Stem cell differentiation and biomaterial processing for the engineering of pulmonary epithelial tissue

A thesis submitted to Imperial College London for the degree of Doctor of Philosophy in the Faculty of Engineering

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Yuan-Min Lin
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Declaration

I hereby certify that all of the works in this thesis, including experiments and research plans, are entirely my own unless otherwise specified in the text.

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Yuan-Min Lin
Abstract

The standard approach of tissue engineering is to develop a cell-seeded scaffold which has morphology and mechanical properties similar to those of the target tissue. The scaffold material should possess surface properties so that the seeded cells can maintain proper activity, function and morphology. In terms of engineering of pulmonary epithelial tissue, a major challenge is to obtain sufficient cells because of the difficulty in isolating and culturing primary pulmonary epithelial cells \textit{in vitro}, especially type II pneumocytes. To overcome this problem, a method to differentiate pulmonary progenitors, which possessed most of the features of type II pulmonary epithelial cells, from murine embryonic stem cells (mESCs) was investigated in this study. The first aim of this project was to increase the differentiation efficiency of type II pneumocyte progenitors from mESCs by enhancing the efficiency of an established differentiation protocol using extracellular matrix (ECM), growth factors and bioactive peptides. In the first study, mESCs were differentiated on tissue culture plastic (TCP) and poly(D,L-lactide) (PDLLA) films coated with ECM proteins, including collagen, laminin, fibronectin and Matrigel. The results demonstrated that all protein coatings can enhance the wettability of the TCP and PDLLA films and, moreover, laminin and Matrigel can enhance the differentiation of mESCs into pulmonary progenitors. In the second study, growth factors that are commonly thought to affect the development of embryonic lung, including fibroblast growth factors (FGFs) 1, 2, 7 and 10, were added to the differentiation culture at various concentrations and the subsequent expression of surfactant protein C, a marker of type II pneumocytes and their progenitors, was measured. It was found that FGF1 alone and FGF10 in combination with Matrigel coating enhanced the differentiation of mESCs into pulmonary progenitors. In another study, mESCs were differentiated on PDLLA films grafted with the bioactive peptides RGD and YIGSR. Preliminary result showed that YIGSR enhanced the differentiation of mESCs into pulmonary progenitors.

The second aim of this project was to develop 2D environments and 3D scaffolds made of PDLLA suitable for the culture of human pulmonary epithelial cells (A549 line). PDLLA has advantages of biocompatibility and biodegradability, but a major
drawback is its hydrophobic nature. To make the surface of PDLLA films hydrophilic, it was modified using a variety of methods, i.e. by grafting the bioactive peptides RGD and YIGSR, by introducing amines using aminolysis and by creating amine-terminated and carboxylic acid-terminated tree-like branched architectures on to the surface. The surface properties of modified PDLLA films were evaluated using various techniques. The culture of A549 cells on PDLLA films demonstrated that surface modifications can affect the attachment, focal adhesion point formation, activity and population size, depending on the type and the concentration of the bioactive peptides or functional groups presented on the surface of PDLLA films.

The challenge of culturing pulmonary epithelial cells in 3D is to generate scaffolds with proper porous structures which allow sufficient medium diffusion in and waste disposal out of the scaffolds. The influence of the preparation conditions, i.e. coarsening time and coarsening period of a liquid-liquid phase separation system and freezing temperature of a solid-liquid phase separation system, on the porous morphology and the subsequent pulmonary epithelial cell culture were examined. Scaffolds that possessed alveolus-like round pores and ladder-like pores were prepared using liquid-liquid phase separation and solid-liquid phase separation, respectively. Culture of A549 cells on the PDLLA scaffolds demonstrated that cell penetration into and activity on the scaffolds are influenced by the pore size and the pore throat size of the scaffolds.

In conclusion, the results of this project demonstrated that the differentiation of mESCs into pulmonary epithelial progenitors can be enhanced by external signals i.e. from ECM proteins, FGFs and bioactive peptides. The responses of pulmonary epithelial cells to the PDLLA scaffolds can be enhanced by surface modifications using bioactive peptides and functional groups and scaffolds that can serve as a culture environment for pulmonary epithelial cells were prepared accordingly. Taken together, the results of these studies provide a basis for future engineering of pulmonary epithelial tissue, an area of tissue engineering that lags behind that of other major organs.
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<td>ADA</td>
<td>N-(2-acetamido) iminodiacetic acid</td>
</tr>
<tr>
<td>PDLLA film (Chapter 6) or PDLLA scaffold (Chapter 7)</td>
<td></td>
</tr>
<tr>
<td>bADA</td>
<td>that possessed carboxylic acid-terminated branched architectures</td>
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<tr>
<td>bADA_n</td>
<td>bADA that have up to n generations of branched architectures, n can be 1, 2, or 3</td>
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<tr>
<td>PDLLA film (Chapter 6) or PDLLA scaffold (Chapter 7)</td>
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<tr>
<td>bTREN</td>
<td>that possessed amine-terminated branched architectures</td>
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<td>bTREN_n</td>
<td>bTREN that have up to n generations of branched architectures, n can be 1, 2, or 3</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CC-10</td>
<td>Clara cell-associated protein 10</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge-coupled device</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<td>CO</td>
<td>Collagen</td>
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<tr>
<td>DCC</td>
<td>N,N'-dicyclohexylcarbodiimide</td>
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<td>DIC</td>
<td>N,N'-diisopropylcarbodiimide</td>
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<td>DMC</td>
<td>Dimethyl carbonate</td>
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<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>EB</td>
<td>Embryoid body</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EDC</td>
<td>1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide</td>
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<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>EHS</td>
<td>Engelbreth-Holm-Swarm (sarcoma)</td>
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<td>Embryonic stem cell</td>
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<tr>
<td>ESCA</td>
<td>Electron spectroscopy for chemical analysis</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>FGF-1</td>
<td>Fibroblast growth factor 1 (also known as acidic FGF)</td>
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FGF-2  Fibroblast growth factor 2 (also known as basic FGF)
FGF-7  Fibroblast growth factor 7 (also known as keratinocyte growth factor, KGF)
FGF-10 Fibroblast growth factor 10
FISH  Fluorescence in situ hybridization
FN    Fibronectin
Foa2   Transcriptional factor Forkhead box a2
GABA  γ-amino butyric acid
GFP   Green fluorescent protein
GN    Gelatin
h     hour
hESCs Human embryonic stem cells
HGF   Hepatocyte growth factor
ICM   Inner cell mass (of blastocyst embryo)
IEP   Isoelectric point
KO-DMEM Knock-out Dulbecco’s modified Eagle’s medium
KO-SR Knock-out serum
KGF   Keratinocyte growth factor
LIF   Leukemia inhibitory factor
LL0M  Scaffolds prepared using liquid-liquid phase separation at coarsening temperature at 24°C for 0 min
LL10M Scaffolds prepared using liquid-liquid phase separation at coarsening temperature at 24°C for 10 min
LL12C Scaffolds prepared using liquid-liquid phase separation at coarsening temperature at 12°C for 30 min
LL15C Scaffolds prepared using liquid-liquid phase separation with coarsening temperature at 15°C for 30 min
LL18C Scaffolds prepared using liquid-liquid phase separation with coarsening temperature at 18°C for 30 min
LL20M Scaffolds prepared using liquid-liquid phase separation with coarsening temperature at 24°C for 20 min
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<tr>
<td>LL60M</td>
<td>Scaffolds prepared using liquid-liquid phase separation with coarsening temperature at 24°C for 60 min</td>
</tr>
<tr>
<td>LN332</td>
<td>Laminin 332 (former name laminin 5[1])</td>
</tr>
<tr>
<td>m</td>
<td>minute</td>
</tr>
<tr>
<td>MEF</td>
<td>Murine embryonic fibroblast</td>
</tr>
<tr>
<td>ML</td>
<td>Matrigel</td>
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<tr>
<td>MES</td>
<td>2-Morpholinoethanesulfonic acid</td>
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<td>mESC</td>
<td>Murine embryonic stem cell</td>
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<td>n%EDiamine</td>
<td>PDLLA film (Chapter 6) or SL-25C (a prepared PDLLA scaffolds as described in Chapter 7) aminolyzed using n% of ethylenediamine in isopropanol for 5 minutes</td>
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<td>NHS</td>
<td>N-hydroxysuccinimide</td>
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<td>Octamer factor 4</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PDLLA</td>
<td>Poly(D,L-lactide)</td>
</tr>
<tr>
<td>PLA</td>
<td>Lactic acid-based polymer</td>
</tr>
<tr>
<td>PLLA</td>
<td>Poly(L-Lactide)</td>
</tr>
<tr>
<td>RGD</td>
<td>Peptide composed of arginine-glycine-aspartic acid</td>
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<tr>
<td>ROP</td>
<td>Ring-opening polymerization</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
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<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
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<td>Acronym</td>
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<td>SABM</td>
<td>Small Airway Basal Medium</td>
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<td>SAGM</td>
<td>Small Airway Growth Medium</td>
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<td>SCID</td>
<td>Severe combined immunodeficiency</td>
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<td>Surfactant protein C</td>
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<td>SPD</td>
<td>Surfactant protein D</td>
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<td>SSEA</td>
<td>Stage specific embryonic antigen</td>
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<td>TCP</td>
<td>Tissue culture plastic</td>
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<td>TGF</td>
<td>Transforming growth factor</td>
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<td>TIPS</td>
<td>Thermal-induced phase separation</td>
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<td>TTF-1</td>
<td>Thyroid transcription factor-1</td>
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<td>TREN</td>
<td>tris(2-aminoethyl)amine</td>
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<tr>
<td>UNG</td>
<td>Uracil-N-glycosylase</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>XPS</td>
<td>X-ray photoelectron spectroscopy</td>
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<tr>
<td>YIGSR</td>
<td>Peptide composed of tyrosine-isoleucine-glycine-serine-arginine</td>
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<tr>
<td>θ_A</td>
<td>Advancing contact angle</td>
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<td>θ_R</td>
<td>Receding contact angle</td>
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<td>ζ</td>
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<td>ζ_plateau</td>
<td>The plateau region of the ζ=f(pH) curve</td>
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<td>PDLLA film grafted with bioactive peptide using peptide solution composed of 0 % RGD and 100 % YIGSR</td>
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<td>PDLLA film grafted with bioactive peptide using peptide solution composed of 25 % RGD and 75 % YIGSR</td>
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<td>100RGD-0YIGSR</td>
<td>PDLLA film grafted with bioactive peptide using peptide solution composed of 100% RGD and 0% YIGSR</td>
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Chapter 1. Introduction

1.1. Objective and scope of this thesis

Pulmonary disease is a major cause of morbidity and mortality in the world. For end stage pulmonary diseases, such as chronic obstructive pulmonary disease (COPD), emphysema, idiopathic pulmonary fibrosis, cystic fibrosis and primary pulmonary arterial hypertension, there is no effective medical treatment except lung transplantation. In 2005, the global reported number of lung transplants reached an all time high of 2169 [2], even so the availability of donor lungs is very limited. In 2006, patients with severe pulmonary disease had to wait an average of 132 days for a suitable lung [3]. For transplanted patients, a big challenge to their long-term survival is immune rejection of the transplant. From 1992 to 2006, approximately 10 % of transplanted patients died due to acute rejection of the transplanted organ, despite receiving immunosuppressive treatments [2].

Tissue engineering is a multidisciplinary science which aims to overcome the limitations of conventional therapies by the ‘application of the principles and methods of engineering and life sciences towards the fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes that restore, maintain or improve tissue function’ [4]. In addition to the general understanding of delivery of immunologically tolerant tissue substitutes into the human body, tissue engineering also involves the understanding of \textit{in vitro, in vivo} and \textit{ex vivo} cell-environment behaviour in an engineered and biological manner. A large number of review papers [5-7] and textbooks [8, 9] has been published in recent years, covering the basis and principles of tissue engineering. In a nutshell, three approaches are commonly used in tissue engineering:

1. Growth of human tissues \textit{in vitro} for later implantation into the body to restore injured tissues.
2. Implantation of cell-loaded or cell-free biodegradable constructs to enhance the regeneration and repair of human tissues. The former is a scaffold loaded with target cells whilst the latter is a bioactive material that can enhance the tissue repair.
3. Design of external or internal cell-containing devices that can augment the physiological function of the impaired organs.
In terms of engineering lung tissue, restoration of the gas-exchange function would be critical. The units of the lung where gas-exchange occurs, the alveoli, are lined by two types of endoderm-derived epithelial cells termed pneumocytes; gas exchange occurs across type I pneumocytes while type II cells secrete surfactant that maintains the patency of the distal airways. In addition, when type I cells are injured, type II cells proliferate and transdifferentiate into type I cells to restore the type I population. At present, it is very difficult to obtain pure populations of primary type II pneumocytes and it is not possible to maintain their phenotype for a long time in vitro [10]. This makes the scale-up of type II pneumocytes in vitro for potential therapeutic applications very difficult.

Stem cells hold the promise as cell sources for many of the approaches of tissue engineering and regenerative medicine in general. Stem cells are defined as clonogenic cells capable of self-renewal and multilineage differentiation [11]. Since the derivation of human embryonic stem cells (hESCs) in 1998 [12], stem cell research has attracted a lot of attention from various research institutes and funding bodies [13-15]. Many cell types, such as osteoblasts, cardiomyocytes and hepatocytes, have been successfully differentiated from ESCs. Colleagues in the Stem Cells and Regenerative Medicine group at Imperial College have successfully differentiated type II pulmonary epithelial cells and their progenitors from murine embryonic stem cells (mESCs) using soluble factors [16], reprogramming of mESCs with cell extracts [17] or co-culture with pulmonary mesenchyme [18], although only 2 - 10 % of type II cells could be acquired. **The first aim of this project, therefore, was to increase the differentiation efficiency of type II pneumocyte progenitors from mESCs by enhancing the established differentiation protocol.** To achieve this, extracellular matrix (ECM) proteins and fibroblast growth factors (FGFs) were introduced to the culture system during the differentiation of mESCs. ECM is the extracellular part of the animal tissue and can provide structure support and other physiological functions. FGFs are a family of growth factors that are heavily involved in the development of the vertebrate embryonic development. Both extracellular matrix (ECM) proteins and FGFs are important factors in the development of embryonic lung.

Cells can not be delivered as a whole into the host body with sufficient mechanical
integrity and adequate physiological activity if they are not cultured on a biomaterial support [19]. The biomaterial supports, which should fulfil several very stringent requirements [5], are called scaffolds. Throughout this thesis, PDLLA, a widely-used biocompatible and biodegradable polymer, was used to construct 2D environments and 3D scaffolds. The mechanical properties of porous PDLLA scaffolds can be tailored to meet the requirements of various tissue engineering applications [20]. Once the cells are seeded onto a scaffold, factors such as surface topography, surface chemistry and the porous structure of the scaffold affect cell attachment, growth and differentiation [21]. Thus, the second aim of this project was to develop 2D environments and 3D scaffolds made of poly(D,L-lactide) (PDLLA) suitable for the culture of pulmonary epithelial cells.

It is possible to modify the surface of a scaffold to improve its interaction with a specific cell type. Strategies to achieve this goal include the introduction of cell recognition ligands for a specific cell type or the introduction functional groups [10, 22-41]. Cell recognition ligands can be found in the ECM. Coating the scaffold surface with a thin layer of ECM protein(s) thus can introduce cell recognition ligands to the biomaterial surface. Furthermore, given the problems associated with the high cost and immunogenicity of ECM proteins, peptides that contain the sequence of cell recognition ligands of ECM have been grafted to the surface of scaffolds [23-30]. Surface modification of PDLLA scaffolds has been carried out to provide appropriate functional groups for various cell types [31, 34-36, 40, 41], e.g. attachment of MC3T3-E1 osteoblast–like cells was 2.5 fold greater on thiol-functionalised surfaces compared with amine-functionalised surfaces. Both strategies were applied in the present study for the culture of pulmonary epithelial cells.

To achieve the two main aims of thesis, the following three questions were addressed:

**Question 1:** Can the differentiation of murine embryonic stem cells into type II pneumocyte progenitor cells be enhanced by

- coating the culture vessel with ECM protein(s)?
- grafting bioactive peptides to the culture vessel?
- adding fibroblast growth factors to the cell culture medium?

**Question 2:** How do the pulmonary epithelial cells respond to the surface of PDLLA
scaffolds grafted with RGD and YIGSR peptides or modified with amines or carboxylic acids and how do the surface-modified PDLLA scaffolds differ from unmodified PDLLA scaffolds?

Question 3: What are the parameters needed to prepare synthetic scaffolds with a pore structure that is suitable for the culture of pulmonary epithelial cells?

These questions are addressed in the experimental chapters of this thesis. This thesis is composed of following chapters: Chapter 1 provides the background information related to the engineering of pulmonary epithelial tissue. In Chapter 2, the materials and methods used for culture and characterization of the pulmonary progenitors and A549 cells, as well as for the preparation and characterization of the 2D environments and 3D scaffolds, are described in detail. Chapter 3 describes how ECM proteins were coated on to tissue culture plastic (TCP) and PDLLA. The ECM coatings on TCP and PDLLA were characterized using water contact angle and ζ-potential measurements, and their effects on the differentiation of mESCs into progenitors of pulmonary epithelial cells studied. In Chapter 4, the effect of fibroblast growth factors (FGFs) on the differentiation of mESCs into pulmonary epithelial progenitors was investigated. The work described in Chapter 5 is an extension of the study in presented in Chapter 3 i.e. the bioactive peptides RGD and YIGSR, mimicking the functions of ECM, were grafted to the surface of PDLLA films. The effect of peptide-grafted PDLLA on the culture of a standard pulmonary epithelial cell (A549) line and the differentiation of progenitors of pulmonary epithelial cells from mESCs were studied. In Chapter 6, PDLLA films were modified by introducing various functional groups on to their surfaces; primary amines were introduced using aminolysis and, in addition, amine-terminated and carboxylic acid-terminated, tree-like branched architectures were created on the surface of the PDLLA films. The surface properties of all surface modified PDLLA films were characterized and the responses of A549 cells to the surface-modified PDLLA films were recorded. Chapter 7 describes the preparation of porous 3D scaffolds using thermally induced phase separation (TIPS). The prepared scaffolds were further modified using the techniques described in Chapter 6. A549 cells were subsequently cultured on the prepared scaffolds and their metabolic activity was investigated. In Chapter 8, the main conclusions and possible future work following on from the findings of this
thesis are described.

1.2. Biology of lung and extracellular matrix

1.2.1. Cells of lung alveolar epithelium: anatomy and physiology

The human lung alveolus, around 200 μm in diameter, consists of several types of cells, including type I pneumocytes, type II pneumocytes, macrophages, fibroblasts and others. Among these cells, type I and type II pneumocytes play major roles in the physiological functions of lung. Only 8% of the total lung alveolar epithelial cells are type I pneumocytes. Despite this low percentage, they cover 92% of the alveolar surface which reflects their physiological function: gas exchange [42]. Thus, the diameter of the type I cell ranges from 50-100 μm [43]. In contrast, type II pneumocytes comprise 15% of total lung epithelial cell numbers and 2-5% of the alveolar surface [44]. The diameter of type II cells is around 10 μm [43]. Type II pneumocytes secrete surfactant [45] which decreases the surface tension in the alveoli and, thus, maintains their shape during respiration. Furthermore, type II cells also serve as type I cell progenitors during development [46] and in the injury repair process [47]. The most distinct characteristic of the type II cell is its cytoplasmic inclusions: lamella bodies. Lamellar bodies contain surfactant phospholipids, surfactant proteins A, B, C and D, and lysozyme [48]. Type II cells also synthesise type IV collagen [49], laminin [50], fibronectin [51], thrombospondin, heparin sulfate proteoglycans and chondroitin sulfate proteoglycans [52]. The synthesis of these molecules is enhanced by transforming growth factor-α, transferrin, epidermal growth factor and retinoic acid.
1.2.2. Type II pulmonary cells: characteristics, derivation and culture

Isolation of type II pneumocytes and maintenance of their phenotype had been a challenge in the 1970s. They were not successfully isolated until the first breakthrough made by Kikkawa et al. [53] in 1974. After that, several techniques, such as IgG-coated plate isolation [54] and isolation using laser flow cytometry with monoclonal antibodies against type II cells [55], were developed to provide a reproducible method for type II cell isolation.

As Table 1-1 shows, type II cells possess a range of morphological and biochemical characteristics, many of which are also expressed by other cells but some are specific; these latter are of importance since they can be used as assessment of type II cell phenotype and condition. Lamellar bodies are the most obvious morphological characteristics of type II cells under phase contrast microscopy or interference contrast microscopy, although it can be difficult to differentiate lamellar bodies from intracellular inclusions of other cell types. Type II pneumocytes also secrete pulmonary surfactant proteins A, B, C and D. Among these four types of surfactant proteins, only surfactant protein C is solely expressed by type II pneumocytes. As a result, the expression of surfactant protein C was frequently used to evaluate the presence of type II pneumocytes in the experimental sections of this thesis. Aquaporins are the water channels existing on the surface of various lung epithelial cells. Type I pneumocytes express aquaporin 4 and 5, whereas aquaporin 3 is only expressed by type II pneumocytes.
Table 1-1 Characteristics of type II pneumocytes (adapted and modified from [56])

<table>
<thead>
<tr>
<th>Morphological characteristic</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamellar bodies</td>
<td></td>
</tr>
<tr>
<td>Microvilli</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Biochemical characteristics</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthesized molecules</td>
<td></td>
</tr>
<tr>
<td>Lipid (phosphatidylglycerol and phosphatidylcholine)</td>
<td></td>
</tr>
<tr>
<td>Surfactant proteins A, B, C and D</td>
<td></td>
</tr>
<tr>
<td>TTF1 (Thyroid transcription factor-1)</td>
<td></td>
</tr>
<tr>
<td>Surface marker</td>
<td></td>
</tr>
<tr>
<td>Heymann nephritis antigen</td>
<td></td>
</tr>
<tr>
<td>Aquaporin 3 [57]</td>
<td></td>
</tr>
<tr>
<td>Cytokeratins</td>
<td></td>
</tr>
<tr>
<td>Cytokeratins 7, 8, 18, 19</td>
<td></td>
</tr>
</tbody>
</table>

Despite success in their isolation, prolonged maintenance of the type II phenotype is still to be achieved; type II cells lose their characteristics within days of being cultured. The synthesis of surfactant protein by primary type II pneumocytes decreases with time [58]. Expression of surfactant protein mRNA decreases within several hours of culture [59]. In addition, the number of lamellar bodies in cultured primary type II cell pneumocytes decreases with time [56]. Evidence indicates that type II cells turn into type I cells in vitro with the increased expression of the type I pneumocyte marker aquaporin-5 [60]. Rice et al. reported the longest period, 7 days, of functional type II pneumocyte culture to date [10]. They used BEGM (bronchial epithelial cell basal medium) with the addition of carbon-stripped FBS (fetal bovine serum) and human KGF (keratinocyte growth factor) as the culture medium for the type II cells grown on 30:70 rat-tail collagen-Matrigel mixture-coated wells. These culture conditions may provide information for stem cell differentiation into type II cells.

