expression level of each mRNA was normalized to that of GAPDH [ΔCT], a housekeeping gene, using the mathematical expression:

$$\Delta CT = C\ (t)_1 - C\ (t)_2$$

$[C\ (t)_1 = \text{GAPDH},\ C(t)_2 = \text{gene of interest}]$

2.2.5 Atomic Force Microscopic Analysis (AFM)

For AFM analysis the A549 cells were seeded on to PVDF, MCE and CA. The cells on the scaffold were fixed using 4% Paraformaldehyde after four days of dynamic culture. The samples were dried in incubator at 37°C, one hour prior to use. Witec 300 A AFM microscope was used.

RESULTS AND DISCUSSION

CHAPTER III

Results and Discussion

The pulmonary epithelium sub serves a wide range of functions from providing a barrier against inhaled particles and pathogens to transmitting signals to sub-epithelial cells. Given its central place in respiratory pathophysiology developing an *in vitro* model to address both the functional and cellular make up of the lungs is a challenge. Tissue engineering is based on the understanding of cell-cell and cell-material interactions resulting in the development of tissue like constructs in the laboratory. Such well studied constructs would be the fore runners for organ or tissue replacement therapy. Such tissue specific three-dimensional constructs would be invaluable to pharmaceutical research, biomaterial and toxicity testing. Ideally, the tissue constructs would have the same structural, physiological, antigenic differentiation features found in the tissue of origin. This is possible only on a thorough understanding of the corresponding interactions of cells and candidate biomaterials. The alveolar epithelial surface area of the lung is composed of only two morphologically distinct types of cells, type I and type II cells. Type I cells are large for gas exchange while Type II pneumocytes are highly specialized alveolar epithelial cells whose primary function is secretion of surfactant, which is a complex mixture of phospholipids and lung specific proteins. Type II cells have been reported to lose the ability to secrete surfactant during culture rapidly.

In this work we hypothesize that a suitable scaffold and dynamic culture conditions would help in the maintenance of cell specific functions specifically that of alveolar type II pneumocytes.

The thesis consists of two parts:

- Part I consists of studies of interactions of A549 cell line with different scaffold materials.
- Part II consists of studies of interactions of primary alveolar cells of which type II pneumocytes is the major cell type with scaffold material.

These interactions were analyzed both morphologically using microscopy and functionally looking at surfactant protein gene mRNA expression.

Part I

Three different scaffold materials PVDF, CA and mixed cellulose esters (MCE) were chosen for the study. They were seeded with A549 cells at a density of $1x10^5$ cells per scaffold. They were incubated both under static and dynamic culture conditions. The cells for dynamic culture were allowed to adhere overnight and then transferred to minucell perfusion system for dynamic culture conditions. The cells had adhered uniformly over the scaffold surface. This data is substantiated by AFM analysis also as seen in Fig: 3.1 which shows distribution of cells on PVDF, CA and MCE scaffolds when A 549 cells were cultured on it for 4 days. PVDF maximally and CA and MCE supported A549 cell attachment and proliferation.

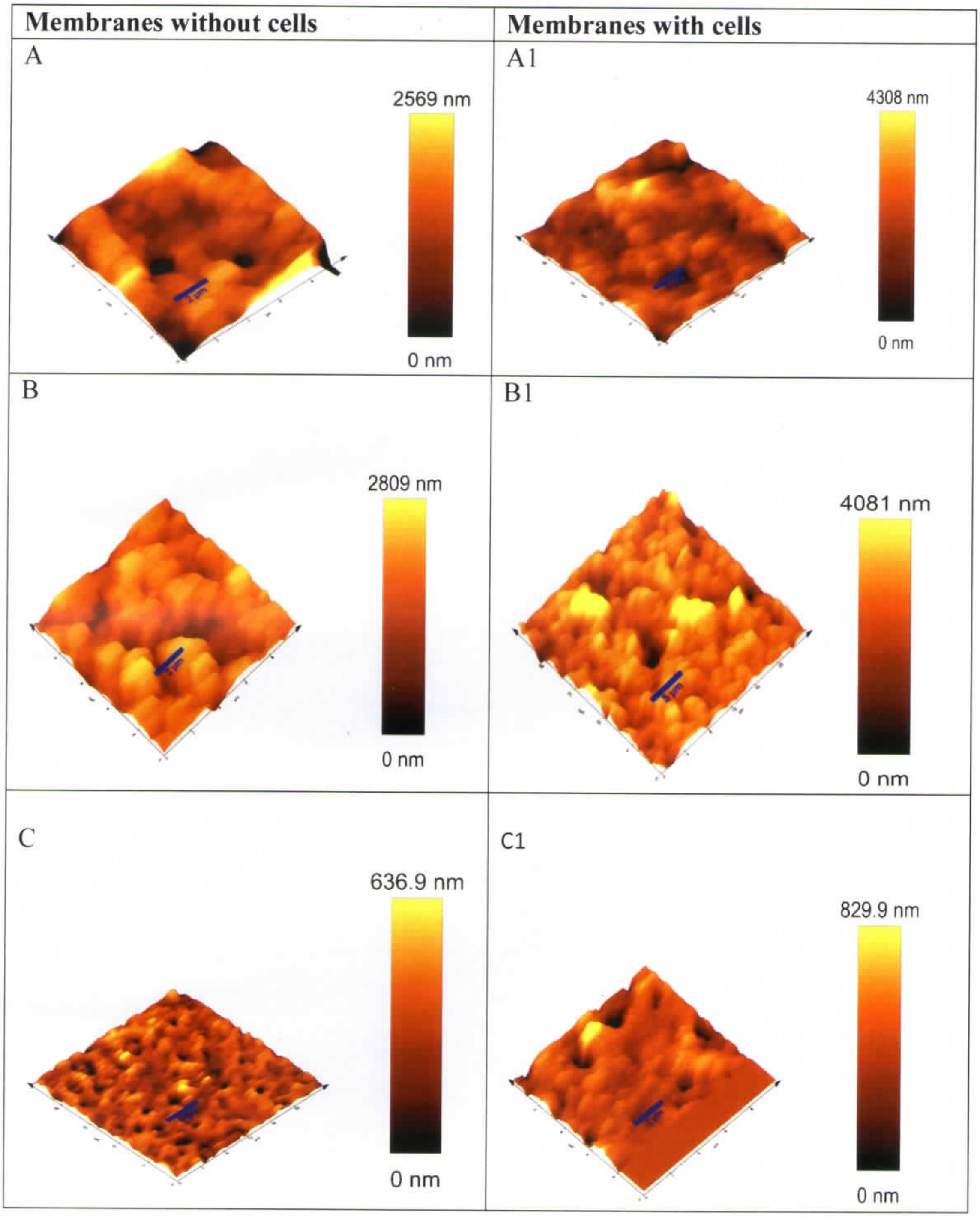
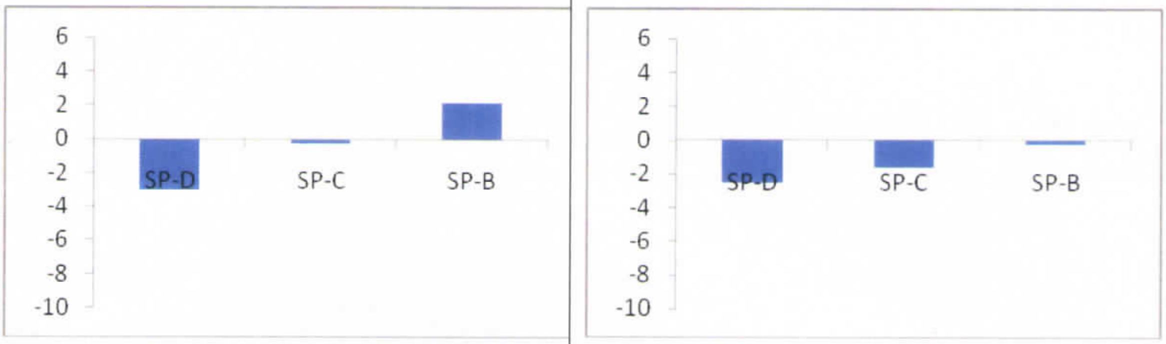