In view of the difficulty in culturing pneumocytes, several cell lines, including A549 [61], L2 [62], MLE-12 [63] and MLE-15 [63] cells, have been developed for in vitro study. The intrinsic weaknesses of these cell lines include the deficiency of certain cell markers; carcinoma-origin and virus-transformation mean that these cells do not correspond fully to the primary cell phenotype.
1.2.3. Lung development

Furthering the understanding of the mechanisms of lung development is not an objective of this study. However, factors that are involved in lung development may also have effects on the in vitro differentiation of pulmonary progenitors from ESCs, and so the development of human lung is briefly reviewed in this section.

Human lung maturation can be divided into several phases [64, 65]. In the embryonic stage, the foregut endoderm develops into a lung bud, which then invades into the splanchnic mesoderm and starts to branch, generating five saccules. Two saccules lie on the left side and three on the right. The five saccules correspond to the future lung lobes. During the pseudoglandular stage, the hierarchical patterns of the blood vessels and preacinar airways develop. The whole bronchial tree is formed, and the airway epithelial cells are presented in the airway earlier than 10 weeks after fertilization. All major elements of the lung have formed at this stage except for the gas exchange unit. Thus, respiration is impossible for those fetuses born during this period. The most distinct characteristic of the canalicular stage is the advent of the terminal saccules (primordial alveoli), the development of vascularization and differentiation of pulmonary epithelium. The terminal bronchioles divide into two or more respiratory bronchioles, which further give rise to three to six alveolar ducts by 24 weeks of gestation. Various transcription factors are known to play important roles in differentiation at this stage; Foxa-2, Gata-6 and TTF-1 are expressed by the alveolar and airway epithelial cells. (Table 1-2). During the final saccular stage, the alveolar epithelium becomes thinner and, thus, the air space becomes larger. Capillaries bulge into the developing lung, forming the so-called blood-air barrier [66]. The blood-air barrier, where gas exchange happens, is the intimate contact between the vascular endothelium and alveolar epithelium. Type I alveolar cells line most of the surfaces of the terminal saccules surface by 26 weeks, whereas type II cells are scattered among the squamous type I cells [67]. Secretion of surfactant protein by type II cells and the presence of lamellar bodies can be identified at this stage. The surfactant counteracts the surface tension forces in the terminal saccules during this stage, thus with intensive care, premature infants may survive if born 24-26 weeks after fertilization. During the alveolar stage, type I pneumocytes attenuate into very
thin cells, so that the surrounding capillaries bulge into the alveolus. The alveolocapillary membrane becomes thin enough to facilitate gas exchange. The increase in number and complexity of alveoli continues until the age of 8.

Table 1-2 Characteristics and molecular control of lung development, adapted and modified from [68]

<table>
<thead>
<tr>
<th>Stage</th>
<th>Duration</th>
<th>Characteristics</th>
<th>Molecular mediators</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryonic</td>
<td>0-5 weeks</td>
<td>Growth of trachea, bronchi of both sides and major airways</td>
<td>HNF-3β, TTF-1, RA ,RAR, Shh, Ptc, Gli2, Gli3, FGF-8, FGF-10, HNF-4, N-cadherin, activin-β, activin R IIA, lefty-1/2, nodal, Ptkx-2</td>
</tr>
<tr>
<td>Pseudoglandular</td>
<td>6-16 weeks</td>
<td>Formation of bronchial tree</td>
<td>GATA-6, N-myc, PDGF, PDGF-R, EGF, EGF-R, FGF, TGF-β, Shh, Ptc, VEGF, BMP-4, RA, RAR</td>
</tr>
<tr>
<td>Canaliclar</td>
<td>26 weeks to birth</td>
<td>Epithelial differentiation, air-blood barrier formation, pulmonary acinus formation, increase of capillary bed</td>
<td>GATA-6, TTF-1, HNF-3β, Mash-1, VEGF</td>
</tr>
<tr>
<td>Saccular</td>
<td>26 weeks to birth</td>
<td>Formation of transitory air space</td>
<td>HNF-3β, TTF-1, NF1, VEGF, VEGF-R</td>
</tr>
<tr>
<td>Alveolar</td>
<td>32 weeks to 8 years</td>
<td>Alveolarization and formation of the secondary septum</td>
<td>PDGF, PDGF-R, FGF, FGF-R, VEGF, VEGF-R, angiopoietins, ephrins, RA, RAR</td>
</tr>
<tr>
<td>Microvascular maturation</td>
<td>Birth to 3 years</td>
<td>Thinning of interalveolar walls, capillary bilayer fuse to single layer</td>
<td>VEGF, VEGF-R, PDGF, PDGF-R, angiopoietins, ephrins</td>
</tr>
</tbody>
</table>
1.2.4. Role of fibroblast growth factors (FGFs) in lung development

Fibroblast growth factors (FGFs) play important roles in the development of lung. Among all FGFs expressed, FGF-1 (also called acidic FGF), FGF-2 (also called basic FGF), FGF-7 (also called keratinocyte growth factor) and FGF-10 are the most studied ones [69-91]. In the early development of the embryo, cells in the foregut endoderm require signals from the cardiac mesoderm to commit the differentiation into liver or lung [92]. This intermesenchymal interaction was found to be necessary because the isolated foregut endoderm alone can not enter the pulmonary lineage in the absence of cardiac mesoderm. Interestingly, when FGF-1 and FGF-2 were added to isolated foregut endoderm, the explants could activate either liver- or lung-specific genes, depending on the concentrations of added FGF-1 and 2 [85].

An important mechanism, called epithelial-mesenchymal interaction, governs embryonic lung development. FGFs are released by the lung mesenchyme and selectively bind to the FGF receptors with different levels of affinity. In the initial stages of lung bud formation and bronchial tree formation, FGF-10 is absolutely necessary; FGF-10 knockout mice fail to develop a lung [83]. The FGF-10 receptor, FGFR2IIIb, can be found throughout the embryonic respiratory epithelium [93] and FGFR2IIIb knock-out mice also fail to develop lung. In vitro culture of pulmonary epithelium explants demonstrated that FGF-1 can stimulate the proliferation of pulmonary epithelium and result in lung bud formation, while FGF-7 promotes the proliferation of pulmonary epithelium and results in the formation of cyst-like structures [69]. FGF-7 also can enhance the secretion of surfactant protein C (SPC) by primary type II pneumocyte cultured in vitro [74, 75].

1.2.5. Extracellular matrix and integrins: an introduction

Extracellular matrix (ECM) is a component of the basement membrane and interstitial connective tissue. The basement membrane of the lung consists of type IV collagen, laminin, entactin, heparin sulfate proteoglycans, fibronectin, nidogen and other proteoglycans. Interstitial connective tissue is composed of fibrillar collagen, elastic fibres and proteoglycans. It has been established that ECM can provide structural
support, affect cell shape and function, alter gene expression, stimulate cell proliferation and induce cell polarity [94]. Cell-ECM interactions are mainly mediated by heterodimeric transmembrane glycoproteins called integrins.

Collagen is the most abundant protein in the body. It provides the tensile strength of tissues. Collagen is mainly made up of left-handed triple-helix chains which consist of Glycine-X-Y. X and Y could be any amino acids, but are most frequently proline and hydroxyproline, respectively. Other amino acids presented in X and Y positions do not follow any repeating pattern. Among all the amino acids in collagen, lysines which participate in the covalent cross-linking between molecules are the site for sugar attachment. Collagens can be divided into fibrillar and non-fibrillar ones. Fibrillar collagens, type I, II, III, V and type XI, comprise most of connective tissues. Type I collagen is the prototype of fibrillar collagen, forming most of the skin, bone and tendon. In contrast to fibrillar collagens, non-fibrillar collagens can be categorised into basement membrane collagens, short-chain collagen and others. Type IV collagen is the major collagen in the basement membrane. Type IV collagen therefore a major component of Matrigel, a commercially-available ECM replacement extracted from Engelbreth-Holm-Swarm (EHS) sarcoma [95].

Laminin is a heterotrimer composed of three polypeptide chains, namely α chain, β chain and γ chain (Figure 1-1), linked together by disulfide bonds [96]. Currently, there five variations of α chains (α1 - α5), three variations of β chains (β1 - β3) and three variations of γ chains (γ1 - γ3) have been identified in either mouse or human [1, 97]. The first laminin identified was from an EHS tumour, laminin 1, which has α1, β1 and γ1 chains [98]. The molecular structure of laminin comprises 4 arms: three short and one long arm. N-terminal regions of α1, β1 and γ1 chains contribute to the short arm, whereas the long arm is formed by three chains in a triple-stranded manner. In the presence of $Ca^{2+}$, laminin self-assembles into oligomers or a large polymer network in vivo. This self-assembly property is thought to be the basis of the structure of the basement membrane. One of the most important functions of laminin is to mediate cell adhesion by interacting with cells on one hand and the ECM on the other. Laminin molecules contain a domain which is similar to that of epidermal growth factor (EGF) molecules. This domain is reported to possess mitogenic activity [99].
Fibronectin (FN) is a heterodimeric glycoprotein with a molecular weight of 500 kDa [100] (Figure 1-2). The two FN monomers are linked by two disulfide bonds at the C-terminus (Figure 1-2). Two major forms of FN are present in vertebrates, including soluble plasma FN and insoluble cellular FN. Plasma FN, synthesised mainly in the liver, is a major component of blood plasma. Cellular FN, secreted by various cell types, is a major component of the ECM. FN is encoded by only one gene, however, alternative splicing of the FN precursor mRNA gives rise to more than 20 forms of FN. Several functional domains can be found along the two FN monomers. The self-association domain is located near the end of the N-terminus of the FN monomer. It is necessary for the initiation of the FN matrix assembly. Another important domain, the cell binding domain, contains the RGD sequence and the synergy sequence. Both the RGD sequence and the synergy sequence form part of the cell binding sites [101].
Cells bind to ECM mainly via integrins. Integrins are cell surface receptors containing two distinct subunits, namely α and β subunits. The different combinations of α and β subunits result in a number of distinct integrins. At the present time, 25 different integrins are identified in mammals. Integrins for collagen binding include integrin α1β1, α2β1 and α3β1. Integrin α6β1 binds to laminin-1, whereas α2β1, α3β1, α6β1 and α6β4 integrins bind to laminin-5 [102]. For the integrin binding to FN, integrin α5β1 and α3β1 bind to RGD sites of FN whereas α4β1 integrin binds to IIICS site of the molecules [100].

1.2.6. Role of extracellular matrix in lung epithelium and lung development
The maturation and development of the lung involves several forces, including transcriptional factors, growth factors, cell-cell as well as cell-matrix interactions. The cell-matrix interaction affects the airway branching, epithelium differentiation, vascularization and other processes during lung development. The most distinct phenomenon of the ECM in alveolar differentiation is the discontinuity of the basement membrane (BM) which coincides with the differentiation of type II pneumocytes [103]. The discontinuity of the BM allows the extension of cytoplasmic processes from differentiating type II cells into interstitial spaces where they associate.
closely with fibroblasts [104]. This physical arrangement reaches a peak after birth and decreases in adult age. As no discontinuity is reported beneath type I cells, the phenomenon is considered as an inseparable process of type II cell differentiation and proliferation. Type IV collagenase expression is also reported to be higher in fibroblasts and alveolar epithelium during discontinuity formation [105].

During lung development, laminin is involved in mesenchymal-epithelial interaction as well as cell adhesion and cell polarization [106]. It is crucial to the development of many solid and glandular organs, such as lung, liver and kidney. During lung development, laminin mRNA levels increase as organogenesis progresses. Three chains of laminin-1 are strongly expressed during lung development, even in the basement membrane of early organogenesis [107]. When the antibody AL-1 was used to block the crossing region of the \( \alpha \) chain of laminin, lung epithelial cells were found to be unable to attach to mesenchymal cell populations, and the proliferation of lung epithelial cells was inhibited [106].

Fibronectin (FN) is synthesised by many embryonic tissues and several adult tissues [108]. Fibronectin-deficient mice die even before the embryonic stage of lung development [109]. As integrin \( \alpha 5 \beta 1 \) can bind to FN, mice that are deficient in the \( \alpha 5 \) integrin subunit also die at an early embryonic stage [110]. Increased expression of FN between 9-18 days of the pseudoglandular stage is followed by subsequent decline at the beginning of alveolar stage in chick lung development [111]. It is also worth noting that FN expression in mesenchyme always increases prior to the appearance of differentiated type II epithelial cells [112]. A similar finding was made by immunostaining FN and integrin \( \alpha 5 \beta 1 \) in developing murine lung. Fibronectin expression was detected in the early pseudoglandular stage in mesenchyme and parabronchial cells, it then increased until the early canalicular stage [113]. To sum up the findings, it seems that an increase in FN expression coincides with branching morphogenesis, thus FN may be related more to branching than alveolarization. However, there is also a parallel between vascular endothelial marker expression and FN expression, suggesting a role of FN in vascularization. The possible role of FN in the differentiation of type II cells has been less addressed. \textit{In vitro}, type II cells, cultured on FN-coated tissue culture plates exhibit better organized, polarized and tightened morphology [114]. An increase in FN expression after birth implies its role
in lung maturation [115].

1.2.7. Extracellular matrix in in vitro lung biology

As mentioned previously (1.2.2), it is well known that type II pneumocytes lose their phenotype soon after being placed in culture. The effects of the ECM on the morphology and physiological function of type II pneumocytes are summarized in Table 1.3. The morphology of type II pneumocytes can be preserved by culturing them on Matrigel-coated tissue culture plastic [116], floating collagen membrane [117] and inactivated fibroblast feeder layers [70].

Matrigel is a solubilized basement membrane extract prepared from Engelbreth-Holm-Swarm (EHS) mouse sarcoma which is rich in extracellular matrix. Matrigel consists of collagen IV, laminin [118], heparin sulfate proteoglycans, entactin as well as nidogen [95, 119]. The original Matrigel also contains some naturally-occurring growth factors, including 0-0.1 pg/ml bFGF, 0.5-1.3 ng/ml EGF, 15.6 ng/ml IGF-1, 12 pg/ml PDGF, <0.2 ng/ml NGF, 2.3 ng/ml TGF-beta and others. For experiments that require highly defined base membrane, growth factor-reduced Matrigel, which contains 0-0.1 pg/ml of bFGF, <0.5 ng/ml EGF, 5 ng/ml IGF-1, <5 pg/ml PDGF, <0.2 ng/ml NGF, 1.7 ng/ml TGF-beta, was developed in order to reduce the effect of the growth factors. Despite so, tract amounts of growth factors still exist in the commercially-available Matrigel.

Individual components of ECM, such as collagen [50] and laminin [120], can also help transient phenotype preservation. With different proportions of ECM, type II pneumocytes exhibit different morphologies. Type II pneumocytes cultured on a layer of protein composed of 90:10 of Matrigel to collagen with the supplement of keratinocyte growth factor (KGF) in the cell culture medium aggregate into colonies whereas they formed a monolayer of cells when cultured on a layer of protein composed of 70:30 of Matrigel to collagen ratio. Under serum-free conditions, primary type II cells form an organoid structure, which is characterised by a lumen and epithelium, within collagen gel [121] and spherical aggregates within Matrigel [116]. Laminin is also reported to contribute to the alveolar-like structure [122]. In terms of surfactant secretion, type II cells cultured on Matrigel-coated plastic and
feeder layers on floating collagen gels synthesized more surfactant phospholipids and proteins than cells grown on TCP alone [123]. The role of ECM in the expression of cell markers, DNA synthesis and cell proliferation is listed in Table 1.3. Among the cell markers, intercellular adhesion molecule-1 (ICAM-1) is expressed in type I rather than type II cells. Increased ICAM-1 expression represents transdifferentiation of type II to type I cells [124].
Table 1-3 Composition effects of ECM on morphology and metabolic activities of type II pneumocytes, adapted from [94]. ND, no significant difference; ↑, increase with time in culture; ↓, decrease with time in culture; + increase compared to tissue culture plastic; -, decrease compared with TCP

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>Morphology</th>
<th>Surfactant phospholipid expression</th>
<th>Surfactant protein expression</th>
<th>Cytokeratin expression</th>
<th>ICAM-1</th>
<th>DNA synthesis</th>
<th>Radiolabeled nuclei</th>
<th>Cell division</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCP</td>
<td>Attenuated</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
<td>5%</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Matrigel</td>
<td>Cuboidal</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>N/A</td>
<td>-</td>
<td>ND</td>
<td>N/A</td>
</tr>
<tr>
<td>Type I collagen</td>
<td>Cuboidal</td>
<td>ND</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>ND</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Type IV collagen</td>
<td>Attenuated</td>
<td>ND</td>
<td>N/A</td>
<td>N/A</td>
<td>+</td>
<td>N/A</td>
<td>ND or +</td>
<td>N/A</td>
</tr>
<tr>
<td>Type I with type IV Collagen</td>
<td>Attenuated</td>
<td>ND</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>ND</td>
<td>N/A</td>
</tr>
<tr>
<td>Laminin</td>
<td>Cuboidal</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>+</td>
<td>N/A</td>
<td>+ Serum free medium</td>
<td>N/A</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Attenuated</td>
<td>N/A</td>
<td>N/A</td>
<td>+</td>
<td>+</td>
<td>N/A</td>
<td>+ N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Type II cell matrix</td>
<td>Attenuated</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Floating collagen gel</td>
<td>Cuboidal</td>
<td>N/A</td>
<td>N/A</td>
<td>-</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Feeder+floating collagen gel</td>
<td>Cuboidal</td>
<td>+</td>
<td>+</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Feeder layers+attached collagen gel</td>
<td>Attenuated</td>
<td>ND</td>
<td>ND</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

1.3. Embryonic stem cells and their differentiation

The first derivation of murine (mESCs) and human embryonic stem cells (hESCs) was achieved in 1981 [125] and 1998 [12], respectively, by isolation of cells from the inner cell mass (ICM) of blastocysts. Both types of ESCs are characterized by their pluripotency, which is the capacity to differentiate into every cell types of endoderm, mesoderm and ectoderm. Recently, hESCs were found to be able to differentiate into cells of the trophoblast [126].

Both types of ESC showed high alkaline phosphatase activity, high telomerase
activity and high nucleus/cytoplasm ratio [127]. Furthermore, both mESCs and hESCs express characteristic surface markers and transcriptional factors. The commonly-used surface markers of mESCs include stage-specific embryonic antigens 1 (SSEA-1) and CD9, while the commonly-used surface markers of hESCs include SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, GCTM2 and CD9. Active transcriptional factors expressed by both mESCs and hESCs include POU domain transcriptional factor Oct 3/4 (POU5F1), homeodomain protein Nanog, homeobox domain transcriptional factor SOX-2 and zinc finger transcriptional factor REX-1.

To maintain the undifferentiated state of ESCs, most lines of mESCs require leukemia inhibitory factor (LIF) and many hESCs require the bFGF and the support of a MEF feeder layer. The MEF feeder layer secretes the factors that worked in conjugation with bFGF to support the undifferentiated state of hESCs. LIF can bind to the heterodimeric LIF receptor and subsequently activate the STAT3 transcription factor via a complicated pathway to control the gene of self-renewal [128]. For the maintenance of hESC pluripotency, feeders layers other than MEF feeder layers, including human skin fibroblasts [129] and immortalized hESCs-derived fibroblasts [130] have been reported. In other studies, KGF, noggin [131] and activin A [132] were also applied to maintain the undifferentiated state of hESCs in a feeder-free culture environment.

Once implanted into Severe Combined Immunodeficiency (SCID) mice, both mESCs hESCs form teratomas which comprise cells from the three germ layers [125, 133]. In vitro, a similar phenomenon can also be observed; upon removal of LIF (for mESCs) or the feeder layer (for hESCs), ESCs start to differentiate into 3D structures known as embryoid bodies (EBs) that are spheroid colonies of ESCs usually grown in suspension culture on bacterial-grade Petri dishes. Markers for endoderm, mesoderm and ectoderm are localised to specific regions of the EBs [134, 135]. This observation led to the hypothesis that EBs may recapitulate the development of the embryo to a certain extent. This is why the relevance of lung development to the current study was highlighted in the previous section.

Most reported research on ESC differentiation starts with EB formation. In contrast, some studies indicate that ESCs can also differentiate into specific lineages on a layer
of ECM protein or on a feeder layer in a 2D manner, that is, without EB formation [136, 137]. Both lines of research involved ECM coating of the culture vessels during stem cell differentiation. Coraux et al. [137] reported the differentiation of mESCs into airway epithelium without the EB formation at an air-liquid interface. This air-liquid interface suggests the importance of the extracellular environment of both in vitro differentiation and natural development processes.

Strategies that have been employed to try to upregulate the differentiation of ESCs into a specific lineage include addition of growth factors to the culture medium [16], reprogramming of ESCs using cytoplasmic or nuclear extracts from the target cells [17], genetic manipulation of ESCs [138] and co-culture of ESCs with the target cells [18]. By adding various growth factors or interleukins to EBs, ESCs can be encouraged to differentiate into specific lineages. For example, retinoic acid induces neuronal formation [139]. Combining IL-3 and other factors stimulates ESCs to turn into macrophages, mast cells or neutrophils [140]. TGF-β guides the ESCs into forming muscle cells [141]. The addition of the growth factors does not exclusively direct ESCs differentiation into a single specific cell type, rather it changes the relative proportion of each cell type [142].

Whether or not the in vitro differentiation of ESCs to a particular lineage has to recapitulate the in vivo development process has yet to be determined but simulation of the natural developmental process and environment may give a good guideline. During embryonic development, embryonic ectoderm of the epiblast forms neuroectoderm and bi-potential mesendoderm, the latter then undergoes epithelial-mesenchymal transition, bringing about mesoderm and endoderm formation. Using soluble factors for stem cell differentiation is stage-specific. For an endodermal lineage, one can increase the relative endoderm population by adding certain factors to culture medium at an early stage of the differentiation of ESCs, then other factors can be added to the culture medium after the endoderm phase to maximise the differentiation of cells of that lineage, such as type II epithelial cells, in the later stage. Activin A is reported to increase mESC differentiation into the endodermal lineage when added to EB culture medium [143]. However, addition of activin A to monolayer cultures of differentiated ESCs dissociated from human EBs increases mesoderm differentiation [142]. Furthermore, adding activin A to hESCs is reported to
help maintain pluripotency of the undifferentiated hESCs in the absence of a feeder layer [132]. These results indicate the time-, species- and quantity-dependent effects of soluble factors.

1.4. Composition, preparation and modification of 2D environments and 3D scaffolds

1.4.1. Lactic acid-based polyesters

Lactic acid-based polymers have been used for medical applications for more than 2 decades [144]. Being one of the smallest optically active materials, lactic acid, also called 2-hydroxypropanoic acid, can be either a L(+) or D(-) stereoisomer. Both of the optically active configurations are produced by the Lactobacilli bacterial systems through the Embden-Meyerhof pathway which converts 1 mole of hexose into as much as 1.8 mole of lactic acid [145]. In contrast, mammalian systems can only produce L-lactic acid. Catalysed by lactic acid dehydrogenase, lactic acid can be synthesised from pyruvate under oxygen-limiting conditions using lactic acid fermentation [145], then the synthesised lactic acid can be neutralised with base followed by filtration, concentration and acidification to obtain pure lactic acid for polymerization [146].

In spite of a large number of publications on lactic acid-based polymers, there are lots of contradictions in their nomenclature. It is known that polymers derived from lactic acid can be prepared by polycondensation or ring-opening polymerization (ROP). In order to differentiate the way they are synthesised, polymers prepared by the former method are generally referred to as poly(lactic acid) whereas the ones prepared by the latter are called poly(lactide) [146]. As the material used in this project is synthesised by ROP, it is proper to refer to the material as poly(D,L-lactide) instead of poly(D,L-lactic acid), which is used extensively in the literature [147, 148]. Furthermore, poly(D,L-lactide) is also generally referred to as PDLLA.

Polycondensation usually generates polymers with a lower molecular weight. An additional chain coupling agent, such as hexamethylene diisocyanate, is usually required to overcome this effect [149]. The low molecular weight of the condensation polymer is due to the viscous polymer melt, the statistical absence of the reactive end
group and the formation of the six-member lactide ring during reaction [150]. Recently, the preparation of poly(lactic acid) by condensation polymerization with a molecular weight of $10^5$ Da was reported [151]. In contrast, PDLLA prepared by ROP has a higher molecular weight ($M_w > 10^5$ Da). The route of ROP includes condensation polymerization of lactic acid into low molecular weight prepolymer followed by the depolymerization of the low molecular weight prepolymer into a dehydrated cyclic dimer (3,6-dimethyl-1,4-dioxane-2,5-dione) (Figure 1-3). The depolymerization is processed by elevating the polycondensation temperature and lowering pressure followed by distilling off the depolymerized cyclic dimer [146]. The cyclic dimer then undergoes ROP to yield a high molar mass polymer. The stereoregularity of the polylactide is therefore dependent on the proportion of lactide of different stereoforms prepared. Among all the metal catalysts commonly used in ROP, tin(II) 2-ethylhexanoante, which later converts into tin(II) alkoxide in reaction with alcohol in ROP, is the most frequently used catalyst despite concerns over its potential toxicity [152]. The use of supercritical $CO_2$ to remove the catalyst and residual solvent is therefore promising for the synthesis of polymer used in biomedical applications [153, 154].

**Figure 1-3 Stereoforms of lactide.** Adapted from [146]

Two configurationally distinct polymers are commonly used in tissue engineering applications, PLLA (poly(L-lactide)) and poly(D,L-lactide) (PDLLA). PLLA is semicrystalline, stereoregular, highly resistant to hydrolysis and possesses high mechanical strength. In contrast, PDLLA is amorphous, racemic and, therefore, more susceptible to hydrolysis. PDLLA undergoes degradation by hydrolysis in an aqueous environment. In an acidic environment, the introduction of water and protons causes the cleavage of the ester bonding. One may see this reaction as the reverse reaction of
the polycondensation. The acidic degradation product of the polylactide further increases the degradation rate by lowering the pH. In a basic environment, the polyester bond breaks because of the attack of the ester bond by hydroxyl ions.

Lactic acid-based polymers usually undergo bulk degradation instead of surface erosion. Bulk degradation occurs by random scission of molecular chains followed by the onset of weight loss and a decrease of chain scission rate [155, 156]. The weight loss becomes significant when the molar mass of the degraded polymer is considerably below the entanglement molar mass, which is around 5170 Da (for number average molecular weight) or 3330 Da (for weight average molecular weight) [157]. Similar results obtained from a dielectric spectroscopy study of PDLLA have also been reported [158]. However, the GPC elution profile of water soluble oligomers diluted from the PDLLA microspheres demonstrates a peak \( M_w \) ranging from 1050 to 1150 Da [159]. It may suggest that polymer chains smaller than the entanglement molar mass undergo further degradation before they can freely diffuse out of the polymer.

PLA features higher autocatalytic effects in the interior than that near the surface. This is because oligomeric degradation products, which possess carboxyl end groups that can catalyse further hydrolysis, can diffuse out of the sample easier from a volume near the surface than those at the centre, resulting in an acidity gradient across the sample from the centre to the surface. As the degradation progresses, the inner part turns into a highly viscous oligomer core followed by further degradation which renders the oligomers small enough to diffuse through to the outer layer of the sample. A hollow structure is therefore formed. However, this process is not applicable to polylactides in any dimension [148]. Lactic acid-based polymer samples smaller than 300 µm in diameter undergo homogeneous degradation, which means the surface material degrades as fast as the material at the core [160]. Furthermore, when the dimensions of the lactic acid-based polymer parts are larger than the so-called “critical thickness”, the degradation mode alters. Figure 1-4 demonstrates the critical thickness of most degradable degradable materials [161]. When the dimension of the polymers is above the critical thickness, polymers undergo surface erosion instead of bulk erosion. PDLLA, which belongs to the polyester group, has a critical thickness of
around 7.4 cm.

The auto-catalytic effect of PDLLA degradation can be lowered by the introduction of basic particles, such as hydroxyapatite and Bioglass®, into the polymer to neutralize the released lactic acid [162]. The porosity of a porous polymer also plays a role in this degradation behaviour. 75:25 poly(DL-lactide-co-glycolide) porous microspheres incorporated with bone morphological protein degraded in three weeks in vivo whereas the non-porous counterpart took more than 20 weeks to degrade [163].

1.4.2. Surface modification of lactic acid-based polyesters

The hydrophobicity of lactic acid-based polyesters, such as PDLLA and PLLA, may inhibit cell responses, e.g. cell attachment and cell proliferation. The easiest way to enhance the PDLLA surface wettability is to apply a protein coating to the polymer surface. This coating process is simply the adsorption and the unfolding of proteins to the materials; no chemical reaction occurs in the coating process. Modifications of polyesters that involve the breakdown of covalent bonds can be classified as either physical or chemical modifications. The former include plasma treatments [41, 164, 165], electron beam [166] and UV irradiation [167]. The latter include hydrolysis [33], aminolysis [33-35, 40, 168] and surface grafting of bioactive molecules [31, 165, 169].

Plasma treatments are the most frequently-used physical surface treatments using oxygen, nitrogen and ammonia plasmas, which have been found to be effective for
lactic acid-based polymer films. Traditional plasma treatments, however, are unable to penetrate into the porous structures of scaffolds for more than a few millimetres [33]. Chemical modification of polymer scaffolds, such as hydrolysis and aminolysis, can be cheap and easier than surface modification of scaffolds using plasma treatment. Hydrolysis introduces carboxylic acid and hydroxyl groups to the surface of polyesters, while aminolysis introduces amines. Hydrolysis can occur under either acidic or basic conditions. Under acidic conditions, hydrolysis occurs when hydrogen ions attack the carbonyl oxygen due to electrophilic attack. Under basic conditions, hydrolysis occurs when hydroxide ions attack the carbonyl carbon due to the nucleophilic attack. Surface modification using hydrolysis is strongly dependent on the reaction pH and temperature, furthermore, it is difficult to restrict the hydrolysis to the polymer surface alone [33, 170]. In contrast, aminolysis has been reported to be more controllable [33]. Aminolysis is commonly used in the textile industry to increase the wettability of synthetic fibres. Its application in biomaterial modification has only recently been investigated [33-35, 40, 168]. The basic mechanism of aminolysis is the nucleophilic attack of the carbonyl carbon in the polymer backbone by amines, generating a tetrahedral intermediate, which then further deprotonates to generate amide bonds in basic aqueous solution or in a polar solvent with high \( \pi \) basicity, such as isopropanol. In this thesis, aminolysis was used to introduce amines to the PDLLA surfaces used as substrates for cell culture and for further surface modification of these pre-treated PDLLA surfaces by grafting bioactive peptides and molecules that possessed multiple functional groups.

A variety of studies has reported the coupling of peptides [25, 26, 28], proteins [35], hydrophilic molecules [168], or molecules possessing multiple functional groups [171] to the biomaterial surfaces using linker molecules. The choice of these linkers is critical to the final product of the grafting reaction. Zero-length linkers, such as 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), N,N'-Dicyclohexylcarbodiimide (DCC) and N,N'-diisopropylcarbodiimide (DIC), mediate the formation of covalent bonds between the surface and the molecule to be coupled without adding spacers or linkers in between. Other linkers mediate the grafting reaction by adding a spacer of several atoms in between the surface and the grafted species.
1.4.3. Extracellular matrix coating on biomaterials

Extracellular matrix (ECM) proteins have been used as biomaterial coatings for decades [172-174]. Cells cultured on protein-coated biomaterials experience more cell-matrix interaction than on TCP. To maintain the bioactivity of the protein coating on the biomaterial surface, it must be appropriately conformed during the coating process.

In water solution, the interactions between the side chains of a protein cause the folding of protein molecules, with hydrophobic residuals preferentially located in the hydrophobic core of the protein molecules [175]. Once a protein is exposed to a polymer surface, adsorption to the material surface occurs [175-177]. This process is driven by electrostatic forces, hydrogen bonds and van der Waals forces between the polymer surface and the protein in solution. When the protein adsorbs, the protein and the material surface should be at least partially dehydrated. Thus, the water molecules associated with the protein are released when the proteins unfolds, consequently, the thermodynamics favours the hydrophobic materials as a better adherent than materials of a more hydrophilic nature. The entropy of the whole system increases when proteins unfold and adsorb to the polymer surfaces.

Fibronectin (FN) is a good model to elucidate the changes in surface properties of a biomaterial after coating. Adsorption of FN on biomaterials can modulate cell behaviour. At low FN concentration, the bioactivity of the FN is substrate-dependent. This is because when FN is at low concentration, the conformation of the unfolded FN on the substrate determines the exposure of bioactive sites of the FN. Once the FN concentration increases, the bioactivity of FN exhibits no differences between substrates [178]. FN exhibits an extended and condensed morphology when coated onto hydrophilic and hydrophobic materials, respectively [179]. The active binding site of FN for integrin measured by immunostaining varies as FN is coated on different substrates [23].
1.4.4. Scaffolds prepared by Thermally-Induced Phase Separation (TIPS)

A tissue engineering scaffold should satisfy the following requirements [5]: The scaffolds should (1) exhibit interconnected porosity with an appropriate porous structure allowing cell penetration and mass transport, (2) have a biodegradation rate that matches the formation rate of the host tissue, (3) possess optimal surface properties to enhance cellular attachment, differentiation and/or proliferation, (4) possess adequate mechanical properties to bear physiological movement and handling, (5) induce no adverse response of the host tissue and (6) be and easy and reproducible to manufacture.

Techniques for scaffold fabrication have been reviewed extensively [5, 7, 20, 21, 180, 181]. Among all the techniques, Thermal-Induced Phase Separation (TIPS), was one of the earliest techniques used to prepare porous polymer membranes [20, 181]. It has since been applied to make porous polymer scaffolds [20, 181] and for polymer/Bioglass® composite scaffolds [182].

The TIPS process can be categorized into solid-liquid phase separation or liquid-liquid phase separation. In a solid-liquid phase separation system, the liquid-liquid phase separation temperature of the polymer solution is lower than the crystallization temperature (freezing point) of the solvent [181]. Therefore, when the polymer solution is quenched or cooled down, the solvent starts to crystallize, causing the polymer to be repelled from the crystallization front of the solvent. The separation of the dissolved polymer from the crystalline solvent therefore is named solid-liquid phase separation. The porous structure can be preserved by lyophilising the frozen-polymer solution to remove the solvent. The solid-liquid phase separation technique typically produces highly porous, sheet-like, anisotropic pore structures, with better mechanical properties along the pore orientation than along the transverse direction [181]. Scaffolds prepared by solid-liquid phase separation can be used for the engineering of tissues with orientated tubular or fibrous bundle architectures, such as nerve, bone, muscle, tendon, ligament and dentin.
Unlike solid-liquid phase separation, in a liquid-liquid phase separation system, the temperature of the polymer solution is much higher than the crystallization temperature (freezing point) of the solvent [20]. The bottom half of the Figure 1-5 illustrates the typical temperature to composition phase diagram of an ideal polymer solution. The dashed, spinodal curve divides the two phase regions; the area below the binodal region is split into a thermodynamically unstable spinodal decomposition region and the metastable nucleation region. The area above the binodal curve is the homogeneous solution area. Phase separation happens as the polymer solution is cooled down from the homogeneous region to the area below binodal decomposition temperature.

Figure 1-5 can also be derived mathematically [183, 184]. Firstly, consider a polymer solution “A+B → Mixture of A and B”. Complete miscibility occurs only when the Gibbs free energy of mixing is lower than the Gibbs free energy of the components A and B.

It is well explained in the literature that the entropy of mixing $\Delta S_{\text{mix}}$ can be derived as [184]:

$$\Delta S_{\text{mix}} = -kN_1 \ln X_1 - kN_2 \ln X_2$$

where $k$ is the Boltzmann constant. $N_1$ and $N_2$ represent the numbers of the molecules of A and B, and $X_1$ and $X_2$ the mole fraction of A and B, respectively. Therefore:

$$X_1 = \frac{N_1}{N_1 + N_2}$$  \hspace{1cm} (1-2)

and

$$X_2 = \frac{N_2}{N_1 + N_2}$$  \hspace{1cm} (1-3)

Upon mixing, there are interactions between the two components A and B. Therefore, consider the enthalpy change on mixing $\Delta H_{\text{mix}}$ [184]:

$$\Delta H_{\text{mix}} = rN_1 X_2 = r(N_1 + N_2)X_1X_2$$

where $r$ is defined as the energy change per molecule in the mixing process and the Gibbs free energy of mixing $\Delta G_{\text{mix}}$ is:

$$\Delta G_{\text{mix}} = -T\Delta S_{\text{mix}} + \Delta H_{\text{mix}}$$

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where \( T \) is the temperature. By combining equation (1-1), (1-4) and (1-5) together, the following equation is obtained:

\[
\Delta G_{\text{mix}} = kT N_1 \ln X_1 + kT N_2 \ln X_2 + r(N_1 + N_2)X_1X_2
\]  

(1-6)

Consider \( X_1+X_2=1 \), equation (1-2) and (1-3), \( \Delta G_{\text{mix}} \) can be rewritten as:

\[
\Delta \bar{G}_{\text{mix}} = kT(X_1 \ln X_1 + (1-X_1) \ln(1-X_1)) + rX_1(1-X_1)
\]  

(1-7)

where \( \Delta \bar{G}_{\text{mix}} = \frac{\Delta G_{\text{mix}}}{N_1 + N_2} \), is the Gibbs free energy of mixing per molecule.

Therefore, the second derivative of the Gibbs free energy of mixing per molecule is:

\[
\frac{\partial^2 \Delta \bar{G}_{\text{mix}}}{\partial X_1^2} = kT \left( \frac{1}{X_1} + \frac{1}{1-X_1} \right) - 2r
\]  

(1-8)

Figure 1-5 Gibbs free energy of mixing \( \Delta \bar{G}_{\text{mix}} \) as the function of \( X_1 \). Adapted from [184].
The top half of Figure 1-5 demonstrates the Gibbs free energy of mixing per molecule $\Delta G_{\text{mix}}$ as the function of the polymer composition $X_1$. At temperature $T_5$ the polymer system is miscible regardless of the solution composition $X_1$. Between temperature $T_C$ and the crystallization temperature of the solvent, the polymer system is partially miscible, and tangent lines that have two contact points with $\Delta G_{\text{mix}}$ can be found. The binodal curve in the bottom half of Figure 1-5 is the projection of the contact points between the $\Delta G_{\text{mix}}$ curve and their tangents to the temperature-composition plane. Similarly, the spinodal curve is the projection of the inflexion points of the

$$\frac{\partial^2 \Delta G_{\text{mix}}}{\partial X_1^2} = 0$$

to the temperature-composition plane. At a certain temperature, say $T_1$, the $\Delta G_{\text{mix}}$ is higher at composition $C_2$ than at $C_1$ or $C_3$ where two co-existing phases exist. As a result, phase separation occurs at composition $C_2$. A special temperature $T_C$, also called the critical solution temperature, is the temperature where

$$\frac{\partial \Delta G_{\text{mix}}}{\partial X_1} = \frac{\partial^2 \Delta G_{\text{mix}}}{\partial X_1^2} = \frac{\partial^3 \Delta G_{\text{mix}}}{\partial X_1^3} = 0.$$  Therefore, a homogeneous phase exists at $T_C$.

When the temperature of an ideal polymer solution is lowered below the binodal curve of the temperature-composition phase diagram, the polymer solution phase separates into polymer-rich and polymer-poor regions. In both metastable regions, phases separate by the nucleation and growth mechanism [185]. In the left metastable region, the polymer solution separates into droplet-like polymer-rich regions dispersed in polymer-poor matrix. Once the solvent is lyophilized, the final polymer product has a beaded structure (Figure 1-6A). In the right metastable region, the final polymer product possesses a closed-pore structure (Figure 1-6C).

In contrast, in the unstable region, phases separate by the spinodal decomposition. If the status of a polymer solution is brought to the unstable region, foams with well-interconnected open-pores can form when the solvent is lyophilized (Figure 1-6B).
The pore size of scaffolds has a huge effect on the behaviour of cells seeded on them [187]. For different cell types, the optimal pore size for cell entry, attachment and matrix deposition varies. For the preparation of a porous scaffold using TIPS, the pore size and the interconnectivity are influenced by many factors, including polymer type, polymer solution concentration, solvent type, cooling rate of the polymer solution and coarsening time and temperature [20, 181, 188]. Coarsening, also referred to as aging, is a time-dependent phase separation process. When the temperature of a polymer solution is brought down to the temperature below the binodal curve, phase separation of the polymer solution occurs. Over time, the tendency to reduce the interfacial energy between the already-separated two phases results in the increased size of the phase-separated droplets and decreased total interfacial area [189]. Therefore, the pore size of the future scaffolds is affected by the coarsening time and coarsening temperature [185, 189, 190]. Moreover, the rate that a polymer solution is cooled down also affects the pore size of the future scaffold. A slow cooling rate of the polymer solution generates scaffolds with larger pore sizes and a more uniform pore structure than a fast cooling rate [188]. This is because during the cooling process, phase separation still progresses. A slow cooling rate, therefore, allows for a longer period of phase separation than a fast cooling rate. Practically, a slow cooling rate can
be achieved using a freezer and a fast cooling rate using liquid nitrogen.

1.4.5. Cell seeding and infiltration of porous scaffolds
Cell penetration into scaffolds is dependent on mass transport, such as oxygen diffusion, nutrition diffusion into and waste removal from the scaffold interior. For those scaffolds with small pore sizes, the ingrowth of cells is limited by the oxygen concentration. Cell penetration from the surface into the bulk of the scaffold has been reported to be limited to within 180 μm for cardiac-like tissues [191], 500 μm for osteoblasts [192] and 5 mm for chondrocytes [193]. These results suggest that cells which bear higher physiological activities might have lower scaffold penetration ability due to the higher requirement of mass transport. In vivo, capillaries which are around 6-8 μm in diameter, cause slow passage of blood through the vessels. The resident time of blood within the vessels is longer than the diffusion time of O₂, CO₂ and nutrients to the surrounding tissues, thus ensuring adequate mass exchange. In addition, all cells except chondrocytes can not survive more than 100 μm away from a blood supply in vivo [194]. This finding is also supported by the avascular structure of cartilage. As far as lung type II epithelial cells are concerned, their penetration and growth on 3D scaffolds were reported in only very few studies [22, 195-199]. Consequently, this is one of the focuses in the present research project.