Fig 3.1: Analysis by AFM A549 adhesion to (A & A1) PVDF (B & B1) Mixed cellulose acetate (C& C1)cellulose acetate after 4 days of dynamic culture.

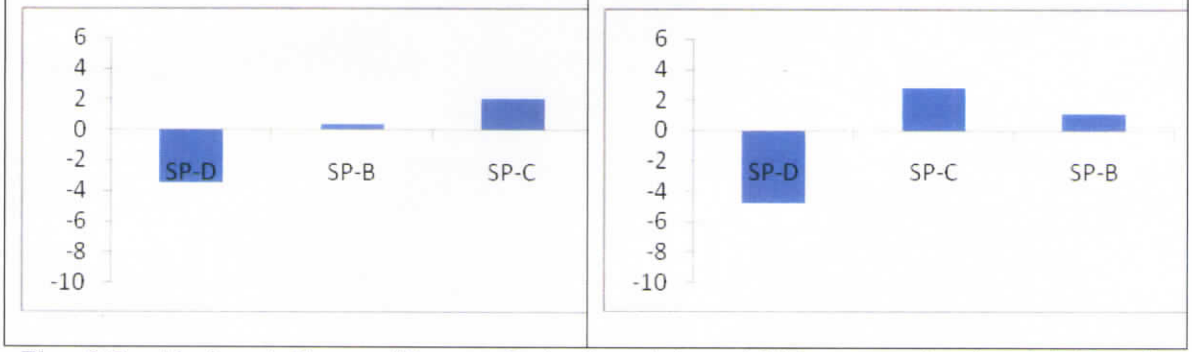


A) PVDF-2 days static

A) PVDF-4 days static

B) MCE-2 days static

B) MCE-4 days static



C) CA-2 days static

C) CA-4 days static

Fig: 3.2 : Under static condition surfactant protein m RNA expression by A 549 cells grown on PVDF (A), CE (B) & CA (C) at Day 2 and day 4. By day4 on PVDF all surfactant mRNA expressions were down regulated while CA & MCE seemed to support surfactant expression better on day 4 under static conditions. The mRNA expression was normalized to housekeeping gene GAPDH mRNA.