1.5. Surface characterization of modified biodegradable polymer scaffolds
Depending on the information required, a variety of techniques can be used to characterise the surface of biomaterials [200]. In this section, background information on some characterization techniques used in this thesis is briefly reviewed.

1.5.1. Wettability of surfaces: contact angle measurements
When a drop of liquid is placed on a surface which has a lower surface energy than the liquid, a clear angle \( \theta \) will form at the three-phase contact point, as Figure 1-7 shows. Young’s equation describes this three phase interfacial equilibrium using the vectorial sum:

\[ \gamma_{LG} \cos \theta = \gamma_{SG} - \gamma_{SL} \]  (1-9)
where $\gamma_{ij}$ is the interfacial energy between the phase $i$ and $j$. Subscript L, G and S refer to the liquid phase, gas in equilibrium with the saturated liquid vapour, and solid phase, respectively. Young’s equation is valid only if the following prerequisites can be satisfied: the surface is smooth, rigid and homogeneous, and there is no interaction between liquid and solid phase.

![Graphical representation of Young's equation](image)

In contrast to the static contact angle or Young’s contact angle described by equation 1-9, commonly two dynamic contact angles can be measured, which are the contact angles when gas-liquid front is advancing, i.e. the liquid wets a dry surface, or receding, i.e. the liquid dewets a wet surface. Practically, adding liquid to an existing water droplet will increase the contact angle of the droplet. The gas-liquid front only moves towards the gas phase when enough water is added. The advancing contact angle $\theta_a$ stands for the largest observed angle during this gas-liquid front movement [200]. Subtracting liquid from the existing water droplet decreases the height and the contact angle of the droplet. The gas-liquid front only withdraws over a pre-wetted surface when enough water is subtracted. The receding contact angle $\theta_r$ stands for the smallest observed angle during this gas-liquid front movement [200]. The movement of the liquid-air front can be controlled by increasing or decreasing the liquid drop (or air bubble) using a microsyringe connected to a syringe pump. The shape of the liquid drop can be recorded using a camera, and the contact angle $\theta$ can be measured by drop shape analysis software. As the dynamic contact angle is governed by the interfacial driving force and the viscous retarding force, it is rate dependent.

Contact angle hysteresis is defined as the difference between $\theta_a$ and $\theta_r$. Factors that determine contact angle hysteresis include the surface roughness, chemical surface heterogeneity and surface contaminations. In a surface composed of hydrophilic and hydrophobic domains, when the water front advances, the hydrophobic domain can
pin the movement of the water front thereby increasing the contact angle. Similarly, when the water front recedes, the hydrophilic domain can hold the water phase of the contact line, decreasing the contact angle.

The dynamic contact angle can be measured in several ways, such as using the Wilhelmy plate technique using a tensiometer or using the sessile or captive bubble method using a goniometer [200]. The latter approach was used throughout the current research.

1.5.2. Information about the surface chemistry obtained from ζ-potential measurements

When an aqueous electrolyte solution is in contact with a solid surface that possesses surface charges, ions in the electrolyte accumulate at the solid-liquid interface. According to the widely accepted GCSG model on the basis of the studies of Gouy, Chapman, Stern and Grahame [201], the ions that accumulate at the solid-liquid interface can be defined as belonging to two layers, namely the diffuse layer and the immobile layer. The immobile layer, also known as the Stern layer, is made up of the inner (IHP) and the outer (OHP) Helmholtz layer or plane. The IHP comprises a monolayer of immobile, partially dehydrated ions adsorbed on the solid surface. The charge of the adsorbed ions depends on the nature of the solid surface. The OHP, in contrast, is composed of immobile, hydrated ions that are opposite in charge to the ions adsorbed in the IHP. The number of charged ions in the diffuse layer decreases when the distance from the solid-liquid interface increases. When the electrolyte solution moves under an applied external force, movement of the ions in the diffuse layer generates a shear plane close to the boundary between the Stern layer and the diffuse layer. The potential at this shear plane is called the ζ potential.
Figure 1-8 Schematic representation of the electrochemical double layer model according to the Gouy, Chapman, Stern and Grahame model (redrawn from [202]). The adsorbed ions in the inner Helmholtz plane are partially dehydrated.

Figure 1-9 Schematic representation of the measurement cell of the streaming potential

Figure 1-9 illustrates the measurement cell used to measure the $\zeta$-potential of flat film substrates using the streaming potential method. This method shears off the ions in the diffuse layer and, therefore, generates a potential when an electrolyte is driven through a channel by a pressure gradient. The polymer samples to be studied line both sides of the streaming channel. If the polymer surface is negatively charged, part of the positive counter-ions accumulates at the right electrode when the liquid is pumped through the streaming channel in the direction of the arrows, generating a potential.
(\Delta U) between the two electrodes. The $\zeta$-potential can be calculated using the Helmholtz-Smoluchowski equation:

$$\zeta = \frac{\eta \cdot \kappa \cdot U_s}{\varepsilon \varepsilon_0 \cdot \Delta p}$$

(1-10)

where $\eta$ is the viscosity of the solution, $\varepsilon$ the dielectricity of the electrolyte, $\varepsilon_0$ the permittivity of vacuum, $\kappa$ the total specific conductivity of the electrolyte solution in the channel in between the samples and $\Delta p$ the applied pressure difference across the channel. The $\zeta$-potential can be measured as a function of pH ($\zeta=f(pH)$). There are two important indicators of the $\zeta=f(pH)$ curve, the isoelectric point (IEP) and the plateau value of $\zeta=f(pH)$ curve ($\zeta_{\text{plateau}}$). IEP is the pH where the measured $\zeta$ potential is equal to zero. The IEP is mainly determined by the number (i.e. concentration) and $pK_a$ of the dissociating acidic and basic functional groups present at the surface. The plateau value of the $\zeta=f(pH)$ is determined by both the ions adsorbed to the solid-liquid interface as well as the number of dissociated surface functional groups at the corresponding pH. Therefore, it is also affected by the wettability of the solid surface, i.e. the more hydrophilic a surface is the more water will be adsorbed to the surface and, therefore, fewer adsorption sites for ions will be available. Another advantage of $\zeta$-potential measurements is that they provide information about the macroscopic properties at the solid-liquid interface in aqueous solution, which resembles the biological environment.

### 1.5.3. X-ray photoelectron spectroscopy (XPS)

X-ray photoelectron spectroscopy (XPS), also known as electron spectroscopy for chemical analysis (ESCA), is a spectroscopic technique that allows to quantify the elemental composition of a material surface in an ultra-high vacuum environment [200]. It provides compositional information about a material in a volume of 1 to 10 nm away from the surface. The sensitivity of a standard XPS instrument is in the parts per thousand range. The basic principle of XPS is to use an X-ray beam to irradiate the material surface and the energy of the X-ray photon is transferred to the core electrons of the atoms in the surface material. The kinetic energy ($E_k$) and the number of the escaped core electrons are measured. As the kinetic energy ($E_k$) of the photoelectron is measurable, the binding energy ($E_b$) of the electron can be calculated by:
\[ E_b = h\nu - E_k \] (1-11)

where \( h \) is Planck’s constant, \( \nu \) the X-ray frequency and \( h\nu \) the incident energy of the X-ray beam.

Once core electrons escape, the vacancy of the core shell is filled by electrons from a shell with higher energy level with energy released in a form of X-ray photons at the same time. If the released energy is high enough, it can be conserved by the ejection of another electron from higher energy level. The second ejected electron is called an Auger electron, which can also be detected by the sensor of the XPS instrument. Therefore, the signals detected by the XPS instrument include photoelectron and Auger electron lines. Each element present in the materials surface can produce a unique set of characteristic peaks that can be used as the “fingerprint” to identify its elemental composition. The intensities of the characteristic peaks are proportional to the concentration of the detected elements in the surface. If the same atom is bound to different chemical species, shifts in the peak positions called chemical shift, ranging from 0.1 eV to 10 eV, can occur. This is because the binding energies of electrons in an atom are affected by the atom’s chemical, physical environment as well as the oxidation state of the atom. For example, C_{1s} binding energy in a hydrocarbon bond is 285 eV, whilst in an ester bond, the C_{1s} binding energy is 289 eV, shifted by 4 eV to higher energy because the presence of the more electronegative oxygen in the vicinity of the carbon draws away electron density form the carbon making it harder to remove even a core electron [200]. Depending on the locations of these bondings in a molecule, the binding energy can vary for a range of ± 0.2 eV. In this way, it is possible to evaluate the chemical state of the element in the materials by comparing the obtained peaks with the records in the XPS peak database [200]. For an insulating material, such as PDLLA that was used in the current project, positive charge can build-up on the material surface when the photoelectrons are emitted from of the sample surface. Consequently, the sample surface acquires a positive potential and the XPS peaks of the core electrons shift to higher binding energies. The surface charge can be neutralised by the electrons generated by an accessorised low-energy electron flood gun of the XPS instrument.
Chapter 2. Materials and Methods

2.1. Introduction
This present thesis can be divided into 2 major areas: (1) derivation of type II pneumocytes from mESCs and (2) processing of biomaterials for the culture of pulmonary epithelial cells. The former focuses more on biological aspects and the latter on material aspects. As each area requires sample preparation and characterization, this chapter therefore is divided into methods for cell culture and characterization of cell culture as well as preparation and surface modification of polymer constructs, characterization of polymer constructs, and finally, culture of cells on biomaterials.

2.2. Cell culture and A549 cell transfection

2.2.1. Cell culture medium
2.2.1.1. Medium for the maintenance of mESCs in the undifferentiated state
Dubelcco’s Modified Eagle’s Medium (DMEM, Invitrogen, Paisley, UK) was supplemented with 10% (v/v) FBS (Invitrogen, Paisley UK), 100 U/mL penicillin and streptomycin (Invitrogen, Paisley, UK), 2 mM L-glutamine, (Invitrogen, Paisley UK), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich, Dorset, UK) and 1000 U/mL Esgro™ LIF (Chemicon, Temecula, CA). For the transfected mESC line, SPC-eGFP mESC, an additional 200 µg/mL Geneticin sulphate (Invitrogen, Paisley, UK) was added to the undifferentiated mES medium.

2.2.1.2. Medium for the differentiation of mESCs into pulmonary progenitors
Three differentiation media were used during pulmonary epithelium differentiation, depending on the time point of the differentiation, including (1) differentiation medium A: DMEM supplemented with 10% (v/v) FBS and 2 mM L-glutamine, (2) differentiation medium B: DMEM supplemented with 10% (v/v) Knockout-serum (KOSR) , 2 mM L-glutamine, plus 10 ng/ml Activin A, (3) differentiation medium C: DMEM supplemented with 10% (v/v) KOSR and 2 mM L-glutamine. No antibiotics were used in any of the differentiation media.
2.2.1.3. Medium for the culture of A549 cells
The medium for the culture of the untransfected A549 cells was composed of Ham’s F12K medium supplemented with 10% (v/v) FBS, 100 U/mL penicillin and streptomycin, and 2 mM L-glutamine. For the culture of EGFP-N1 transfected A549 cells (Chapter 2.2.4), 300 μg/ml of geneticin was added to the A549 medium during expansion and subsequent culture in order to maintain the EGFP expression.

2.2.1.4. Medium for the culture of 804G cells
DMEM was supplemented with 10% (v/v) FBS, 100 U/mL penicillin and streptomycin and 2 mM L-glutamine.

2.2.2. Culture of murine embryonic stem cells
Three important stages in murine embryonic stem cell culture are (1) culture of undifferentiated mESCs, (2) formation of embryoid bodies and (3) the three-step differentiation into type II pneumocytes.

2.2.2.1. Culture of undifferentiated mESCs
The murine embryonic stem cell (mESC) line E14-Tg2a (a kind gift from Prof. Austin Smith, University of Edinburgh, UK) stably transfected with a 4.8kb murine SPC promoter/EGFP construct using Lipofectamine™ 2000 (Invitrogen, Paisley, UK), as described previously [17], was used through this study. Untransfected E14-Tg2a cells were also cultured in parallel as a negative control for the flow cytometry. mESCs were maintained and feed everyday in an undifferentiated state on 0.1% gelatin-coated tissue culture plates (TCP, Corning Inc., Acton, MA) with fresh mESC maintenance medium in a humidified incubator set to 37°C and 5% CO2. Undifferentiated mESCs were passaged every 2-3 days when they reached 30-50% confluency.
2.2.2.2. Formation of embryoid bodies (EBs)

It requires careful manipulation to form EBs from mESCs. Roughly day 2 or 3 after passaging, EBs (Figure 2-2) were formed when undifferentiated mESCs reached around 50% confluency. In order to obtain a high undifferentiated to differentiated cell ratio, cells were used 2 to 3 passages after defrosting from liquid nitrogen.

Briefly, cells were trypsinized for 3 min and mESC maintenance medium was added to stop the function of the trypsin/EDTA. Trypsinized cells were centrifuged for 3 min at 800 rpm. The supernatant medium was aspirated and cells resuspended from the pellet using differentiation medium A with very gentle pipetting to form cell clumps comprising around 20 cells. Great care was taken in this step because clumps with too or too few cells form larger or smaller EBs, respectively, resulting in variations of the experimental results. A T75 flask of mESCs generated three 90 mm bacteriological grade Petri dishes (Bibby Sterilin, UK). 10 ml of differentiation medium was used for
each 90 mm petri dish.

Figure 2-2 Light microscopic image of EBs three days after their formation captured using an inverted microscope (IX70, Olympus, Southall, UK). Scale bar indicates 500 μm. Variations in the sizes of the EBs can be observed.

2.2.2.3. Original three-step differentiation of mESCs into pulmonary progenitors

The three-step differentiation protocol [203] was used to differentiate mESCs into pulmonary progenitors as illustrated in Figure 2-3. Three different media, namely differentiation medium A, B and C, were used to feed cells in sequence (Chapter 2.2.1.2). EB formation is referred to as day 1 in the three-step differentiation protocol. On day 1 and 2, EBs were fed with differentiation medium A. On day 3, the medium was changed to differentiation medium B with fresh medium being given again on day 5. On day 7, differentiation medium C was used to fed EBs. The adherent culture period starts on day 11 when EBs were plated on 6 well plates coated with gelatin. One Petri dish of EBs generated two 6 well plates (or one 6-well plate and one 24 well plate). During day 11 to day 20, the differentiation medium C was used to culture adherent EBs with medium changed for every 4 days. From day 21 to day 28, SABM
(Cambrex Corporation, East Rutherford, NJ, USA) was used for 8 days with medium changed for every 4 days.

![Diagram](image)

Figure 2-3 The original 3 step differentiation of mESCs to pulmonary progenitors

2.2.2.3.1. Differentiation of mESCs into pulmonary progenitors on ECM protein-coated TCP and PDLLA films

The effects of the ECM proteins and ECM replacement Matrigel coating of TCP and PDLLA films on the differentiation of the mESCs into pulmonary progenitors were investigated in Chapter 3. The experimental protocol was modified from the original three-step differentiation protocol [201](Figure 2-3). The only difference between the modified and original protocols was that, on day 11 of the modified protocol, EBs were seeded and differentiated onto ECM protein-coated polymer substrate. The ECM proteins and polymer substrates used are listed in Table 2-1. The control used for this experiment was TCP coated with gelatin (TCP-GN), as was used in the original three-step differentiation protocol [203].
Table 2-1 The polymer substrate and coating of the 6 well-plate used for differentiation culture

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Polymer substrate</th>
<th>Coating</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCP-GN (the experimental control)</td>
<td>TCP</td>
<td>Gelatin (GN)</td>
</tr>
<tr>
<td>TCP-CO</td>
<td>TCP</td>
<td>Collagen (CO)</td>
</tr>
<tr>
<td>TCP-LN</td>
<td>TCP</td>
<td>Laminin 332 (LN)</td>
</tr>
<tr>
<td>TCP-FN</td>
<td>TCP</td>
<td>Fibronectin (FN)</td>
</tr>
<tr>
<td>TCP-ML</td>
<td>TCP</td>
<td>Matrigel (ML)</td>
</tr>
<tr>
<td>PDLLA-GN</td>
<td>PDLLA</td>
<td>GN</td>
</tr>
<tr>
<td>PDLLA-LN</td>
<td>PDLLA</td>
<td>LN</td>
</tr>
<tr>
<td>PDLLA-ML</td>
<td>PDLLA</td>
<td>ML</td>
</tr>
</tbody>
</table>

2.2.2.3.2. Differentiation of mESCs into pulmonary progenitors by the addition of growth factors

The effects of the fibroblast growth factors (FGFs) 1, 2, 7 and 10 added to the differentiation medium for the differentiation of mESCs into pulmonary epithelial progenitors were investigated in Chapter 4. The experimental protocol was modified from the original three-step differentiation protocol [201] (Figure 2-4). The only difference between the modified and original protocols was that during day 11-14 (period A in Figure 2-4) and day 15-18 (period B in Figure 2-4) of the modified protocol, FGF-1, 2, 7 and 10 were added to the differentiation medium C. The FGFs were added at the beginning of the day 11 or day 15, and the medium was not changed for 4 days. The details of the FGF supplementation in periods A and B are listed in Table 2-2.

Figure 2-4 The differentiation protocol. Three differentiation media were used during pulmonary epithelial differentiation.
Table 2-2 Conditions used for periods A and B. The differentiation medium C in this table is that shown in Figure 2-4.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Period A (day 11-14)</th>
<th>Period B (day 15-18)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group 1: Addition of FGF-1 and FGF-2 to cells cultured on gelatin-coated plates</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-1</td>
<td>Differentiation medium C</td>
<td>Differentiation medium C</td>
</tr>
<tr>
<td>1-2</td>
<td>500 ng/ml FGF-1</td>
<td>Differentiation medium C</td>
</tr>
<tr>
<td>1-3</td>
<td>1000 ng/ml FGF-1</td>
<td>Differentiation medium C</td>
</tr>
<tr>
<td>1-4</td>
<td>100 ng/ml FGF-2</td>
<td>Differentiation medium C</td>
</tr>
<tr>
<td>1-5</td>
<td>500 ng/ml FGF-2</td>
<td>Differentiation medium C</td>
</tr>
<tr>
<td>1-6</td>
<td>500 ng/ml FGF-1 + 100 ng/ml FGF-2</td>
<td>Differentiation medium C</td>
</tr>
<tr>
<td><strong>Group 2: Addition of FGF10 to cells cultured on gelatin-coated plates</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-1</td>
<td>Differentiation medium C</td>
<td>Differentiation medium C</td>
</tr>
<tr>
<td>2-2</td>
<td>20 ng/ml FGF-10</td>
<td>Differentiation medium C</td>
</tr>
<tr>
<td>2-3</td>
<td>60 ng/ml FGF-10</td>
<td>Differentiation medium C</td>
</tr>
<tr>
<td>2-4</td>
<td>100 ng/ml FGF-10</td>
<td>Differentiation medium C</td>
</tr>
<tr>
<td>2-5</td>
<td>200 ng/ml FGF-10</td>
<td>Differentiation medium C</td>
</tr>
<tr>
<td>2-6</td>
<td>Differentiation medium C</td>
<td>20 ng/ml FGF-10</td>
</tr>
<tr>
<td>2-7</td>
<td>Differentiation medium C</td>
<td>60 ng/ml FGF-10</td>
</tr>
<tr>
<td>2-8</td>
<td>Differentiation medium C</td>
<td>100 ng/ml FGF-10</td>
</tr>
<tr>
<td>2-9</td>
<td>Differentiation medium C</td>
<td>200 ng/ml FGF-10</td>
</tr>
<tr>
<td><strong>Group 3: Addition of 60 ng/ml FGF10 to cells cultured on Matrigel-coated plates</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-1</td>
<td>Differentiation medium C</td>
<td>Differentiation medium C</td>
</tr>
<tr>
<td>3-2</td>
<td>60 ng/ml FGF-10</td>
<td>Differentiation medium C</td>
</tr>
<tr>
<td>3-3</td>
<td>Differentiation medium C</td>
<td>60 ng/ml FGF-10</td>
</tr>
<tr>
<td><strong>Group 4: Addition of 10 ng/ml FGF-7 to cells cultured on Matrigel-coated plates</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-1</td>
<td>Differentiation medium C</td>
<td>Differentiation medium C</td>
</tr>
<tr>
<td>4-2</td>
<td>10 ng/ml FGF-7</td>
<td>Differentiation medium C</td>
</tr>
<tr>
<td>4-3</td>
<td>Differentiation medium C</td>
<td>10 ng/ml FGF-7</td>
</tr>
</tbody>
</table>

2.2.2.3.3. Differentiation of mESCs into pulmonary progenitors on peptide-grafted PDLLA films

The effects of the bioactive peptide-grafted PDLLA films on the differentiation of the mESCs into pulmonary progenitors were investigated in Chapter 5. The experimental protocol was modified from the original three-step differentiation protocol (Figure 2-3). The only difference between the modified and original protocols is that at the
beginning of the day 11 of the modified protocol, EBs were seeded and differentiated on PDLLA films grafted with bioactive peptides. Three different grafted-peptide layers were prepared, i.e. the grafted-peptide layer composed of only RGD, RGD and YIGSR, or only of YIGSR. The method of the peptide-grafting was explained in Chapter 2.5.4.

2.2.3. Culture of A549 and 804G cell lines

The A549 cell line A549 (ATCC CCL no. 185, Rockville, MD) was originally isolated from a human alveolar cell carcinoma [61]. It possesses the features of type II pneumocytes, including cytoplasmic inclusion bodies (lamellar bodies) and synthesis of lecithin containing a high percentage of disaturated fatty acid. Its migration and adhesion repertoire has also been used as a model for lung injury [204].

804G cells (kind gift of Prof. Richard Lubman, University of South California, CA, USA), originally isolated from a bladder carcinoma, can secrete matrix containing laminin-related components that participate in hemidesmosome assembly formation [205]. Medium conditioned by 804G cells contains laminin 332 that can be used as a coating for pulmonary epithelium culture [206]. A standard cell culture protocol can be applied to both A549 and 804G cells with medium changed every other day using A549 medium and 804G medium, respectively. Cultures of either cell type can be split once 80% confluency is reached.

2.2.4. Transfection of CMV-EGFP construct to A549 cells

In order to see A549 cells on fluorescence microscopy, they were transfected with pEGFP-1 vector using Lipofectamine 2000 reagent (Invitrogen, Paisley, UK). pEGFP-N1 (CMV-promoter, Clontech, Heidelberg, Germany) is a commonly-used plasmid that carries cytomegalovirus (CMV) promoter, CMV enhancer and the coding sequence for enhanced green fluorescence protein (EGFP). The CMV promoter is active in A549 cells [207], therefore pEGFP-N1-transfected A549 cells can stably emit green fluorescence when excited at the wavelength of 395 nm using a fluorescence microscopy.

First of all, A549 cells at 90% confluency were cultured in 24 well plates using A549
culture medium excluding antibiotics. *E. coli* strand TOP10s carrying pEGFPN-1 in glycerol stock was defrosted and expanded in liquid LB broth with kanamycin prepared using imMedia™ Kan liquid (Invitrogen, Paisley, UK). Plasmids were extracted using Wizard® Plus SV Minipreps DNA Purification System (Promega, UK) according to manufacturer’s protocols. Once plasmid DNA was extracted, a complex containing DNA (μl) and Lipofectamine 20000 (μl) at ratio of 1:2.5 was prepared. Briefly, pGFPN-1 vector was added to 50 μl DMEM. Lipofectamine™ 2000 was also diluted in 50 μl DMEM followed by 5 min incubation at room temperature, then the diluted DNA was mixed with the diluted Lipofectamine™ 2000 gently followed by 20 min incubation at room temperature.

100 μl of complex was added to 24 well plates containing cells and medium, mixed thoroughly by rocking the plate back and forth. A549 cells were incubated at 37°C in 5% CO₂ for 24 h. Transient EGFP expression can be observed at this time (Figure 2-5). The transfection efficiency is 6.8%, determined using manual microscopy. Cells were then passaged at a 1:10 ratio into fresh A549 medium. Geneticin (G418) at 1100 μg/ml was then added to the A549 medium on the following day, with medium and geneticin changed every other day for 1 week, after which only cells that stably expressed EGFP survived. The best colonies were picked up carefully and expanded using the standard A549 cell culture protocol. In order to maintain the EGFP expression, 300 μg/ml of geneticin was added to the A549 medium during expansion and subsequent culture.

![Figure 2-5 Transient and stable EGFP expression of A549 cells captured using an inverted microscope (IX70, Olympus, Southall, UK) equipped with a fluorescence source (excitation wavelength 395nm). Left: EGFP expression 24 h after transfection of A549 cell line. Right: A stable EGFP-A549 cell line after selection with Geneticin](image-url)
2.3. Characterization of cell culture

2.3.1. Microscopy

Light and fluorescent microscope images were captured using an IX70 inverted microscope (Olympus, Southall, UK) equipped with F-View II, a black and white high resolution charge-coupled device (CCD) camera (Soft Imaging Systems GmbH, Germany), or a BX60 upright (Olympus Southall, UK) microscope equipped with an axiocam (Zeiss, Germany) digital camera. Confocal images were taken using an upright Leica SP2 confocal microscope (Leica, Germany) and processed using LCS lite software (Leica, Germany).

2.3.2. Conventional and realtime PCR for mRNA expression analysis

For Chapter 3, conventional PCR was performed to evaluate the expression of mRNAs for genes coding for the pneumocyte markers SPC, SPD, and aquaporin 5, the housekeeping genes GAPDH and beta-actin in the differentiating mESCs on day 28. For Chapter 4, the expression of mRNAs for these genes plus those coding for Clara cell protein 10 (CC10), a marker for distal airway epithelium progenitors and mature Clara cells, and the hepatocyte marker, albumin, was analysed on day 28 of the differentiation culture using conventional PCR. Furthermore, on day 28, the expression of SPC and GAPDH mRNAs by differentiating cells treated with 20 ng/ml, 60 ng/ml, 100 ng/ml and 200 ng/ml of FGF-10 during day 11-14 or 15-18 in the presence of gelatin coating and by differentiating cells treated with 60 ng/ml of FGF-10 during day 11-14 or 15-18 in the presence of Matrigel was analysed using conventional PCR.

Realtime PCR was performed to quantify the RNAs for the endoderm markers SOX17, Foxa2 and the mesoderm marker brachyury at the end of day 14 of the differentiation culture, right after addition of FGF-1 and 2 for 4 days, as described in Chapter 4. In the same chapter, realtime PCR was also performed to evaluate the expression of SPC mRNA by cells of the differentiation cultures treated with (1) 500 ng/ml and 1000 ng/ml of FGF-1 during day 11-14, (2) 20 ng/ml, 60 ng/ml, 100 ng/ml and 200 ng/ml of FGF-10 during day 11-14 or 15-18 in presence of gelatin coating, (3)
0 ng/ml of FGF-10 during day 11-14 or 15-18 in presence of matrigel or (4) 10 ng/ml of FGF7 during day 11-14 or 15-18 in presence of Matrigel.

2.3.2.1. RNA isolation
Total RNA was isolated using the RNeasy Mini Kit (Qiagen, UK) according to the manufacturer’s instructions. Briefly, a cell monolayer was harvested directly using 350 µL of buffer RLT pre-added with 1% (v/v) of 14.3 M β-mercaptoethanol. Cell lysates were transferred to QIAshredder spin columns placed in 2 mL collection tubes and centrifuged for 2 min at 13000 rpm. 350 µL 70% ethanol was added to the homogenized lysate and mixed well by pipetting. 700 µL of the sample, including any precipitate, was transferred to the RNeasy mini columns placed in 2 mL collection tubes and centrifuged for 15 s at 13000 rpm. After discarding the flow-through, 700 µL Buffer RW1 was added to the RNeasy columns followed by centrifugation for 15 s at 13000 rpm. The flow-through and the collection tubes were discarded. RNeasy columns were transferred into new 2 mL collection tubes and 500 µL Buffer RPE pre-added with 4 volumes of ethanol added onto the RNeasy columns followed by centrifugation for 15 s at 13000 rpm and again the flow-through was discarded. Another 500 µL buffer RPE was added to the RNeasy columns then centrifugation was performed for 2 min at 13000 rpm. The RNeasy columns were transferred to new 1.5 mL collection tubes and 50 µL RNase-free water was directly pipetted onto the RNeasy silica-gel membrane. Samples were centrifuged for 1 min at 13000 rpm to collect the total RNA. The concentration and purity of the total RNA were determined using a spectrophotometer (Eppendorf BioPhotometer, Germany) at 260/280 nm with a dilution rate of 5 µl of sample to 145 µl of RNase and DNase-free water. Samples were stored at −80°C until further use.

2.3.2.2. Reverse transcription
A thermoscript RT-PCR system (Invitrogen, Paisley, UK) was used to form cDNA according to the manufacturer’s protocol. 12 µl mixtures containing 1 µl of primer, 2 µl of dNTPs, up to 5 µl of RNA and DEPC-treated water together were transferred to a to 65°C pre-heated thermal cycler for 5 min and quenched on ice. The second 8 µl mixtures containing 4 µl of 5x buffer, 1 µl of 0.1 M DTT, 1 µl (40U) of RNaseOUT, 1µl of DEPC-treated water and 1 µl (15U) of reverse transcriptase were mixed with
first mixtures and transferred to a thermal cycler at 25°C for 10 min, 50°C for 55 min, 85°C for 5 min and a hold at 4°C at the end of the temperature profile.

2.3.2.3. Conventional PCR amplification

Conventional PCR primers were designed using Primer 3 software (Primer3 (v. 0.4.0) Pick primers from a DNA sequence, link in: http://frodo.wi.mit.edu/ accessed on 06.01.2009). PCR cycles were performed using a thermal cycler (Eppendorf Mastercycler, Hamburg, Germany) with cycle conditions as followed: 94°C for 2 min, amplification phase (denaturation at 94°C for 15 s, annealing for 30 s, elongation for 30 s at 72°C) for different cycles and final incubation at 72°C for 7 min. Optimal cycle numbers were predetermined (see Appendix B1 for the determination of optimal cycle number of various primers).

Table 2-3 The primers and the corresponding reaction conditions of conventional PCR reactions

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Conventional PCR primer</th>
<th>Annealing temp</th>
<th>Cycle</th>
<th>Product Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>F:AACCTGGGCTGACTGTGTAC R:GCAATATGTGTCATAAAG</td>
<td>57</td>
<td>36</td>
<td>162bp</td>
</tr>
<tr>
<td>Aquaporin 5</td>
<td>F:ACTGCCACAGCTCAGACCTCA R:AACGCCAACCCTGAAATACC</td>
<td>58</td>
<td>36</td>
<td>267bp</td>
</tr>
<tr>
<td>β-actin</td>
<td>F:GTGGGCCCGCTTCAAGCCTCAA R:CTCTTATGTCACGACGATTTCC</td>
<td>59</td>
<td>33</td>
<td>540bp</td>
</tr>
<tr>
<td>CC10</td>
<td>F:GCTCTCCAACTTACCATGA R:CTCTTATGTCACGACGATTTCC</td>
<td>55</td>
<td>42</td>
<td>241bp</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F:TGTGTCCCCTGACGATCTGA R:CCTGTCCACCCTCCTTCCA</td>
<td>59</td>
<td>30</td>
<td>76bp</td>
</tr>
<tr>
<td>SPC</td>
<td>F:CAGCTCCAGGAACTGACTGA R:CACAGCAAGGCTTAGGAAAG</td>
<td>63</td>
<td>36</td>
<td>200bp</td>
</tr>
<tr>
<td>SPD</td>
<td>F:GAGGTGGCCTTCTCCCCTTCCCTA R:CCACAAGCCCTTATTCCTTCCA</td>
<td>55</td>
<td>35</td>
<td>374bp</td>
</tr>
</tbody>
</table>

2.3.2.4. Realtime PCR amplification

Realtime PCR amplification was performed using SYBR Green PCR Core Reagents with GeneAmp® 5700 Sequence Detection System (both from AB Applied Biosystems, Warrington, UK). Each test was performed in triplicate in 25 μl reaction volumes containing 2.5 μl of 10X SYBR green PCR buffer, 3 μl of 25 mM MgCl₂, 2 μl of 2.5 mM dNTP blend, 0.125 μl of 5 U/μl AmpliTaq Gold polymerase, 0.25 μl of 1
U AmpErase UNG (uracil-N-glycosylase), 2.5 µl of forward primer, 2.5 µl of reverse primer, cDNA template equal to 25 ng of RNA input and water which made up the final total volume to 25 µl. A matrix of combinations of different primer concentrations ranging between 50-900 nM was tested for SPC mRNA expression according to manufacturer’s protocol (See Appendix B2). The concentration and sequences of other primers were listed in Table 2-4. The melting temperature of all realtime PCR primers were considered to be between 58-60°C.

It is worth noting that, in realtime PCR reactions, the dNTP mixture does not contain dTTP, instead, it contains dUTP, dCTP, dGTP and dATP. The advantages of this design is that any carryover from previous realtime PCR product can be degraded using AmpErase UNG before the start of any realtime PCR reaction. This decontamination process is of special importance because realtime PCR is highly sensitive. Therefore, in each realtime PCR reaction, the temperature profile starts at 50°C for 2 min, which is optimal for AmpErase UNG activity. 94°C for 10 min was then used to activate the AmpliTaq Gold DNA Polymerase. This was followed by 40 cycles of template denaturation (95°C for 15 s) and primer annealing and extension (60°C for 1 min). Melting curve (dissociation curve) analysis was performed at the end of all realtime PCR cycles to ensure that the fluorescence light was emitted by the SYBR® green dye binding to the amplicon instead of primer dimer. Pure PCR product only produces one sharp and narrow peak [208]. If there is primer dimer in the reaction mixture, a lower and broader peak can be seen at lower temperature, mostly around 80°C, than the main peak [209].

In addition to the samples to be tested, three No Template Controls (NTCs) and three No Amplification Controls (NACs) were also used. The former, also known as a negative control in conventional PCR, contains everything except cDNA in order to rule out any cDNA contamination in the mastermix or water. NAC control contains samples but no enzyme. It is used to rule out any fluorescence contamination present in the sample or heating block.
Table 2-4 The primers and conditions of realtime PCR reactions

<table>
<thead>
<tr>
<th>Primers</th>
<th>Primer concentrations</th>
<th>Product length</th>
</tr>
</thead>
<tbody>
<tr>
<td>murine GAPDH primer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>TGTGTCCGTCGGATCTGA</td>
<td>300 nM</td>
</tr>
<tr>
<td>Reverse</td>
<td>CCTGCTTCACCATTTGTTGA</td>
<td>300 nM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>77</td>
</tr>
<tr>
<td>murine Brachyury primer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>F: CATTACACACCCTAGCAG</td>
<td>300 nM</td>
</tr>
<tr>
<td>Reverse</td>
<td>R: AGAAGACGAGGCGTGGCA</td>
<td>300 nM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>51bp</td>
</tr>
<tr>
<td>murine Foxa2 primer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>CATCTCGCTCATACATGG</td>
<td>900 nM</td>
</tr>
<tr>
<td>Reverse</td>
<td>CAGCGTCAGCATCTGTGTTG</td>
<td>900 nM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>55</td>
</tr>
<tr>
<td>murine Sox17 primer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>AGTCGCTGGTCTACTATGG</td>
<td>300 nM</td>
</tr>
<tr>
<td>Reverse</td>
<td>GGTCGGCAACCGTCGATGG</td>
<td>300 nM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>51</td>
</tr>
<tr>
<td>murine SPC primer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>CAGCTCCAGGAACCTACTGC</td>
<td>900 nM</td>
</tr>
<tr>
<td>Reverse</td>
<td>GCGAAAGCCTCAAGACTAGG</td>
<td>900 nM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>70</td>
</tr>
</tbody>
</table>

The efficiency of the amplification of primer pairs was determined by realtime PCR tests of serial dilution of sample cDNAs. After obtaining the amplification curves of all diluted cDNAs, a horizontal line was drawn across the linear regions of all the amplification curves. The cycle number where the horizontal line crosses the amplification curve is called Ct. A linear regression line can be plotted against Ct of each diluted sample [208]. The efficiency can be obtained using the equation 

\[ E = \frac{1}{10^{(S/E) - 1}} \]

where \( E \) is the efficiency of the amplification and \( S \) the slope of the regression line. The gene expression of the target gene was calculated using Pfaffl’s equation:

\[ \text{ratio} = \left( \frac{E_{\text{target}}}{E_{\text{reference}}} \right)^{\Delta C_{\text{target}}/\Delta C_{\text{reference}}} \]

where \( \Delta C \) is the \( C_{\text{control}} - C_{\text{treated}} \) [208].

2.3.2.5. Agarose gel electrophoresis

Aliquots of 10 μl of amplified cDNA were mixed with 2 μl of 6x loading buffer. Gel electrophoresis was performed on 26 well 2% (w/v) agarose gels made up of 2.4 g of agarose gel, 120 ml of 1X TBE and 0.001 μg/mL ethidium bromide. 10 L of 1X TBE
can be diluted from 1 L of 10X TBE made up of 108 g Trizma® base, 54 g Boric acid, 40 ml 0.5 M EDTA (pH 8), 960 ml double distilled H₂O. When exposed to UV illumination, the gel should show a single band in each lane. Multiple bands in a single lane imply nonspecific binding or poor PCR primer design. In this case, a higher annealing temperature or a new primer should be used. Images of the gel were captured digitally using a BioRad Fluor-S multi-imager (BioRad/Zeiss, UK).

2.3.3. Immunocytochemistry

Immunostaining was performed in Chapters 3, 4, 5 and 6. For Chapter 3, immunostaining was performed on EBs differentiated for 10 days. The expression of the endodermal marker Foxa2, early lung and thyroid marker thyroid transcription factor 1 (TTF1) and basement membrane component laminin was examined in this study. For Chapter 4, differentiating cells that were fed with FGF-1 and 2 from day 11 to day 14 were immunostained for TTF-1 at the end of day 14. For Chapter 5 and 6, immunostaining was performed to localize focal adhesion points in the A549 cells 24 h after seeding on various substrate surfaces.

Negative controls were performed for every sample stained and for every method in every run. Negative controls were stained using a normal immunostaining procedure in which the primary antibody was replaced by the normal serum from the species of the first layer antibody. Negative controls were used to test the specificity of the antibodies involved in the staining procedure. Positive controls were also performed to ensure that all the reagents were in working condition and all the immunostaining protocols applied correctly. Positive controls were specific tissue or cells that were already proven to contain antigens tested. The positive control tissue or cells used for each antibody in the experiments were listed in Table 2-5.

The step by step protocol of immunostaining, modified from the book “Introduction to immunocytochemistry” [210], is described in Appendix A1. In addition to staining antigens using antibodies, staining of F-actin in A549 cells using Phalloidin-TRITC (1:50, P1951, Sigma-Aldrich, Dorset, UK) was also performed in Chapter 5 and 6.
Table 2-5 Details of antibodies

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Primary antibodies</th>
<th>Secondary antibodies</th>
<th>Positive controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPC</td>
<td>rabbit anti-mouse (diluted 1:500, kind gift from Professor Jeffrey A Whitsett, Cincinnati Children's Hospital Medical Centre, US)</td>
<td>goat anti-rabbit conjugated with Fluorescein (diluted 1:100; F9887, Sigma-Aldrich, Dorset, UK)</td>
<td>Sectioned murine lung tissue</td>
</tr>
<tr>
<td>TTF-1</td>
<td>mouse anti-mouse (diluted 1:100, NCL-L-TTF1, Novacastra, New castle upon Tyne, UK)</td>
<td>rabbit anti-mouse conjugated with TRITC (diluted 1:100; R0270, Dako, Copenhagen, Denmark)</td>
<td>Sectioned murine lung tissue</td>
</tr>
<tr>
<td>FOXA-2</td>
<td>goat anti-mouse (diluted 1:50; SC-9187, Santa Cruz, Berkeley, CA)</td>
<td>rabbit anti-goat conjugated with rhodamine (diluted 1:100; AP106R, Chemicon international, Temecula, CA)</td>
<td>Sectioned murine lung tissue</td>
</tr>
<tr>
<td>Laminin</td>
<td>rabbit anti-mouse (1:100; L9393, Sigma-Aldrich, Dorset, UK)</td>
<td>goat anti-rabbit conjugated with rhodamine (diluted 1:100; AP132R, Chemicon)</td>
<td>MLE12 cells</td>
</tr>
<tr>
<td>Vinculin</td>
<td>mouse anti-mouse (1:100; V4505, Sigma-Aldrich, Dorset, UK)</td>
<td>rabbit anti-mouse conjugated with FITC (diluted 1:100; F0261, Dako, Copenhagen, Denmark)</td>
<td>MLE12 cells</td>
</tr>
</tbody>
</table>

2.3.4. Flow cytometry

For Chapter 3, flow cytometry was performed on day 28 on the experimental groups and negative controls, which are E14-Tg2a mESCs without SPC-eGFP transfection cultured on gelatin in parallel with experimental groups. 400 μl of trypsin/EDTA (T/E) was added to each well in a six-well plate for 15 min to dissociate the adherent cell clumps. In order not to damage the cells during the subsequent pipetting, chicken serum was added at a pre-determined dilution ratio of 1:50 to slow down the cytotoxic effects of trypsin. Cells were pipetted vigorously more than 60 times after T/E dissociation. Dissociated cells were spun down at 800 rpm for 3 min and resuspended with 1 ml of PBS with 2μg/ml of propidium iodide (PI). Flow cytometry was performed with a FACalibur® flow cytometer and CellQuest™ software (Becton Dickinson, CA, USA). Data were analysed with FCS express 3.0 software (De Novo Software, Ontario, Canada). Gating was done to exclude dead cells and cell debris.
(Figure 2-6; Left) in the low forward scattering region. Necrotic cells were also ruled out by excluding cells expressing high fluorescence in the FL3 (PI) channel (Figure 2-6; Middle). A threshold was then applied in the FL1 channel to make 0.05% of the negative control scores positive (Figure 2-6; Right) [4]. Relative populations of SPC-eGFP-positive cells were compared using one way analysis of variance (ANOVA) with LSD post-hoc tests at a significance level of $P < 0.05$ using SPSS software (SPSS Inc., Chicago, USA).

Flow cytometry was also performed to evaluate the immediate effects of FGF-1 and 2 on the number of TTF-1 immunoreactive cells, at the end of day 14, right after the addition of FGF-1 and 2 for 4 days as described in Chapter 4. The attached EBs were dissociated into single cells using collagenase and further stained for TTF-1 for flow cytometry. Briefly, attached EBs were washed thoroughly with PBS and treated with 0.25% (w/v) collagenase for 60 min in an incubator. Cells were passed through 21G needles for 3 times and an equal volume of medium containing 5% (v/v) FBS was added to stop the reaction of the collagenase. Dissociated cells were immunostained with TTF-1 antibody using the same protocol as described in the immunostaining method section (Chapter 2.3.3). The negative control used here was the samples that are only stained with the secondary antibodies. The frequencies of TTF-1-immunoreactive cells were evaluated using FACScalibur system (BD bioscience, Oxford, UK) equipped with CellQuest™ software (Becton Dickinson, CA, USA). Collected data were analysed with FCS express 3.0 software (De Novo Software, Ontario, Canada). A size gate was applied to the negative control sample to exclude the cell debris in the low forward scattering region (Figure 2-7A). A threshold
was then applied to the negative control sample (Figure 2-7B) in the FL1 channel to make 0.1% of the sample scores positive [203].