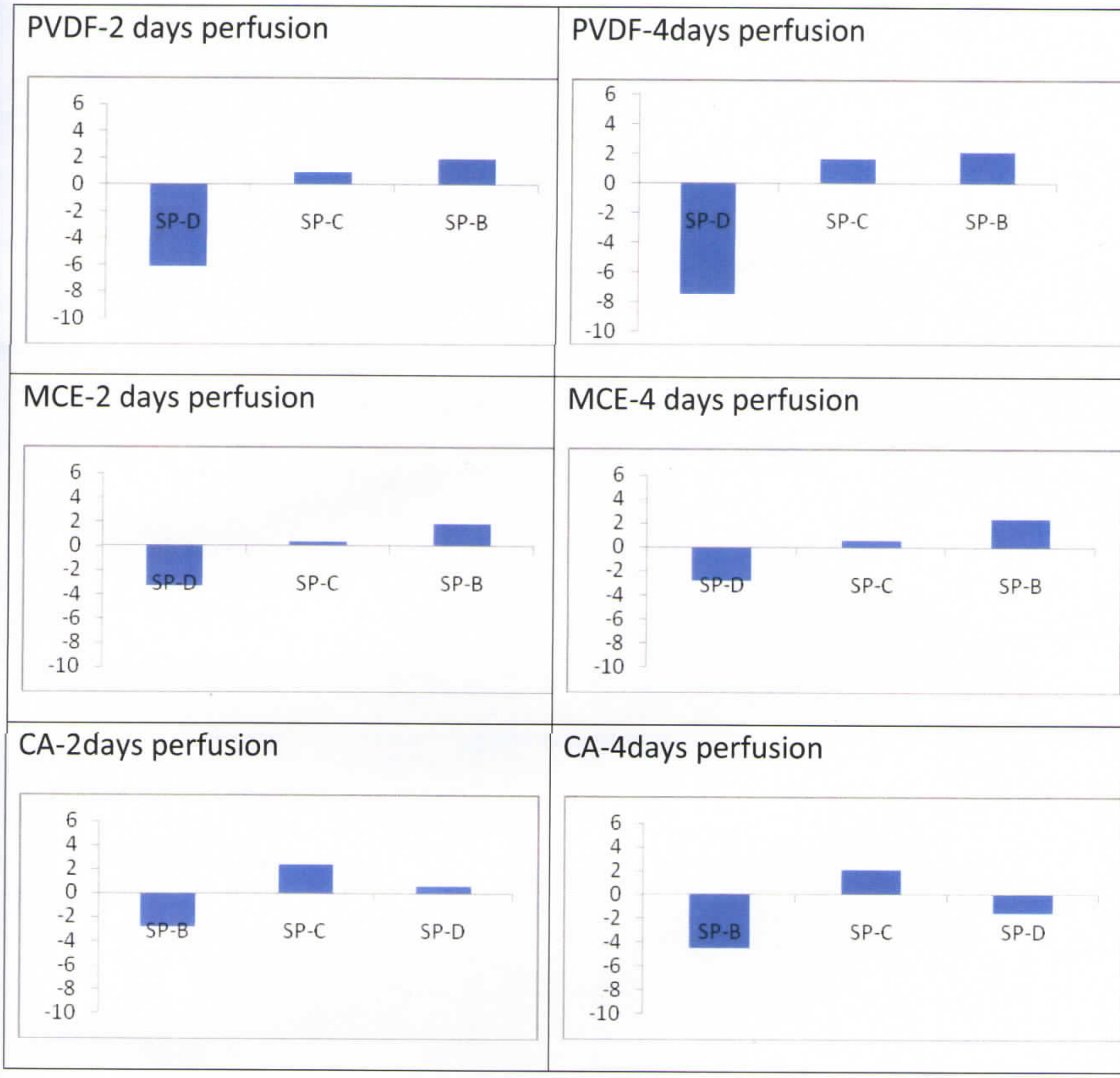


Fig 3.3: qRT –PCR of mRNA of SPC, SPB & SPD done on day 2 &4 under dynamic culture conditions for PVDF, CA and MCE. The mRNA expression was normalised to mRNA of GAPDH a house keeping gene. Under dynamic condition surfactant protein expression was retained on day 4 as in day 2.