Figure 2-7 Applied gates for the flow cytometry of the TTF-1 positive cells. Left: debris was excluded to gate 1. Right: a gate applied in the FL1 channel (green) to make 0.1% of the negative control scores positive

2.4. Preparation of Polymer Constructs

2.4.1. Preparation of polymer films

PDLLA films were prepared for Chapters 3, 5 and 6. The conditions for the preparation of PDLLA films were the same for all three chapters. 5% PDLLA (w/v) solution was prepared by dissolving PDLLA powder with inherent viscosity of 1.53 dl/g (Purasorb®; Purac biochem, The Netherlands) completely in dimethyl carbonate (DMC) (Sigma-Aldrich, Dorset, UK). 1 ml of the dissolved polymer solution was coated onto circular borosilicate glass coverslips (15 mm or 32 mm diameter to fit into either 24 or 6 well tissue culture plates, respectively), which have previously been washed in acetone and ethanol. The PDLLA solution was allowed to dry slowly for 72 h under minimal air-flow in order to obtain smooth polymer films. The polymer-coated coverslips were fixed on the base of the wells of either 24 or 6 well tissue culture plates by adding several drops of 5% PDLLA-DMC solution, then all plates containing PDLLA films were dried in a fume hood for 1 week and sterilized under UV light for 45 min at 0.120 J and 80 W (BLX-254, Vilber Lourmat, France). UV-sterilized plates were further washed several times with 0.1 M phosphate-buffered saline solution (PBS; Invitrogen Ltd., Paisley, UK) to ensure complete removal of foreign bodies.
2.4.2. Preparation of scaffolds using liquid-liquid phase separation and solid-liquid phase separation

Three groups of 3D porous scaffolds were prepared using different conditions for Chapter 7. The details of the experimental conditions are provided in Table 2-6. Scaffolds prepared were given a sample name for convenience and clarity. The prefix LL stands for scaffolds prepared using liquid-liquid phase separation and SL for solid-phase separation. The suffixes M and C, the abbreviation of minutes and degrees Celsius, stand for coarsening time and coarsening temperature or freezing temperature, respectively.

Table 2-6 Scaffolds prepared by various methods

<table>
<thead>
<tr>
<th>Group 1: Scaffolds prepared by changing coarsening time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymer solution</td>
</tr>
<tr>
<td>5% PDLLA dissolved in 13% water and 87% dioxane and coarsened at 24°C for different times</td>
</tr>
<tr>
<td>Coarsening time</td>
</tr>
<tr>
<td>0 min 10 min 20 min 30 min 60 min</td>
</tr>
<tr>
<td>Sample name</td>
</tr>
<tr>
<td>LL0M LL10 M LL20 M LL30 M LL60 M</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group 2: Scaffolds prepared by changing coarsening temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymer solution</td>
</tr>
<tr>
<td>5% PDLLA dissolved in 13% water and 87% dioxane and coarsened for 30 min at different temperatures</td>
</tr>
<tr>
<td>Coarsening temperature</td>
</tr>
<tr>
<td>27°C 24°C 21°C 18°C 15°C 12°C</td>
</tr>
<tr>
<td>Sample name</td>
</tr>
<tr>
<td>LL27C LL24 C LL21 C LL18 C LL15 C LL12 C</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group 3: Scaffolds prepared by solid-liquid phase separation using two quenching temperatures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymer solution</td>
</tr>
<tr>
<td>5% PDLLA solution (in DMC)</td>
</tr>
<tr>
<td>Quenching temperature</td>
</tr>
<tr>
<td>-25°C (freezer)</td>
</tr>
<tr>
<td>-196°C (liquid nitrogen)</td>
</tr>
<tr>
<td>Sample name</td>
</tr>
<tr>
<td>SL-25C SL-196C</td>
</tr>
</tbody>
</table>

2.4.2.1. Preparation of scaffolds by thermally-induced liquid-liquid phase separation of the dioxane-water-PDLLA ternary system

5% (w/v) of PDLLA with an inherent viscosity of 1.53 dl/g (Purasorb®; Purac biochem, The Netherlands) was dissolved in 100 ml of solution containing 87% dioxane and 13% double distilled water under magnetic stirring for 6 h. Two factors, coarsening time (Table 2-6, Group 1) and coarsening temperature (Table 2-6, Group 2), were used to control the morphology of the scaffold by thermally-induced phase separation [20, 181]. 10 ml of the polymer solution was transferred into a 30 ml glass
bottle prewarmed to 50\(^\circ\)C and held for 1 h, then coarsened in a water bath (Haake K15, Germany) for different times or at different temperatures. The chosen temperatures were below the cloud point of the PDLLA solution, 30 ± 2\(^\circ\)C, which was determined in preliminary experiments. The glass bottles were then quenched in a beaker pre-cooled in liquid nitrogen. After 2 h, the frozen PDLLA solution in the glass bottles was freeze-dried using a custom-made freeze dryer (Chapter 2.4.2.3).

2.4.2.2. Preparation of scaffolds by thermally-induced solid-liquid phase separation using the DMC-PDLLA binary system

Two types of scaffolds were prepared by TIPS using the DMC-PDLLA binary system [20, 181] (Table 2-6). 5% (w/v) of PDLLA in DMC was prepared and stirred overnight. 80 ml of polymer solution was transferred into a Petri dish and either quenched in liquid nitrogen for 2 h or frozen in a freezer kept at -25\(^\circ\)C overnight. The petri dishes were then freeze-dried using a custom-made freeze-dryer (Chapter 2.4.2.3). The exterior temperature was kept at -15\(^\circ\)C.

2.4.2.3. Freeze-drying of polymer solution using a custom-made freeze-dryer

The frozen polymer solution kept in glass bottles or Petri dishes as described in sections 2.4.2, 2.4.2.1 and 2.4.2.2 was placed in a vacuum desiccator (Figure 2-8, 1) and connected to a vacuum pump (Leyhold Trivac D8) at 10\(^{-2}\) Torr (Figure 2-8, 4) via a cold trap (Figure 2-8, 2). The temperature of the ethylene glycol bath was kept at -15\(^\circ\)C (Figure 2-8, 5). The solvent was collected in the cold trap for at least 96 h. The residual solvent was then removed at room temperature until the scaffolds reached a constant weight.
2.5. Surface modification of polymer constructs

2.5.1. Protein adsorption

4 different ECM proteins, type I collagen (Sigma-Aldrich Co., Dorset, UK), laminin 332 (obtained from a conditioned medium as described below), fibronectin (Roche Diagnostics GmbH, Germany) and Matrigel (BD Biosciences, Bedford, USA) and, as an experimental control, gelatin (Sigma-Aldrich Co., Dorset, UK) were used as protein coatings.

Gelatin

0.1% (w/v) gelatin was prepared by dilution of 2% (w/v) gelatin stock solution in PBS. 200 µl of the solution was plated per centimetre square of the culture surface and incubated for 20 min at 37°C. Gelatin was then aspirated and the exposed plate was allowed to dry in a sterile laminar flow hood for 20 min.
**Fibronectin**

Fibronectin lyophilizate was first reconstituted in water to yield a stock solution at a concentration of 1 mg/ml. Incubation for 30-60 min at 37°C without agitation was necessary for dissolution. The stock solution was further dissolved in PBS to make a working solution at concentration of 50 µg/ml. 100 µl of the working solution (50 µg/ml) was applied per centimetre square of culture surface (final concentration of 5 µg/cm²) using rubber cell scrapers. The vessel containing fibronectin was incubated at RT for 45 min. The plate was used immediately after aspiration of the protein solution.

**Collagen I**

One vial of lyophilized collagen was dissolved in sterile 0.2% (v/v) acetic acid to make the final concentration 2 mg/ml. 2.5 µl of the collagen solution was applied to substrate surface at concentrations of 2.5 µl/cm² (5 µg/cm²) using rubber cell scrapers and air-dried at room temperature for 1 h in a fume-cupboard.

**Laminin 332**

Laminin 332 was obtained from DMEM conditioned by a laminin 332-producing rat bladder cell line (804G) (a kind gift from Prof. Richard Lubman, University of South California, USA) [205, 211, 212]. In order to eliminate the competitive adsorption effect of serum proteins, especially albumin and vitronectin, the 804G cells were fed with DMEM only when they reached 70% confluence, then the conditioned medium was collected 24 h after addition followed by 0.22 µm filter sterilization. 200 µl per cm² of 804G cell-conditioned medium was added to substrate surface at 37°C for 1 h. The plates were used immediately for adherent culture after aspiration of conditioned medium.

**Matrigel™ Basement Membrane Matrix**

Stock solution of growth-factor-reduced Matrigel was first thawed at 4°C overnight to prevent gelation. Pre-cooled pipettes, plates and tubes were used in the preparation of coating the culture surface. Thawed Matrigel was diluted 1:30 in serum-free medium (DMEM supplemented with 10% (v/v) KOSR, 2 mM L-glutamine and 0.1 mM 2-mercaptoethanol) and 200 µl/cm² of the diluted solution was applied to the substrate
surface, incubated at RT for 1 h, after which unbound material was aspirated.

2.5.2. Introduction of primary amines to PDLLA using aminolysis

Primary amines were introduced into the surface of PDLLA films (for Chapters 5 and 6) and PDLLA scaffolds (for Chapter 7) using aminolysis (Figure 2-9). Ethylenediamine was diluted in isopropanol to yield a solution with concentrations (v/v) ranging from 1% to 20%. PDLLA films and scaffolds were immersed into the prepared solutions and continuously agitated for 5 min using an orbital shaker. The samples were then rinsed with excess water to remove any free, unreacted solution and stored in a vacuum desiccator until use.

PDLLA films prepared in Chapter 5 were treated with only 4% ethylenediamine solution. PDLLA films prepared in Chapter 6 were treated with 1%, 2%, 4%, 6%, 8%, 10%, 15% and 20% of ethylenediamine solution. PDLLA scaffolds prepared in Chapter 7 were treated with only 4% ethylenediamine solution. PDLLA films (Chapter 6) or scaffolds (Chapter 7) treated with \( n \)% ethylenediamine in isopropanol were abbreviated as \( n \)%EDiamine, where \( n \) is the concentration of the ethylenediamine used for aminolysis.
2.5.3. Preparation of amine-terminated and carboxylic acid-terminated branched architectures

Amine-terminated and carboxylic acid-terminated branched architectures were introduced to the surface of PDLLA films in Chapter 6 and PDLLA scaffolds (SL-25C) in Chapter 7 by repetitively grafting spacer and “trifunctional molecule” to the 4% ethylenediamine-treated PDLLA surface, which was described in Chapter 2.5.2. 4% ethylenediamine (v/v) was used to treat PDLLA films and scaffolds first because the preliminary study suggested that PDLLA films treated with ethylenediamine concentrations higher than 4% were too fragile for further branching modifications. Using either tris(2-aminoethyl)amine (TREN) (Figure 2-10) or N-(2-acetamido)iminodiacetic acid (ADA) (Figure 2-10) as “trifunctional molecules”, which possess three functional groups, it is possible to generate amine-terminated (bTREN) and carboxylic acid-terminated (bADA) branched architectures. Branched architectures up to three generations were prepared. In the following text, $bTREN_n$ and $bADA_n$ stands for branched architectures that have maximal $n$ generations, where $n$ can be 1, 2 or 3. The choice of spacers was not identical for all branched architectures. In brief, succinic acid (Figure 2-10) was used as spacer for all generations of bTREN, while succinic acid was used as spacer for the first generation only and $\gamma$-amino butyric acid (GABA) (Figure 2-10) for the subsequent generations of bADA.
The linkers 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were used to link the carboxylic acid of one molecule, i.e. the spacer or the trifunctional molecule, to the primary amine of another molecule, i.e. the trifunctional molecule or the spacer. Once mixed with carboxylic acid, EDC can react with NHS to form a semi-stable amine-reactive NHS ester with life times of several hours, making two step grafting reactions possible.
Figure 2-11 Schematic of the reaction of EDC and NHS. EDC can react with carboxylic acid to form an unstable amine-reactive O-acylisourea intermediate which can reactive with amine to form a stable amide bond, or hydrolysed by water to regenerative the carboxylic acid, or reactive with NHS to form a semi-stable amine-reactive NHS-ester. Comparing to O-acylisourea which has a life time of only a few minutes, NHS-ester have a life time for several hours in aqueous solution and for several months when samples are stored in a dry environment, therefore it is more suitable to further react with primary amines to form an amide bond ([213]).

Five different reactions were used to prepare branched architectures (Figure 2-12). The choice and the sequence of the reactions for the preparation of certain branched architectures were listed in Table 2-7. All reactions composed of 2 steps. The first step was the activation of the carboxylic acid of one molecule, and the second was the coupling reaction of amine of another molecule to the activated carboxylic acids. Step 1 and step 2 were performed at pH 5 and pH 8, respectively. In reaction 1 4 mg of EDC and 11 mg of NHS were added per ml of 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES) buffer containing 20 mM succinic acid at pH 5 and stirred for 15 min at RT. The final concentrations of EDC and succinic acid in MES buffer were 20 mM and 50 mM, respectively. 10 M NaOH was then added to adjust the solution pH to 8. 4% ethylenediamine-treated films were then immersed and agitated continuously for 2 h in 2 ml of the activated succinic acid solution followed by a thorough water rinse. For reaction 2 and 3 (Figure 2-12), PDLLA films were immersed and agitated in 2 ml of solution containing 20 mM EDC and 50 mM NHS in MES buffer at pH 5 for 15 min, thoroughly washed with water afterwards, then immersed in 2 ml of 4% (w/v) TREN in water at pH 8 (Figure 2-12, reaction 2) or 50 mM ADA in PBS at pH 8.
(Figure 2-12, reaction 3) for 2 h under continuous agitation and finally rinsed thoroughly with water. For reaction 4 and 5, each PDLLA film was activated in the same way as for reaction 2 or 3, rinsed and immersed in 2 ml of 50 mM GABA in PBS at pH 8 (Figure 2-12, reaction 4) or 50 mM ADA in PBS at pH 8 (Figure 2-12, reaction 5) for 2 h under continuous agitation. Samples were washed with large quantities of distilled water after each reaction. All grafting reactions were performed at RT.
Figure 2-12 Reaction scheme used to prepare the 2 branched architectures (bTREN and bADA). Reaction 1: succinic acid activated by EDC and NHS was grafted to 4%EDiamine. Carboxylic acid (circled by dashed line) indicates a site of possible reaction to the adjacent surface amine. Then TREN (reaction 2) or ADA (reaction 3) was grafted to the grafted succinic acid. Amines (circled by dashed line) in reaction 2 indicate possible reaction sites to adjacent carboxylic acid of grafted succinic acid. Reaction 4: GABA was grafted to ADA. Reaction 5: ADA was grafted to GABA.
Table 2-7 The sequence of reactions (for Figure 2-12) for the preparation of branched architectures

<table>
<thead>
<tr>
<th>Branched architectures</th>
<th>Sequence of reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>bTREN1</td>
<td>1→2</td>
</tr>
<tr>
<td>bTREN2</td>
<td>1→2→1→2</td>
</tr>
<tr>
<td>bTREN3</td>
<td>1→2→1→2→1→2</td>
</tr>
<tr>
<td>bADA1</td>
<td>1→3</td>
</tr>
<tr>
<td>bADA2</td>
<td>1→3→4→5</td>
</tr>
<tr>
<td>bADA3</td>
<td>1→3→4→5→4→5</td>
</tr>
</tbody>
</table>

2.5.4. Grafting of laminin peptides onto surface amines of aminolyzed PDLLA films

Bioactive peptides were grafted to the surface amines of the aminolyzed PDLLA films as described in chapter 5. Firstly, PDLLA films were treated with 4% (v/v) ethylenediamine solution as described in section 2.5.2. The peptides arginine-glycine-aspartic acid (RGD, Sigma-Aldrich, Haverhill, UK) (Figure 2-13) and Tyr-Ile-Gly-Srg-Arg (YIGSR, Bachem, St. Helens, UK) (Figure 2-13) were dissolved at concentrations of 0.02 mg/ml and 0.034 mg/ml, respectively, in 0.1 M PBS. Both peptide solutions were adjusted to pH 8 using 10 M HCl. Cocktails consisting of RGD and YIGSR in molar ratios of 100:0 (100RGD-0YIGSR), 75:25 (75RGD-25YIGSR), 50:50 (50RGD-50YIGSR), 25:75 (25RGD-75YIGSR) and 0:100 (0RGD-100YIGSR) were grafted to PDLLA surfaces using a 2 step protocol (Figure 2-14). In step 1, 4 mg of 1-ethyl3(3dimethylaminopropyl) carbodiimide hydrochloride (EDC, Pierce Biotechnology, Northumberland, UK) and 11 mg of N-hydroxysuccinimide (NHS, Acros Organic, Geel, Belgium) were added per ml of 0.1M 2-(N-morpholino)ethanesulfonic acid (MES) buffer containing 20 mM succinic acid at pH 5 and stirred for 15 min at RT. The final concentrations of EDC and succinic acid were 20 mM and 50 mM, respectively. 10 M NaOH was then added to adjust the solution pH to 8. Films pretreated with 4% (v/v) ethylenediamine in isopropanol for 5 min were then immersed and shaken continuously in 2 ml of the activated succinic solution for 2 h followed by thoroughly water rinse. In step 2, each succinic acid-grafted PDLLA film was immersed and agitated in 2 ml of solution containing 20 mM mg EDC and 50 mM of NHS in a MES buffer at pH 5 for 15 min, then each activated film was immersed in 2 ml of the peptide cocktail at pH 8 for 2 h
under continuous agitation followed by thorough washing using large quantities of water.

![Figure 2-13](image1.png) Structures of peptides RGD and YIGSR. Amines circled by dashed lines are the locations where conjugations occur.

![Figure 2-14](image2.png) Schematic of the peptide grafting reactions. The primary amines on the PDLLA were introduced by aminolysis using 4% ethylenediamine in isopropanol for 5 min prior to the grafting reaction.

2.6. Characterization of polymer films and scaffolds

2.6.1. Surface character of polymer films: \(\zeta\) potential Measurements

The \(\zeta\)-potentials of uncoated TCP, protein-coated TCP, uncoated PDLLA films and protein-coated PDLLA films as described in Chapter 3, and surface-modified PDLLA films (4%EDiamine, bTREN3 and bADA3) as described in Chapter 6, were measured using an Electrokinetic Analyser (EKA, Anton Paar KG, Graz, Austria) based on the streaming potential method [214]. To eliminate any errors that could be caused by
polymer swelling, the $\zeta$-potential change over time, $\zeta=f(t)$, was recorded at constant ionic strength (1 mM KCl) prior to conducting the $\zeta=f(pH)$. After the $\zeta$-potential stabilised with time, it was subsequently recorded as a function of pH ($\zeta=f(pH)$) in a 1 mM KCl supporting electrolyte solution. The streaming potential was generated by applying a steadily increasing pressure difference ($\Delta p$) from 30 to 250 mbar across a channel which was created by stacking a PTFE film with a preformed channel between two sample films. The pH was controlled by adding either 0.1 M HCl or 0.1 M KOH using a remote titration unit (RTU, Anton Paar KG, Graz, Austria) at a constant temperature of 20°C.

### 2.6.2. Dynamic contact angle measurements

The relative levels of hydrophilicity displayed by uncoated TCP, uncoated PDLLA films, protein-coated TCP and protein-coated PDLLA films as described in Chapter 3 were assessed by dynamic contact angle measurements using the Drop Shape Analysis System (DSA 10 Mk2; Krüss GmbH, Hamburg, Germany) using sessile drop or captive bubble methods. The wettability of the modified PDLLA films as described in Chapter 5 and Chapter 6 was assessed only by dynamic captive bubble contact angle measurements using the same equipment. At least 5 independent measurements of advancing and receding contact angles were performed per sample.

For sessile drop method, advancing and receding contact angles were measured from digital images of a drop of de-ionised water in direct contact with a dry surface. The volume of the droplet was controlled by a motor driven microsyringe. For the captive bubble method, the polymer films were immersed in distilled water overnight before the measurements, allowing the polymer to equilibrate, i.e. to swell and for the reorientation of polymer chains. An air bubble of 60 $\mu$l was created at a rate of 20 $\mu$l/min beneath the polymer film immersed in de-ionized water. The volume of the bubble was controlled by a motor driven microsyringe. The contact angle $\theta$ was measured into the water phase. At least 5 independent measurements of advancing and receding contact angles were performed per sample. All measurements were made in an air-conditioned room at a constant temperature of 20 °C.
2.6.3. Morphological analysis of the scaffolds using scanning electron microscopy

For the morphological analysis of scaffolds as described in Chapter 7, scaffolds prepared using liquid-liquid phase separation were sectioned along the vertical axis of the scaffolds, and scaffolds prepared using solid-liquid phase separation were sectioned both along and transverse to the direction of the ladder-like pores using a razor blade. The sectioned scaffolds were examined with a Jeol JSM-840A scanning electron microscope. Prior to SEM, the samples were coated with an 11 nm thick Au layer using a sputter coater (Emitech Ltd., Ashford, UK) in an argon atmosphere. The size of the pores was analysed using Image-pro plus software (Media Cybernetics, Silver Spring, Maryland, USA).

2.6.4. Fluorescamine assay for quantification of primary amine functional groups

The fluorescamine assay was originally used to quantify proteins by measuring the amount of the primary amines in a protein [22, 23] and later applied to quantify the amine amount on a polymer surface [28, 215]. A fluorescamine molecule can react with a primary amine to generate a fluorescent primary amine adduct (Figure 2-15). The fluorescence was measured by dissolving the aminolyzed PDLLA films (Chapter 6) in the fluorescamine solution. Thus, the fluorescamine assay measured the bulk concentration of the primary amines of the modified PDLLA films. However, if the amines are located only at the surface, the amine concentration on the surface of PDLLA films can be estimated. The fluorescamine working solution was prepared by dissolving 30 mg of fluorescamine in 90 ml of acetone. 2 cm² of aminolysis-treated PDLLA films were completely dissolved in 1 ml of fluorescamine solution for 30 min. 100 μl of the assay solution containing the samples was transferred to a white 96 well plate and tested in a fluorescence plate reader using an excitation wavelength of 390 nm and emission wavelength of 475 nm. In order to determine the concentration of amines in the fluorescamine solution containing samples, a calibration curve of the fluorescence intensity against primary amine concentration was established by serial dilution of ethylene diamine in the fluorescamine assay solution (see Appendix B3).
Figure 2-15 Fluorescamine reacts with primary amine groups to form primary amine adducts which absorb light at 390 nm, and emit light at 475 nm.

2.6.5. Surface topography and roughness of modified PDLLA: Atomic Force Microscopy (AFM)

1 μm² of PDLLA films grafted with bioactive peptides, as described in Chapter 5, and 1 μm² of functionalized PDLLA films, as described in Chapter 6, were imaged using a Multimode V Atomic Force Microscope with Nanoscope 6 software (Veeco, USA) operated in the tapping mode. The surface roughness parameter Ra, the average deviation of distance between all points of a roughness profile and a mean line, was calculated using the open source software Gwydion V2.9 (Link in: http://gwyddion.net/ assessed on 06.01.2009) according to ISO 4287/1-1997 standard. At least 4 different areas of each sample were imaged to obtain the average Ra of the unmodified and modified PDLLA films.

2.6.6. Surface composition of modified PDLLA: X-ray photoelectron spectroscopy (XPS)

The surface composition of the PDLLA films modified with amine-terminated and carboxylic acid-terminated branched architectures, as described in Chapter 6, were determined using X-ray Photoelectron Spectroscopy (XPS) (Escalab 220iXL, Thermo VG Scientific, UK). Measurements of surface elemental composition were performed using an Al Kα monochromated X-ray source and quantified in CasaXPS (Casa software Ltd). To reduce the charging of the polymer surface, a silver mask was placed on top of the polymer sample and the X-ray beam stroke the sample through the slot of the silver mask. Furthermore, an electron flood gun was used to compensate charging effects of the samples. For all the samples, both survey and high resolution spectra were recorded for precise evaluation of the chemical composition.
For the quantification of the elemental composition of the modified PDLLA films, a frequently-used background subtraction algorithm, Shirley background, was applied for the asymmetric background subtraction before the curve fitting was employed.

2.7. Culture and evaluate cells on biomaterials

2.7.1. Seeding A549 cells onto scaffolds

Scaffolds with different porous structures, as described in Chapter 7, were cut into cubes with the dimensions 2 mm × 2 mm × 4 mm using a razor blade. The scaffolds were sterilized under UV light for 45 min at 0.120 J and 80 W (BLX-254, Vilber Lourmat, France).

It should be noticed that upon exposure to UV, the surface properties of a biomaterial altered. UV radiation could break the polymer chain near the surface of polymer, generating free radicals and new chemical groups. The free radicals can induce further reaction, such as crosslinking and grafting, if other reactive groups were presented. The newly-formed chemical groups can reduce the water contact angle and improve the biocompatibility. The scission of the polymer chains caused by UV lowered the molecular weight of the material, thus accelerate the degradation. As a result, the surface roughness of the biomaterial can be affected by the UV sterilization. Despite so, only extended UV exposure, e.g. more than 5 h as reported by recent studies [216, 217], can the UV sterilization change the cell responses to a material.

Before cell seeding, the scaffolds were immersed in A549 medium, placed in a desiccator and vacuum pumped to remove all air trapped from the scaffolds until no apparent bubbles could be seen. Scaffolds sank to the bottom of the flask when most bubbles were removed. 2 μl of the medium soaked within the scaffold was removed using a pipette, then 2 μl of high density A549 cell suspension (1000 cells/μl) was seeded on the scaffolds, which were placed on non-tissue culture-treated polycarbonate Petri dishes and incubated in 5% (v/v) CO₂ atmosphere at 37°C for 4 h. The scaffolds were then transferred to 24 well plates and 500 μl of culture medium added to each well gently in order not to remove the unattached cells from the scaffolds. A549 cells were cultured for 16 days with medium changed every other day.
2.7.2. The penetration of A549 cells into scaffolds evaluated using fluorescence and scanning electron microscopy

Scaffolds (LL27C to LL12C, SL-25C and SL-196C) cultured with A549 cells for 16 days, as described in Chapter 7, were examined under an Olympus IX70 inverted fluorescence microscope (Olympus, Helperby, UK). Scaffolds were further cut in half along the direction of initial cell seeding in order to investigate whether the A549 cells penetrated into middle of scaffolds. Cells were fixed in situ with 4% (w/v) paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) for 40 min at room temperature. Serial dehydration of fixed A549 cells was then performed to completely remove the water from the cells. Briefly, samples were thoroughly rinsed twice with PBS and dehydrated sequentially in 25, 50, 70 and 90% (v/v) ethanol for 5 min once, 100% ethanol for 5 min twice which was followed by incubation in hexamethyldisilane (Sigma-Aldrich, Dorset, UK) for 5 min. The dehydrated cells on scaffolds were coated with Au and examined using a Jeol JSM-840A scanning electron microscope as described earlier.

2.7.3. Evaluation of attachment and population size of A549 cells by measurement of DNA content using Hoechst dye

The adhesion of A549 cells to the peptide-grafted PDLLA films (Chapter 5) and functionalized PDLLA films (Chapter 6) was assessed by measurement of the DNA
content using Hoechst 33258 dye. Hoechst 33258 dye can penetrate the cell membrane and bind to the minor groove of DNA. The fluorescence emitted by Hoechst 33258 dye is therefore proportional to the DNA amount. 80000 cells were seeded onto 24 well plates containing samples fixed to the bottom of each well. After 2 h, the culture medium was aspirated followed by one gentle rinse with PBS to remove unattached cells. The 2 h period, determined in preliminary tests, was found to be an appropriate period for the cell attachment test. 500 μl of proteinase K in K₂HPO₄ (pH 8) at a concentration of 50 μg/ml was added to each well and incubated in 5% CO₂ incubator at 37°C overnight followed by heating at 90°C for 10 min to inactivate the enzyme. Proteinase-treated samples were then pipetted vigorously and centrifuged at 10000 RPM for 10 min. 100 μl of the supernatant were then transferred to a 24 well plate, mixed with 100 μl of Hoechst 33258 dye (2 μg/ml) and vortexed for 2-3 min. 100 μl of each mixture containing supernatant and Hoechst dye solution was transferred to a white 96 well plate and the emission fluorescence was measured at 458 nm with the excitation wavelength of 365 nm. A standard curve of absorbance versus known cell number was also plotted as a reference for the experimental groups. (Appendix A4, Figure A-9)

The population of A549 cells cultured on the peptide-grafted PDLLA films (Chapter 5) and functionalized PDLLA films (Chapter 6) for 3 and 4 days was also evaluated using Hoechst 33258 dye. The protocol used to determine the A549 cell population was the same as the protocol used to determine A549 attachment.

2.7.4. Evaluation of cell activity using WST-1 assay

The viable cell assay, that uses WST-1 reagent (Roche Applied Science, Herts, UK), is a colourimetric assay to evaluate metabolic activity, proliferation and viability of the cells. Similar to MTT [218], XTT and MTS assays, the tetrazolium salt WST-1 cleaves into formazan by the action of a cellular enzyme, mitochondrial dehydrogenase, causing the colour to change from slightly red to dark red. The level of the colour change correlates with the augmentation of the total enzyme activity of the tested cells. The colour change can be quantified using a spectrophotometer with a measurement wavelength of 420-480 μm and a reference wavelength of over 600 μm. Compared with other tetrazolium salts, WST-1 salt offers a wider linear range in
response to cell number and cell proliferation. In addition, it also cleaves into soluble
products like XTT and MTS, thus the addition step of solubilization is not required.

The WST-1 assay was used throughout this thesis (Chapters 3, 5, 6 and 7). In Chapter
3, in order to test the effect of extracellular matrix on the proliferation of the
differentiating mESCs, 400 µl of the homogenized EB suspension was seeded onto
24-well plates pre-coated with different ECM proteins. On days 11, 12, 16, 20, 24 and
28 of the differentiation culture, 400 µl of fresh culture medium (serum-free medium
or SABM) containing 10% (v/v) WST-1 reagent was added to each well and to 4
blank wells as background controls. The plates were incubated in a humidified
atmosphere (37°C, 5% (v/v) CO₂) for 3 h and shaken gently every 30 min. After 3 h,
100 µl of the medium was transferred to 96 wells plate and sampled using Dynes
MRX multi-well plate reader with the test filter at 450 nm and the reference filter at
650 nm.

For the evaluation of the activity of A549 cells cultured on peptide-grafted PDLLA
films (Chapter 5) and functionalized PDLLA films (Chapter 6), 5000 A549 cells were
seeded onto 24 TCP wells, unmodified PDLLA films and modified PDLLA films
fixed to the bottom of each well containing 500 µl A549 medium changed every other
day. On days 2 and 4, 500 µl A549 medium containing 5% (v/v) WST-1 solution was
added to each well. After 1 h, 100 µl of medium was transferred to a transparent 96
well plate and sampled using the same multi-well plate reader.

For the evaluation of the activity of A549 cells cultured within the scaffolds as
described in Chapter 7, 500 µl fresh A549 medium containing 5% (v/v) of WST-1
solution was added to each well containing unmodified or modified scaffolds 4, 8, 12
and 16 days after A549 cell seeding. After 1 h, 100 µl of medium was transferred to
96 well plates and sampled using the same multi-well plate reader.

2.8. Statistical analysis
Statistical analysis of all experimental data was conducted using SPSS16 software
(SPSS Inc., Chicago, USA). To evaluate whether the average of each independent
experimental groups are equal, one-way ANOVA (analysis of variance) followed by
LSD post-hoc test was performed. The probability value $P < 0.05$ between two samples was considered significantly different.
Chapter 3. The effect of culture substratum on the derivation of pulmonary epithelial progenitors from murine embryonic stem cells

3.1. Introduction

Respiratory diseases are debilitating and are the leading causes of morbidity and mortality worldwide. Pulmonary disease such as pulmonary fibrosis is current irreversible and no effective treatment is available. From a tissue engineering point of view, restoration of lung function of the above-mentioned pulmonary disease may be achieved by implanting pneumocytes on an appropriate scaffold into the gas-exchange area of the impaired organ. This area of the lung is comprised of air sacs, or alveoli, lined by two types of epithelial cells; gas exchange occurs across type I pneumocytes while type II pneumocytes secrete surfactant proteins and act as local progenitors for type I cells. At present, it is very difficult to obtain or expand pure populations of primary type II pneumocytes and they do not maintain their phenotype for prolonged periods in vitro [10]. Thus, stem cells have been explored as a source for potential regenerative medicine strategies for the lung. Pulmonary progenitors which can express the features of type II pneumocytes, have been differentiated from both murine and human embryonic stem cells in a 2D environments using soluble factors [16, 203, 219, 220] or cell extract-based reprogramming [17] and, in 3D, using coculture [18].

Cell-matrix interactions are well known to affect lung development, including type II cell function, surfactant protein synthesis and secretion [221], e.g. laminin is essential for alveolar formation and its mRNA increases during lung development [107]. The extracellular matrix (ECM) associates with lung development and also plays roles in maintaining both the type II pneumocyte phenotype in vitro and lung repair in vivo. The morphology of type II pneumocytes and the secretion of surfactant proteins, such including surfactant protein C (SPC) and surfactant protein D (SPC), by type II pneumocytes retained best on collagen membranes [117], laminin [120] or Matrigel-coated plastic [116] with or without growth factors or external forces.
Recently, collagen-GAG [196] and Matrigel hydrogel [22] were used as coatings for scaffolds seeded with primary pneumocytes. A basic principle of tissue engineering is to deliver appropriate cells loaded on scaffolds resembling the in situ tissue both mechanically and physiologically [19]. These constructs should fulfil several stringent requirements [5]. Poly(D,L-lactide) (PDLLA), a hydrophobic polymer synthesized from lactic acid, has been widely used as a scaffold material for different tissue engineering applications and is appropriate for lung tissue engineering in particular because of its elasticity and its biodegradability when processed into scaffolds. The surface properties of a material are known to have significant effects on cell attachment, growth and differentiation. As PDLLA is hydrophobic, which may not be ideal for cell culture, it is essential to render its surface compatible with cells. In addition, in working towards a simplified, easily reproducible system for the production of lung tissue constructs an attempt is being made to both differentiate and grow ESC-derived pneumocytes on the scaffold. The study was performed in two steps. Firstly, the effects were studied of different ECMs coated onto tissue culture plastic (TCP) on the differentiation of pulmonary epithelial progenitors from mESCs. The pulmonary progenitors, derived using the three-step differentiation protocol [203], express the features of the type II pneumocytes. The protocol starts by forming EBs from the undifferentiated mESCs. The EBs were then differentiated for 10 days before being seeded on to the protein-coated TCP. Day 10 EBs were cryosectioned and immunostained to investigate the spatial distribution of the endodermal markers and the basement membrane. On day 28, the differentiating culture was analysed for expression of type II pneumocyte markers. Based on the results obtained, protein coatings that were found to induce higher expression of type II pneumocyte markers in the differentiating mESCs were further investigated as coatings on PDLLA films, in order to select an appropriate coating for future 3D constructs of distal lung.

Protein coating can change the surface properties of the coated materials dramatically. The change in the surface properties can alter cell responses, such as attachment, migration, proliferation and even the phenotype. Therefore, the surface properties of ECM-coated TCP and PDLLA were also studied using contact angle and ζ-potential measurements. The former unveiled the wettability of the protein-coated surfaces and the latter was used to evaluate the overall chemistry of the protein-coated surface [214].
3.2. Methodology

Five different proteins, including gelatin (GN), collagen (CO), laminin (LN), fibronectin (FN) and ECM replacement Matrigel (ML), were coated to TCP (Chapter 2.5.1.) The water contact angles (Chapter 2.6.2) and the \( \zeta \)-potential (Chapter 2.6.1) of the protein-coated TCP were recorded. For the derivation of pulmonary progenitors, embryoid bodies were generated from undifferentiated mESCs for 10 days (Chapter 2.2.2.2). On day 11, some EBs were cryosectioned and stained for the expression of TTF1, laminin and Foxa 2 (Chapter 2.3.3). Other differentiated EBs were seeded on protein-coated TCP, and the differentiation progressed for next 18 days (Chapter 2.2.2.3.1). The preliminary experiments demonstrated that EBs did not attach to uncoated-TCP or uncoated PDLLA, so these two surfaces were not further evaluated using differentiation culture. Fluorescence microimages were taken on day 21 and 28 of differentiation (Chapter 2.3.1). On day 28, mRNA expression of SPC, SPD, Auqaporin 5, GAPDH and beta-actin were analyzed using RT-PCR (Chapter 2.3.2). On the same day, SPC-eGFP positive cells were counted using flow cytometry (Chapter 2.3.4). To evaluate the metabolic activity of the differentiation culture, WST-1 assay was performed on day 11, 12, 16, 20, 24 and 28 (Chapter 2.7.4).

Basing on the results obtained, LN, ML and the experimental control, GN, were further coated to PDLLA (Chapter 2.6.2). The surface properties of protein-coated PDLLA films were recorded using water contact angles and the \( \zeta \)-potential. The same differentiation experiments and characterizations were performed on mESCs differentiated on protein-coated PDLLA films.

3.3. Results

3.3.1. Transmitted light and fluorescence microscopy

To assess the spatial distribution of the endoderm and possible formation of pulmonary lineages in day 10 EBs, Foxa2 and TTF-1 were immunostained. Numerous Foxa2-immunoreactive cells were found, with the vast majority lying at the periphery of the EBs (Figure 3-1A). A smaller population of TTF1-immunoreactive cells was also present in the same area (Figure 3-1B). Immunostaining of laminin revealed basement membrane formation just beneath the outermost cell layer of the EBs (Figure 3-1C), although it did not appear to be contiguous. The discontinuity of
basement membrane was also observed by examination of other five sectioned EBs.

At the end of day 21 differentiation culture, SPC-eGFP-expressing cells were observed in all experimental groups (Figure 3-1D-K), with their frequency varying greatly between different colonies grown under the same experimental conditions, therefore, it is not possible to evaluate the percentage of SPC-eGFP positive cells in the whole population under fluorescence microscopy. Most SPC-eGFP-positive cells lay at the margin of the adherent EB clump and tended to form clusters. In many colonies, the SPC-eGFP positive cells lined the characteristic cyst at the core of the EB (Figure 3-1H, J). At the end of day 28, cells exhibited some autofluorescence which obscured the SPC-eGFP-positive cells and, therefore, these fluorescent images were not representative (Figure 3-2). The autofluorescence was the result of SABM, which only “selected” the pulmonary progenitors and had chronic toxicity to other cells. Those dying cells therefore exhibit strong autofluorescence.
Figure 3-1 A-C: left: immunostaining of cryosectioned EBs at the end of day 10, right: the negative control of each antibody. (A) Foxa2-immunoreactive cells (red) lying around the border of the cell cluster. (B) TTF1-immunoreactive cells (red). Positive cells are also seen at the periphery of the EB but in lower numbers than those expressing Foxa2. (C) Immunoreactivity for laminin (red) appears to form a layer under the outermost ring of cells. Nuclei (blue) stained with DAPI in all cases. D-K: left: SPC-eGFP positive cells at the end of day 21; middle: transmitted light photograph of the same field; right: the overlay of fluorescence images and transmitted light photograph. Transcription of the SPC gene, and therefore differentiation of pulmonary progenitors, was demonstrated by SPC-eGFP fluorescence (green) in differentiating EBs grown on (D) TCP-GN; (E) TCP-CO; (F) TCP-LN; (G) TCP-FN; (H) TCP-ML; (I) PDLLA-GN; (J) PDLLA-LN; (K) PDLLA-ML. More than 4 images of independent samples from the same conditions were analyzed.
Figure 3-2 Microscopic images taken at the end of day 28 differentiation. (A) Left: transmitted light photograph of TCP-GN. Right: Green fluorescence channel from the same sample. (B) Left: transmitted light photograph of PDLLA-LN. Right: Green fluorescence channel from the same sample. Strong autofluorescence was observed from the green fluorescence channel, obscuring the SPC-eGFP positive cells.

3.3.2. Evaluation of the mRNA expression of differentiated cells using RT-PCR

Figure 3-3A shows the mRNA expression profiles of differentiated cells cultured on protein-coated TCP at the end of day 28. Three independent samples of each condition were shown. It appeared that expression of SPC by the cells was influenced by the substratum upon which they are cultured, with levels of expression appearing to be higher on TCP-LN and TCP-ML than on TCP-GN. Cells grown on TCP-CO or TCP-FN expressed less SPC mRNA than those grown on TCP-GN. All other mRNA expression levels, except those for GAPDH mRNA which was used as housekeeping gene, showed similar trends as those seen for SPC mRNA expression. Cells grown on TCP-CO had the lowest levels of each mRNA assayed except for that of GAPDH. GAPDH mRNA expression was very consistent in every experimental group compared with beta-actin mRNA. On protein-coated PDLLA, the mRNA expression
profile was different. Cells grown on PDLLA-LN showed the highest expression of each gene tested except housekeeping GAPDH (Figure 3-3B). Cells cultured on PDLLA-ML also had mRNA expression levels higher than those obtained for the GN control. Beta-actin and SPD mRNA expression showed a similar tendency to that of SPC mRNA.

![Figure 3-3 Results of RT-PCR analysis of mRNA expression profiles of differentiated cells grown on coated (A) TCP and (B) PDLLA at the end of day 28 differentiation. Expression of markers for type I (aquaporin 5) and type II (SPC, SPD) pneumocytes was seen in EBs grown on TCP coated with GN, CO or LN but the strongest expression occurred in EBs grown on LN-coated PDLLA. The symbol – stands for negative control. The symbol + stands for positive control, which used cDNA from MLE-12 cells, an immortalized murine lung epithelial cell line.]