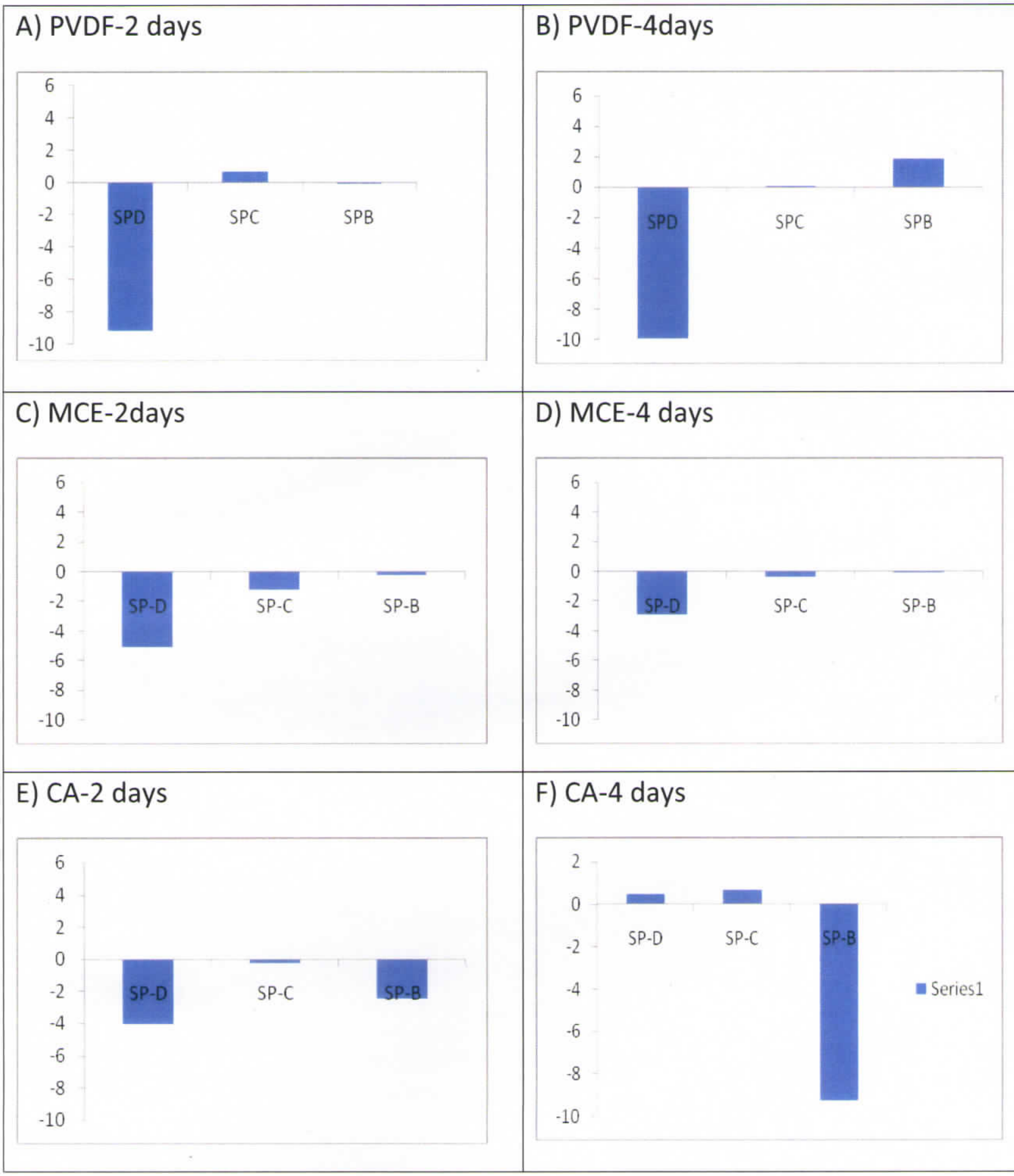


Fig: 3.4: Relative expression of surfactant protein m RNA by A 549 cells grown o PVDF (A &B), CE (C & D) & CA (E &F) at day 2 and day 4 static versus dynami condition.

Surfactant secretion for reducing surface tension at the air-liquid interface to facilitate respiration and prevent alveolar collapse is the primary function of type II pneumocytes. So, mRNA analysis of surfactant protein C (SPC), SPB & SPD genes was done on day 2 and day 4 both under static and dynamic culture conditions.

Figure 3.2 shows that under static condition on PVDF, only surfactant protein B expression by A549 cells was there on day2 while SPC & SPD were not expressed. By day 4 A549 cells had lost surfactant mRNA expression of SPC, SPB & SPD under static culture conditions on PVDF. Mixed cellulose ester (CE) supported surfactant protein expression under static culture conditions as Fig 3.2 (B) shows and it increased by day 4 in comparison to day2. SPB was maximally expressed while there was minimal expression of SPD by day 4. Cellulose acetate (CA) on the other hand maintained expression of SPC, SPB while SPD was not expressed (Fig 3.2 (C))

Fig 3.3 shows that under dynamic culture conditions in the perfusion system PVDF promoted functional expression as observed from mRNa expression of the surfactant proteins. Both SPC and SPB mRNA expression was maintained. MCE supported surfactant protein expression under dynamic conditions during the period of culture. As far as CA was concerned expression of SPC only was supported under dynamic culture conditions. Fig 3.4 shows relative expression of the different surfactant proteins static versus dynamic condition. SPC & SPB are the surfactants primarily responsible for reduction of surface tension and facilitating respiration. SPD on the other hand is responsible for first line of defence on challenge with an infectious agent and is responsible for phagocytosis. Over here, SPD mRNA is down regulated, probably because there is no challenge from an infectious agent.

PART II

Of the three scaffold candidate materials studied in Part II only PVDF supported, adhesion and uniform spreading of A549 cells on the surface. A549 cells cultured on PVDF under dynamic culture conditions expressed both SPB & SPC. So in order to understand the interactions of primary alveolar cells to scaffold materials, PVDF was the scaffold of choice.

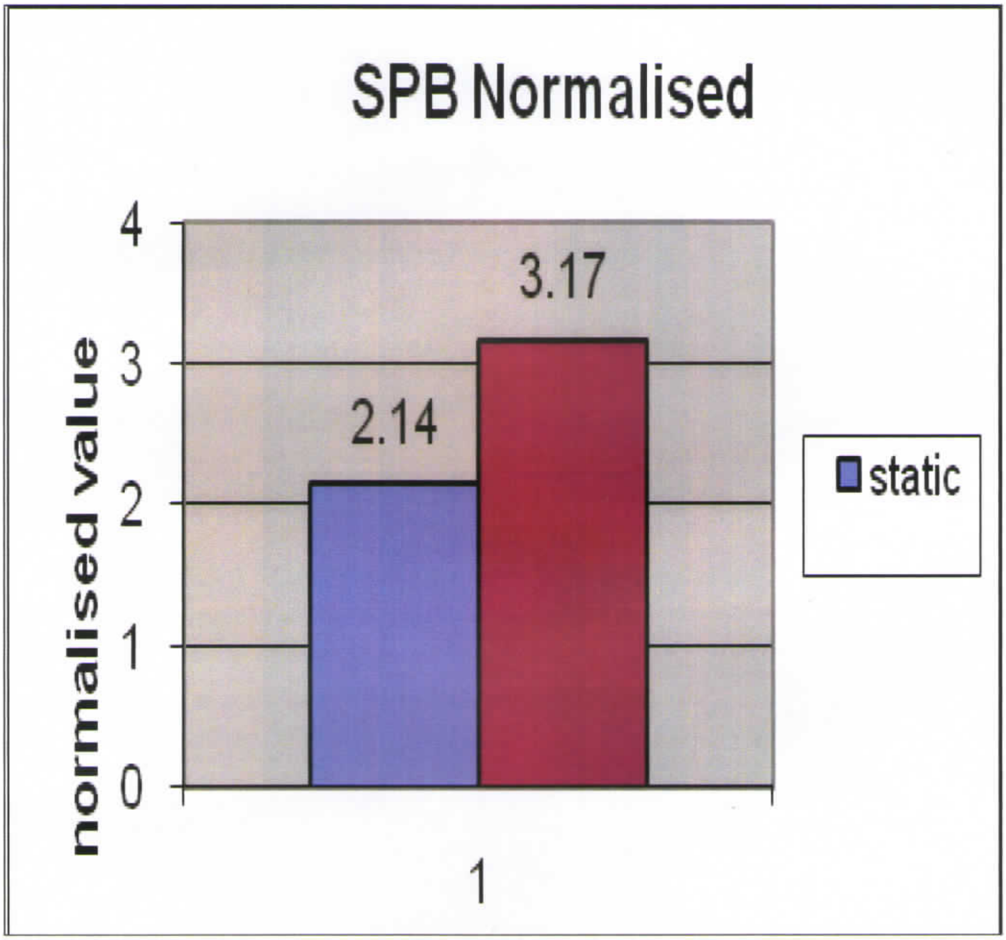


Fig 3.5: qRT PCR of mRNA expression by Primary alveolar epithelial cells cultured on PVDF. The culture was heterotypic with both Type I & Type II pneumocytes and fibroblast. Surfactant protein B mRNA was analysed after 4 days of culture both under static and dynamic culture conditions. The surfactant protein mRNA expression was normalized to mRNA of GAPDH, the house- keeping gene.

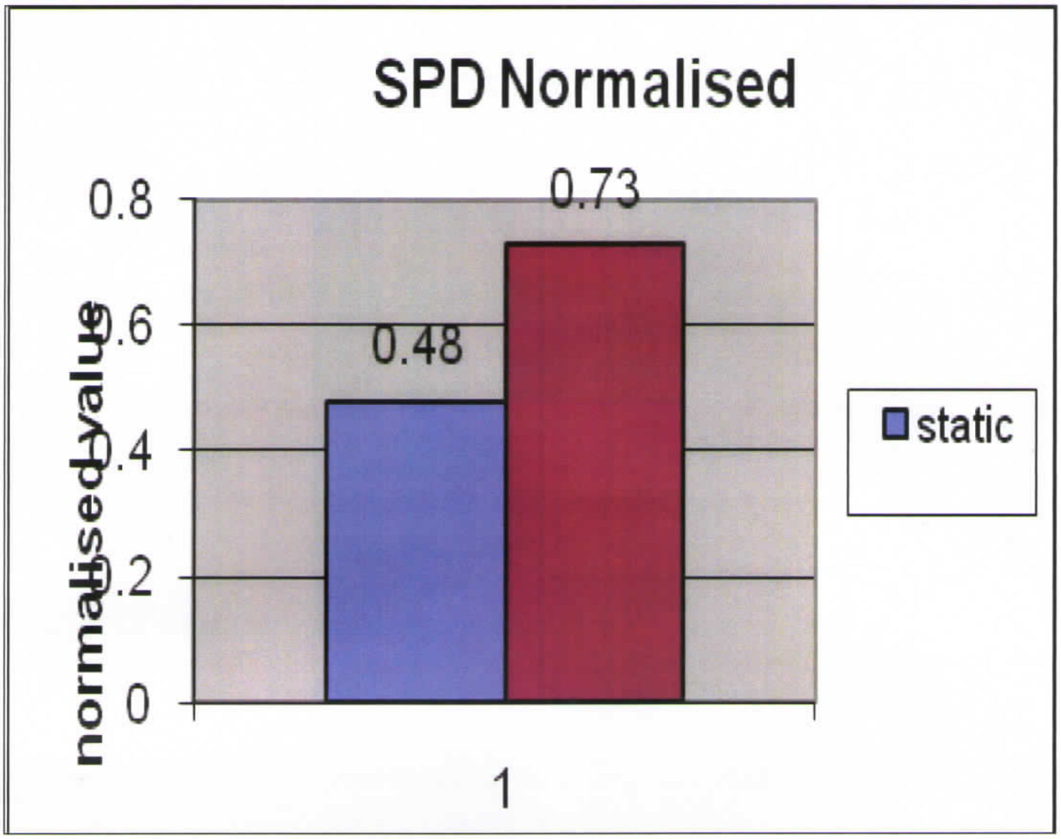


Fig 3.6: qRT PCR of mRNA expression by Primary alveolar epithelial cells cultured on PVDF. The culture was heterotypic with both Type I & Type II pneumocytes and fibroblast. Surfactant protein D mRNA was analysed after 4 days of culture both under static and dynamic culture conditions. The surfactant protein mRNA expression was normalized to mRNA of GAPDH, the house- keeping gene.

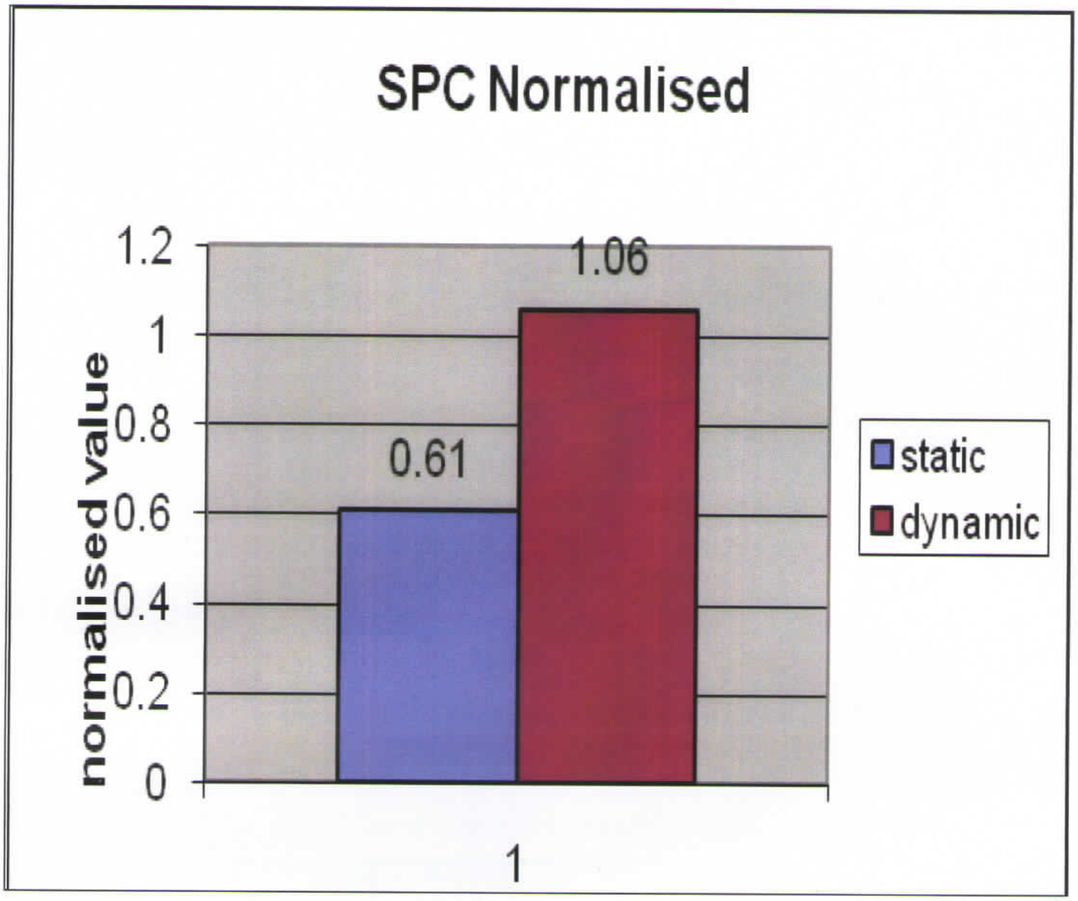


Fig 3.7: qRT PCR of mRNA expression by Primary alveolar epithelial cells cultured on PVDF. The culture was heterotypic with both Type I & Type II pneumocytes and fibroblast. Surfactant protein C mRNA was analysed after 4 days of culture both under static and dynamic culture conditions. The surfactant protein mRNA expression was normalized to mRNA of GAPDH, the house- keeping gene.

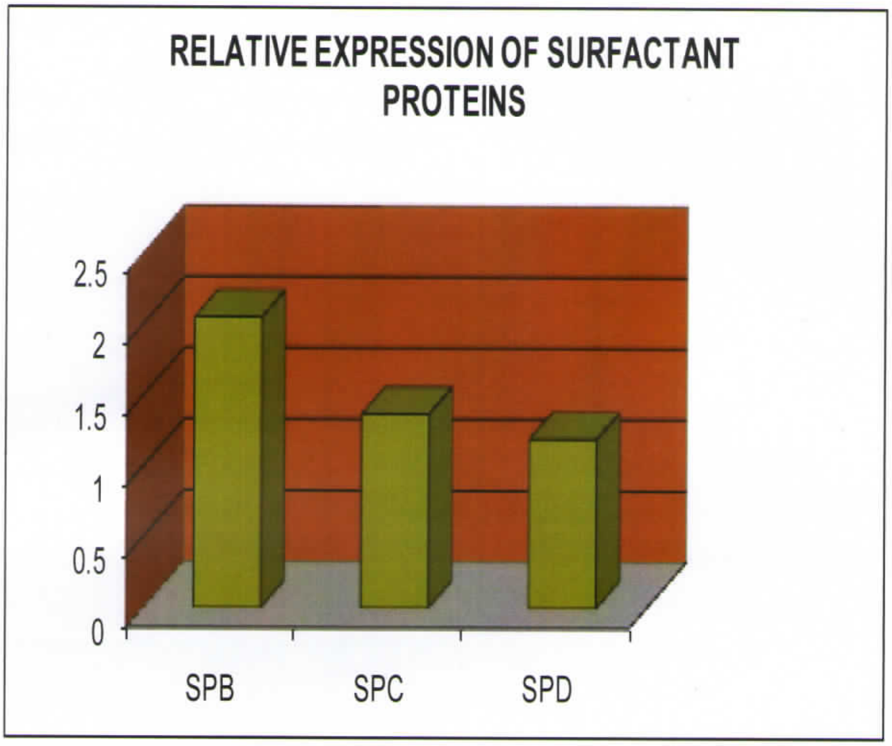


Fig 3.8: Relative expression of the different surfactant proteins under dynamic cultur day 4 of culture. The primary alveolar cells had been cultured of PVDF scaffold.

The lung is a unique organ in terms of its anatomic structure and physiological role. The lung is subjected to several complex physical forces including breathing, pulmonary blood flow and surface tension. The pulmonary homeostasis and functional integrity are a balance between several of these parameters. So there are various factors which are responsible for maintaining cell specific characters which need to be duplicated *in vitro* for functional lung construct. Cell architecture a direct reflection of cell shape, culture conditions are a few of the parameters that need to be understood. Earlier studies have proved the importance of cell shape in the maintenance of cell specific function especially in epithelial cells exhibiting apical – basal polarity (Shannon etal 1992& 1998, Nandkumar etal 2002). The relationship between cuboidal cell shapes and differentiated cell function expression has been pointed out in several papers. When type II cells are cultured on plastic cell culture dishes, it results in cell spreading and dedifferentiation as determined by decreased expression of surfactant proteins and altered patterns of phospholipid biosynthesis, implying that the cuboidal shape and thereby a permissive cyto-architecture are a prerequisite for maintaining type II cell differentiation and surfactant protein mRNA stability. Shannon and colleagues, Nandkumar etal have proved that type II cell differentiation was improved when cells were allowed to regain their native cuboidal shape. A perfusion culture system ensures removal of metabolic waste and continuous supply of nutrients another limitation for a three dimensional tissue construct. Here scaffold and perfusion system were used to evaluate surfactant proteins expression in lung cells during the period of culture. Dynamic culture system promoted continued expression of surfactant proteins.

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

Given the lungs central place in respiratory physiology developing an *in vitro* model to address both the functional and cellular make up of the lungs is a challenge. The alveolar epithelial surface area of the lung is composed of only two morphologically distinct types of cells, type I and type II epithelial cells or pneumocytes. Type I cells are squamous cells with large surface area facilitating gas exchange while Type II pneumocytes are highly specialized alveolar epithelial cells whose primary function is secretion of surfactant. Type II cells have been reported to lose the ability to secrete surfactant rapidly during culture. Our work is based on the premise that a suitable scaffold and dynamic culture conditions would help in the maintenance of cell specific functions specifically that of alveolar type II pneumocytes.

The result of the following studies point that a perfusion culture resembles our physiological system more closely than the usual static system. The q-RT PCR results brings out the fact that in a dynamic system the cells are metabolically more active compared to the static system. The surfactant gene expression quantification results show that surfactant protein B (SP-B) was expressed at a high rate followed by surfactant protein C (SP-C) and then the surfactant protein D (SP-D). Infact SPD was down regulated in PVDF seeded primary and A549 cells, probably because there is no challenge from an infectious agent as SPD is primarily responsible for phagocytosis the primary immune response.

PVDF was established to be the scaffold of choice among the three used. There was an increase in the surfactant gene mRNA expression in cells seeded on PVDF than mixed cellulose ester and cellulose acetate

The q-RT PCR results also supported the view that the perfusion system was favourable for cellular growth functional integrity of the cells than the static one.

Optimal results in biomaterial testing and tissue engineering under *in vitro* conditions can be achieved only when the generated construct resembles the original tissue as closely as possible. At present there is no system available which incorporates all the cell types in the lung to give a tissue engineered hybrid artificial lung model. In this project we have tried to figure out a suitable scaffold from which tissue engineered hybrid artificial lung model can be developed.

We have analysed the effect of scaffolds in dynamic and static monocultures of A549 and primary culture. Characterizations have been done using AFM and Contact Angle Measurement. The characterisation by AFM revealed that cell spread was more on PVDF followed by MCE and CA. RT-PCR results showed the differences in the pattern of SPC, SPB and SPD gene expression in case of monoculture of A549 and primary culture.

In conclusion, it can be stated that PVDF was a suitable scaffold for alveolar cells and dynamic system of culture helped in the maintenance of alveolar epithelial cell specific properties in culture.

BIBLIOGRAPHY

Reference

Adamson I.Y and Young L. Relationship of alveolar epithelial injury and repair to the induction of pulmonary fibrosis. *Am. J. Pathol.*1988; 130: 377-383.

Ballard PL .Hormones and Lung Maturation. *Berlin: Springer-Verlag*. 1986; 4:520-525.

Bangham AD and Horne RW .Negative staining of phospholipids and their structural modification by surface active agents as observed in the electron microscope. *Journal of Molecular Biology* 1964; 8: 660-668.

Brown E.S *etal*. Isolation and assay of dipalmitoyl lecithin in lung extracts. *Am J.Physiol.*1964; 207:402-406.

Cockshutt A and Possamayer F. Pulmonary surfactant-associated protein A enhances the surface activity of lipid extract surfactant and reverses inhibition by blood proteins in vivo*", Biochemistry.*1990; 29: 8424-8429.

Coleman, W. and Baseman, J. B. Selective isolation and culture of a proliferating epithelial cell population from the hamster trachea. *In Vitro.*1984*;* 16: 313-319.

Crouch E and Wright JR .Surfactant proteins A and D and pulmonary host defence. *Annual Review of physiology.*2001; 63:521-554.

Dobbs LG, Pain MS, Maglio M, Dumars S and Allen L. Maintenance of differentiated type II cell phenotype by culture with an apical air surface. *Am. J. Physiol.*1997; 273: 347-354.

Eugene Rannels.D, Gap junction communication in alveolar epithelial cells. *Am J Physiol Lung Cell Mol Physiol* .2001; 280: L1083-L1084.

Evangelina Mascaró et al, Reductive coupling of carbonyl compounds promoted by cobalt and titanium nano particles. *Organic chemistry in Argentina*, 2009; 7:312-326.

Gebb, S. A. and Shannon, J. M. Tissue interactions mediate early events in pulmonary vasculogenesis. *Dev. Dyn*, 2000*;* 217: 159-169.

Henry M and Jobe. A. Surfactant protein B in newborn rabbit. *Biochimica et Biophysica Acta. 1996;* 1300: 97-102.

Hildebran JN *etal*. Pulmonary surface film stability and composition. *Journal of Applied Physiology.*1979; 47: 604-611.

Holm BA *etal* .Contents of dipalmitoylphosphatidylcholine in lung surfactant: Ramification for surface activity*. Pediatric Research.*1996; 39:805-811.

HolgateS.T *etal*. Epithelial damage and response. *Clinical and Experimental Allergy*. 2000; 30.

Ian Y. R *etal.* Alveolar type II cell growth on a pulmonary endothelial extracellular matrix *.Blood.*1987; 22:361-370.

Johannson J and Crustedt T. Molecular structure and interactions of pulmonary surfactant components. *European Journal of Biochemistry.*1997; 244: 675-693.

Kimberly A. Foster et al. Characterization of the A549 cell line as a type II pulmonary epithelial cell model for drug metabolism. *Experimental Cell Research*, 1998; 243:359-366.

King RJ.Pulmonary surfactant, *Journal of Applied Physiology*. 1982; 53: 1-8.

Lammert, E and Melton, D. Induction of pancreatic differentiation by signals from blood vessels. *Science*, 2001; 294: 564-567.

Marchock, *eta*l. Induction of squamous metaplasia (Vitamin A deficiency) and hyper-secretory activity in tracheal organ culture. *Lab. Invest.*1975; 33:451-460

Matsumato et al. Pancreas organogenesis-From bud to plexus to glands. *Developmental Dynamics* 2011; 240 (3):530–565.

Maya A. Nandkumar *et al*. Two-dimensional cell sheet manipulation of heterotypically co-cultured lung cells utilizing temperature-responsive culture dishes results in long-term maintenance of differentiated epithelial cell functions. *Biomaterials.*2002; 23: 1121–1130.

Moise´s Selman and Annie Pardo. Role of Epithelial Cells in Idiopathic Pulmonary Fibrosis from Innocent Targets to Serial Killers. *Proceedings of American thoracic society*. 2006; 3.

Muller F.A *etal*. Cellulose based scaffold materials for cartilage tissue engineering. *Biomaterials*, 2006; 3955-63.

Petra Lynen *etal.* Surgical Mesh as a Scaffold for Tissue Regeneration in the Esophagus. *Eur Surg Res* 2008; 36:104–111.

Possmayer F *eta*l .The Perinatal Lung. Biochemical development of the Fetus and Neonate .*Elsevier Biomedical Press*: 1982; 337-391.

Paur H R *et al. In Vitro* Exposure Systems and Bioassays for the Assessment of Toxicity of Nanoparticles to the Human Lung. *Journal für Verbraucherschutz und Lebensmittelsicherheit.*2008; 3:319-329.

Richard M Effros, Goyal and Shekhar. http://www.nature.com/gimo/fig/index.html.

Richerson *etal.* Growth, Morphological and Immunocytochemical Analysis. *J. Cell Physiol.*1974; 133**:** 228–234.

Rooney SA etal. Regulation of surfactant secretion. *Comparitive Biochemistry and Physiology: Part A.* 2001; 129: 233-243.

Rodrigues MT *etal.* PVDF membrane induced cellular proliferation and differentiation in static and dynamic condition. Material science forum. 2008; 11:72-76.

Shannon JM etal. Functional differentiation of alveolar type II epithelial cells in vitro: effects of cell shape, cell-matrix interactions and cell-cell interactions. *Biochim. et Biophysica. Acta.*1987; 931: 143-156.

Shannon MJ *etal.* Modulation of alveolar type II cell differentiated function in vitro. *Am J Physiol* (lung cell Mol Physiol 6).1992; 262: L427-L436.

Shannon JM *eta*l. Influence of the cytoskeleton on surfactant protein gene expression. *Am J Physiol* (lung cell Mol Physiol) 1998; 18: L 87- L96.

Smith BT and Fletcher WA. Pulmonary epithelial – mesenchymal interaction: beyond organogenesis. *Human Pathology.*1979; 10(3): 248-250.

Smith RS. Annual health burden due to indoor air pollution in India. *PNAS.* 2000; 97(24): 13286-13293.

Stephen.S *etal*. Dynamic seeding and *in vitro* culture of hepatocytes in a flow perfusion system. *Tissue engineering*. 2000; 6(1): 39-44.

Tang F *etal* .Latgé JP Aspergillus fumigatus: Saprophyte or pathogen? *Curr Opin*.1994; 22:55-61.

Valsamma Abraham *etal*. Multimeric connexin interactions prior to the trans-Golgi network, J *Cell Sci*. 2001; 114:4013-4024.

Wellington V. Cardos *eta*l**.** Regulation of early lung morphogenesis: questions, facts and controversies. *Developmen*t. 2006; 133: 1611-1624.

Whitsett JA and Weaver TE .Hydrophobic surfactant protein in lung function and disease. *New England Journal of Medicine*, 2002; 26: 2141-2148.

Wood, H. B *etal*. CD34 expression patterns during early mouse development are related to modes of blood vessel formation and reveal additional sites of hematopoiesis. *Blood* .1997*;* 90: 2300-2311.

www.lungusa.org, Lung Disease Data.2008.

Yang *eta*l. Different mitogenic and phenotypic responses of human breast epithelial cells grown in two versus three dimensions. *Exp. Cell. Res*.1986*;* 167**:** 563–569.

Yu SH and Possamayer F .Comparative studies on the biophysical activities of low-molecular-weight hydrophobic proteins purified from bovine pulmonary surfactant. *Biochimica et Biophysica Acta*. 1988; 961(3): 337-350.

Annexure-1

LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
cDNA	Complementary Deoxyribo nucleic acid
CT	Cholera toxin
DMEM	Dulbeco's modified Eagle's Medium
DNA	Deoxyribo nucleic acid
EDTA	Ethylene diamine tetra acetic acid
EMEM	Eagle's Minimal Essential Medium
F	Cortisol
F12	Ham's F12 medium
F12 k	Ham's F12 medium with kaighn's modification
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehydes -3-phosphate dehydrogenase
I	Insulin
mRNA	Messenger Ribonucleic acid
ng	nanogram
PBS	Phosphate buffer saline
RNA	Ribonucleic acid
RPMI 1640	Roswell Park Memorial Institute 1640 medium
SDS	Sodium dodecyl sulphate
SPA	Surfactant protein A

SPB	Surfactant protein B
SPC	Surfactant protein C
SPD	Surfactant protein D
µg	Microgram
µl	microlitre
PVDF	Polyvinylidene flouride
MCE	Mixed cellulose esters
CA	Cellulose acetate
FITC	Fluorescein isothiocyanate