3.3.3. EBs attachment, cell activity and cell spreading from EBs
The effect of different ECM proteins on cell attachments and activities was assessed with the WST-1 assay. Initial EB adhesion to TCP-LN, TCP-FN and TCP-ML was better than that to TCP-GN and TCP-CO (Figure 3-4A). Clearly, some EBs did not attach to TCP-GN and TCP-CO at the end of day 12, resulting in a drop in optical density (OD; directly proportional to cell activities) at 450 nm wavelength. As for protein-coated PDLLA, drop in OD was observed on PDLLA-GN and PDLLA-LN (Figure 3-4B). Whether or not that EBs can attach to protein coated materials was found determined by the cell spreading from the EBs (Figure 3-4C-J). The cell spreading on day 12 were in agreement with the OD obtained from WST-1. Cell
activities were previously found to increase in this system from day 11 to day 20 [203]. Similarly, cells in all conditions demonstrated slight increase in overall cell activities from day 12 to day 20, which may be the result of increased total cell number. On day 21, the serum-free medium was replaced with SABM. From day 20-24, the overall cell activity in all conditions stayed at a similar level or dropped. It was very likely due to the chronic toxicity of SABM to non-pulmonary cells. From day 24-28, activities of cells on protein-coated TCP dropped, whereas activities of cells on protein-coated PDLLA increased, with ML-PDLLA showed the highest increase. The addition of SABM resulted in increased numbers of dying and dead cells, as observed under light microscopy (Figure 3-5A). SABM can also be the cause of strong autofluorescence of cells differentiated for 28 days (Figure 3-2 and Figure 3-5B) as mentioned before.
Figure 3-4 (A-B) Results of WST-1 assay of cell activity. The activity of cells on ECM-coated TCP or PDLLA were found to increase slightly from day 11 to day 20. A different pattern was noted after day 20 when the overall cell activities on ECM-coated TCP stayed at the same level (day 20-24) then fell (day 24-28) while those on ECM-coated PDLLA stayed or fell (day 20-24), and recovered (day 24-28). 5 independent samples in each condition were evaluated. The WST-1 experiments were repeated for 3 times. C-J: Light microscopy of cells growing on ECM-coated TCP and PDLLA at the end of day 12.
Cells are shown growing on (C) TCP-GN; (D) TCP-CO; (E) TCP-LN; (F) TCP-FN; (G) TCP-ML; (H) PDLLA-GN; (I) PDLLA-LN and (J) PDLLA-ML. On PDLLA, cells were seen to extend from EBs to the greatest extent when grown on Matrigel-coated PDLLA film (J). Scale bar corresponds to 500µm.

3.3.4. Determination of eGFP-positive cells by flow cytometry

Gating of target cells in the differentiating culture was complicated, especially when
they comprised only a low percentage of the whole population. As there were many dead cells, as well as debris from both cells and the protein coating, after dissociating the adherent EBs by trypsin/EDTA, size gating and propidium iodide uptake were used for exclusion. The size of the SPC-eGFP-positive population assessed by flow cytometry generally in agreement with the SPC mRNA expression profile (Figure 3-3A-B, and Figure 3-6D-E). The percentage of SPC-eGFP positive cells was ranged from 1.2% (TCP-CO) to 5.6% (TCP-ML). TCP-LN and TCP-ML increased the frequency of SPC-eGFP positive cells 1.9 and 2.3 fold, respectively, as compared with those differentiated on TCP-GN. However, there is no significant difference between TCP-LN and TCP-ML. PDLLA-LN and PDLLA-ML increased the frequency of SPC-eGFP positive cells 2.7 and 4.3 fold, respectively, as compared with those differentiated on PDLLA-GN. Similarly, there was no significant difference between PDLLA-LN and PDLLA-ML.

![Flow cytometry graphs](image)

Figure 3-6 Flow cytometry of SPC-EGFP-expressing cells performed at the end of day 28. (A) Gating was done to exclude small cell debris in the low forward scattering region. (B) Propidium iodide was used to rule out necrotic cells which have around 100 times higher fluorescence level in the FL3 channel. Necrotic cells were often found in the terminal differentiation culture, especially in the middle of adherent EBs. The peak in the high FL3-H region stood for the PI that binded to the DNA of dead cells (C) A threshold was set in the green fluorescence channel FL1 of the negative control of flow cytometry (non-SPC-eGFP-transfected mESCs differentiated in parallel with the transfected mESCs). (D) The percentage of SPC-eGFP-positive cells cultured on protein-coated TCP was compared with cells grown on the control (TCP-GN). (E) The percentage of SPC-eGFP-positive cells cultured on
protein-coated PDLLA was compared with cells grown on the control (PDLLA-GN). The numbers shown in each column stand for the percentage of SPC-eGFP positive cells of the whole population. Asterisks specify statistically significant difference from value of control (TCP-GN and PDLLA-GN) (P<0.05). 4 independent samples in each condition were evaluated. The flow cytometry were performed for 3 times.

### 3.3.5. Surface properties of the ECM-coated TCP and PDLLA films

All the protein-coated surfaces except TCP-FN had lower advancing and receding contact angles than the uncoated ones, as measured by either the sessile drop or captive bubble method (Table 3-1). Some contact angles measured using the two methods differed significantly. For example, $\theta_A$ of TCP-ML measured by the sessile drop method was comparable to that of TCP-CO. However, $\theta_A$ of TCP-ML measured by captive bubble methods was much lower than that of TCP-CO. This difference between contact angles obtained by sessile drop and captive bubble was due to the intrinsic discrepancy of the measuring principals: contact angles measured using the sessile drop method are characteristic of the “dry” polymer surface while contact angles measured using the captive bubble method are characteristic of the “wet” or hydrated materials surface. Therefore, the positive effects of the coating on enhancing the wettability could be observed clearly in the results obtained using captive bubbles, especially in the receding contact angles. Despite the enhanced wettability with protein coatings, the SPC expression of the differentiated cells did not parallel the wettability, suggesting that the SPC expression is independent of the wetting behaviour of the substrate. Figure 6A-B shows the $\zeta$-potential courses of the protein-coated TCP and PDLLA films as a function of pH ($\zeta=f(pH)$). Both TCP and PDLLA films contain mainly Brønsted acidic function groups at the surfaces, which resulted in an isoelectric point (iep), where $\zeta=0$, at a low pH and a plateau ($\zeta_{plateau}$) in the alkaline region. This was understandable because most TCPs were oxygen plasma-treated and, thus, contained many acidic functional groups. All the different protein-coatings on TCP, except TCP-FN, were found to have a less acidic surface than uncoated TCP itself, according to the shift in IEP to a higher pH. Introduction of proteins, therefore, increased the numbers of basic groups, such as amines. For protein-coated PDLLA, all samples had a higher IEP, also suggesting a less acidic surface. TCP and PDLLA coated with the same proteins, except for LN, showed
similar values for IEP and $\zeta_{\text{plateau}}$. PDLLA-LN had a higher IEP and lower $\zeta_{\text{plateau}}$ than TCP-LN. The former showed that PDLLA-LN contains fewer acidic functional groups and the latter that there were more dissociating acidic functional groups at high pH. The difference in the IEP and $\zeta_{\text{plateau}}$ of laminin 332 coating on different materials suggested dissimilar unfolding conformations of laminin which subsequently altered the mESC responses.
Table 3-1 Water contact angles of TCP- and PDLLA-coated with/without ECM proteins. 6 independent samples in each condition were evaluated. The contact angle measurements were repeated twice.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Contact angle measured by sessile drop method (dried surface)</th>
<th>Contact angle measured by captive bubble method (wet surface)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Advancing</td>
<td>Receding</td>
</tr>
<tr>
<td>TCP</td>
<td>64.3±1.1</td>
<td>13.6±1</td>
</tr>
<tr>
<td>TCP-GN</td>
<td>43.3±4.6</td>
<td>14.0±1.3</td>
</tr>
<tr>
<td>TCP-CO</td>
<td>50.7±1.5</td>
<td>12.7±0.3</td>
</tr>
<tr>
<td>TCP-LN</td>
<td>57.1±0.8</td>
<td>14.8±0.6</td>
</tr>
<tr>
<td>TCP-FN</td>
<td>68.2±2.2</td>
<td>13.8±1.1</td>
</tr>
<tr>
<td>TCP-ML</td>
<td>51.7±1.4</td>
<td>16.5±0.7</td>
</tr>
<tr>
<td>PDLLA</td>
<td>73.6±2.3</td>
<td>57.0±1.9</td>
</tr>
<tr>
<td>PDLLA-GN</td>
<td>62.2±1.2</td>
<td>21.0±1.7</td>
</tr>
<tr>
<td>PDLLA-LN</td>
<td>54.3±4.4</td>
<td>13.3±1.8</td>
</tr>
<tr>
<td>PDLLA-ML</td>
<td>56.3±2.7</td>
<td>16.3±0.9</td>
</tr>
</tbody>
</table>

Figure 3-7 ζ-potentials of TCP and protein-coated TCP (A) as well as PDLLA and protein-coated PDLLA (B) measured as function of pH in 0.1 mM KCl supporting electrolyte. All protein-coatings on both TCP and PDLLA, except TCP-FN, were found to reduce surface acidity as shown by the IEP for each (pH at zero potential [0mV]). 2 independent samples in each condition were evaluated. The ζ-potential measurements were repeated for 3 times.

3.4. Discussion

This study aimed to test the effects of different extracellular matrix (ECM) proteins on the differentiation of mESCs cells to pulmonary epithelium. A previously published
three step differentiation protocol [203] was used to induce the differentiation of pulmonary progenitors from mESCs on TCP and PDLLA coated with a range of ECMs. Differentiating mESCs appeared to express more pulmonary epithelial markers on laminin and Matrigel than on gelatin on day 28. Higher frequency of SPC-eGFP positive cells were observed on laminin and Matrigel than the gelatin at the end of the differentiation course. As laminin and Matrigel can unfold in different conformations which may affect the mESC differentiation into pulmonary progenitors when adsorbed to PDLLA, Laminin 332 and Matrigel were further investigated as a coating for PDLLA. The results demonstrated that laminin 332 induced higher SPC expression than Matrigel and the control (gelatin) did, suggesting that the bioactive motifs exposed by laminin 332 adsorbed to the PDLLA films are favoured for the differentiation of mESCs into pulmonary progenitors.

When the mESCs formed embryoid bodies (EBs), activin A was added to the culture medium to enhance the formation of the endodermal lineage [143, 222]. Colleagues have shown previously that expression of the endodermal markers Foxa2 and Sox17 reaches a peak within EBs on day 10 of differentiation [203]. Therefore, EBs were seeded EBs onto protein-coated substrata at this stage. The peak of Foxa2 expression at day 10 is consistent with the current observation that Foxa2-immunoreactive cells formed a large proportion of the day 10 EBs. The smaller percentage formed by TTF1-immunoreactive cells also agrees with the previous finding made by colleagues that the expression of TTF1 just becomes detectable from day 11 [203].

ECM proteins were introduced to the culture at the adherent culture stage (day 11-20). Serum-free medium was added to induce the random differentiation of mESCs after endoderm formation. Thus, if a certain ECM protein plays an important role in pneumocyte formation, there would be increased expression of the marker for type II pneumocytes, surfactant protein C (SPC), by mESCs differentiating on it. The mESCs used in the study were transfected with a 4.8kb murine SPC promoter/EGFP construct so that transcription of the SPC gene and, thus, differentiation to the type II pneumocyte phenotype, was marked by expression of green fluorescence. SPC-eGFP positive cells were observed at the end of the adherent culture stage. Early stage EBs have been reported to show endodermal cells on their outer surface and primitive ectodermal cells in the middle [223, 224]. The current finding that SPC-EGFP cells
located mainly at the borders of the adherent EBs is in agreement with this observation. It was also observed that the basement membrane beneath the monolayer of the EB surface was discontinuous. The monolayer is likely to be visceral endoderm [224], which will not differentiate into the pulmonary lineage. The enhanced expression of SPC by mESCs differentiated on LN and ML suggested that either an indirect effect was made by ECM on the monolayer cells which further affected the definitive endoderm or that some of the definitive endoderm cells had direct contact with the ECM coating due to the discontinuity of the basement membrane.

Most SPC-EGFP cells were seen to form clusters, reflecting the necessity of cell-cell interactions during ESC differentiation. On day 28 of culture, following the switch to small airway basal medium (SABM) (day 21-28), a high level of autofluorescence was seen in the cells. SABM has been noted previously to increase cell death in differentiating mESC cultures [16] and these cells, in addition to those dying naturally during the terminal stages of differentiation, are likely to have contributed to the autofluorescence. Coating TCP with LN or ML gave higher levels of SPC expression than coating with GN. This corresponds with the recent finding that ML induces high endoderm gene expression whereas collagen has lower inductive potential for differentiation [225]. As the regulation of SPC expression occurs independently of that of other surfactant proteins [226], the fact that increased SPD expression was also seen suggests that a higher degree of type II pneumocyte differentiation, through to the mature phenotype, occurred on TCP-LN, TCP-ML and PDLLA-LN than on other surface-coating combinations. A characteristic feature of type II pneumocytes is their transdifferentiation into type I pneumocytes in vitro, so the increased expression of the type I cell marker, aquaporin 5, also leads to the same conclusion.

It was recently reported that ECM proteins can induce EB differentiation by seeding randomly differentiated 5 day EBs onto collagen gel that incorporated FN and LN. FN was reported to induce dose-dependent endothelial differentiation and LN was shown to guide the differentiation of the cardiac lineage [227]. Comparing these observations with the current finding that LN induces the differentiation of pneumocytes from EBs with enhanced endoderm formation suggests that the effect of exogenous ECM proteins on stem cells might be stage-specific. The effect of the ECM proteins on embryonic development is well known to be stage-specific. For example, expression
of LN subunit α1 is limited to the first trimester in human embryos, while LN subunit α5 expression is detectable from early fetal lung development and thereafter [228]. The LN used in the present study, LN 332, is composed of subunits α3, β3 and γ2. Immunoreactivity for LN 332 has been found on the basement membranes of airway epithelium and alveolar parenchyma throughout the development of the human lung [102, 229]. However, a recent report indicates that LN γ2-deficient mice still showed normal branching morphogenesis and epithelial differentiation until the saccular stage [230]. Its effect on alveolarization is still unknown because the LN γ2-deficient mice died before the alveolarization stage. The increased number of SPC-eGFP-positive cells observed differentiating on TCP-LN implied a positive effect of LN 332, either on the differentiation of the lung epithelial lineage from endoderm or on type II pneumocyte differentiation. In vivo, type I collagen-deficient mice can still develop a lung with normal structure [231], suggesting that either type I collagen is crucial to the process or its function in lung may be shared by other collagens. This might explain why TCP-CO was found in this study not to enhance SPC expression and, thus, type II pneumocyte differentiation. In embryonic lung development, FN is more involved in branching morphogenesis and airway formation [232]. Its role in type II cell differentiation is less obvious. In vitro, FN is unable to maintain the phenotype of type II cells [233]. The relative lack of involvement of FN in pulmonary alveolar differentiation may explain the lower SPC expression found in cells grown on TCP-FN. Matrigel is an EHS sarcoma-derived basement membrane extract. Its main component, LN 111 (formerly named LN 1) [95], has a critical role in lung development. Antibodies to LN 111 reduce the growth of embryonic lung explants and their branching morphogenesis in vitro [234]. Disruption of LN 111 polymerization causes failure of airway basement membrane formation [235]. The increased SPC expression observed in mESC differentiating on TCP-ML agrees with this finding. In this research, only a single exogenous ECM protein was applied to the differentiation culture after endoderm formation; more tests are needed to determine the stage-specific or the synergistic effects of the ECM.

It has been reported previously that beta-tubulin, a cytoskeleton protein, is not an appropriate internal control for stem cell differentiation [236]. In this study, results were obtained that confirmed this observation. Beta-actin expression was highest in cells grown on ML or LN coating on TCP and PDLLA, respectively. As beta-actin is
part of the cytoskeleton, its expression is also strongly related to the substratum [237]. Beta-actin regulation, which is mediated by integrin and focal adhesion protein assemblies, can be activated by ECM [238]. The regulation of beta-actin by the substratum is at the transcription level [239]. The integrin signalling pathway and ECM work in a positive feedback mechanism which induces the cells themselves to produce more ECM [240]. It is also worth noting that the strong correlation between the expression of SPC, SPD and beta-actin mRNAs. This is in agreement with the literature on primary type II cell culture in vitro [241], which suggests that the cytoskeleton can regulate SPC mRNA expression and maintain type II cell differentiation. Regulation of SPC mRNA is very likely mediated by polysomes which associate with 3’ tail of mRNA and, thus, can transport and stabilize the mRNA to a specific location within the cell [242]. Type II cells cultured in vitro on attached collagen lost surfactant protein secretion and phenotype, but both characteristics recover after detaching the attached gel so that it floated, suggesting the involvement of the cytoskeleton in type II differentiation [70, 117].

WST1 cell proliferation assay was used to evaluate the overall cell activities. An interesting finding of largest increase in metabolic activity of cells on PDLLA-ML from day 24 to day 28 suggesting that ML, when coated on PDLLA, may help the differentiating mESCs to recover from the stressed “selecting effect” caused by SABM. It may be one of the reasons why the number of SPC-eGFP positive cells induced by PDLLA-ML was 4.3 folds higher than by PDLLA-GN.

ECM proteins have been used for decades as coatings to render a material surface bioactive [172-174]. Once an ECM protein solution comes into contact with a solid surface, a dynamic, non-specific protein adsorption process occurs in which the protein changes its conformation to the lowest energy state [176]. This process is affected by the chemistry of the solid surface and, therefore, in this report, the overall surface chemistry was evaluated by two methods, contact angle measurements using sessile drops and the captive bubble technique and ζ-potential measurements. Using the sessile drop technique alone to determine the contact angle a cell culture material could generate misleading information. For example, θₐ of “dry” TCP-CO is amongst the lowest of all protein-coated surfaces but the θₐ of “wet” TCP-CO was amongst the highest. As PDLLA films were immersed in cell culture medium during
differentiation, the captive bubble technique is more representative.

It can be expected that different level of exposure of cell-binding ligands were presented on both coatings. This difference may contribute to the fact that SPC expression of cells on PDLLA-LN was much higher than PDLLA-ML whereas the SPC expression of cells on TCP-ML was slightly higher than that of TCP-LN.

ζ-potential measurements allow determination as to whether two surface are similar by measuring the IEP and ζ\text{plateau} of the materials compared. The IEP shifts to higher pH if the surface becomes less acidic after protein coating. ζ\text{plateau} is governed by two factors: the dissociating functional group and the hydrophilicity of a surface. ζ\text{plateau} becomes more negative if the dissociating functional groups are more acidic. In addition, ζ\text{plateau} is less negative in a more hydrophilic surface due to the higher interaction between water and materials surface, i.e. the more hydrophilic a surface the more water will adsorb removing potential adsorption sites for ions [243]. The curves of ζ=f(pH) of TCP-GN and PDLLA-GN are alike, as are TCP-ML and PDLLA-ML. This implied similar protein coating on both materials. In contrast, TCP-LN and PDLLA-LN have distinct IEPs, ζ\text{plateau} and the slope of the ζ=f(pH), implying rather dissimilar coating properties, despite the same protein were coated. This difference may contribute to the higher SPC expression on PDLLA-LN due to the different level of exposure of cell-binding ligands. To conclude, IEP and ζ\text{plateau} do not correlate with the SPC expression of the differentiated cells, but both factors were good tools to evaluate protein coatings.
3.5. Conclusion

The potential of ECM proteins to affect the differentiation of ESCs into pulmonary progenitors was investigated using the three-step differentiation protocol. The current differentiation protocol was modified and improved by introducing laminin or Matrigel into the differentiation culture. Another important progress for the differentiation of pulmonary progenitors was the introduction of degradable PDLLA, which was used widespread in the field of tissue engineering. It was also found that laminin when coated onto PDLLA films can induce higher pulmonary epithelial marker of the differentiating mESCs than gelatin and Matrigel. If the differentiation protocol can be further refined, this differentiation system could have widespread potential in tissue engineering, developmental studies and the creation of cell-scaffold constructs that can be used for pharmacological and toxicological screening.
Chapter 4. The effect of fibroblast growth factors on the derivation of pulmonary progenitors from murine embryonic stem cells

4.1. Introduction

Stem cells were frequently used as *in vitro* models to study the embryonic development of certain organ [11, 133-135, 244, 245]. In other words, factors that play roles in the development of embryonic lung are very likely to affect the differentiation of mESCs into pulmonary progenitors. Organogenesis of lung involves several forces including transcriptional factors [68, 246], growth factors [87, 247-249], cell-cell interactions and cell-matrix interactions [96, 104, 106, 249]. These forces also work *in vitro*, particularly when stem cells were used as a model to recapitulate the *in vivo* organogenesis. In the previous chapter, the importance of extracellular matrix proteins in the differentiation of pneumocytes from ESCs was highlighted. FGFs play a key role in early lung development and FGF-1, 2, 7 and 10 in particular are expressed during epithelial-mesenchymal interactions in the developing lung [247, 249]. FGFs also participate in alveogenesis and the lungs of transgenic mice with FGF receptor knockout lack alveolar formation [82]. FGF is active during the pseudoglandular stage of embryonic lung development (formation of the bronchial tree between 6-16 weeks in human embryos) and during the crucial time of alveolarization [68]. FGF-1 and 2 and 7 appear to play an integral role in the transformation of the tracheal epithelium [250]. Acting through the FGFR2 receptor, FGF-1 has been detected by immunohistochemistry uniformly throughout the embryonic lung mesenchyme of embryonic day 13 rat lungs [83]. FGF-1 has been found to increase expression of SPA, SPB and SPD [84] in differentiated culture. In addition, FGF-1 induces epithelial budding in early rat embryonic lung cultures [251]. In the absence of cardiac mesoderm, ventral foregut endoderm explants have been observed to respond to exogenous FGF-1 and FGF-2 in a dose-dependent manner, with lower concentrations activating liver specific genes and high concentrations activating lung specific genes [85]. FGF-10 was reported to be essential for early lung formation; FGF-10 knockout-mice do not generate pulmonary structures [87].
In view of the low frequency of target cells obtained using current protocols [203], modulating the composition of the culture medium by supplementation with growth factors may increase the yield of type II pneumocytes from mESCs. As a result, in this report, the effects of FGF-1, 2, 7 and 10 on the efficiency of this \textit{in vitro} differentiation pathway were tested.

\textbf{4.2. Methodology}

In this chapter, the effects of FGF-1, 2, 7, 10 on the differentiation of mESCs into pulmonary progenitors using the 3-step differentiation protocol was studied. High concentrations of FGF-1 and FGF-2 were supplemented to mESCs differentiated on gelatine-coated TCP from day 11 to day 14 (Chapter 2.2.2.3.2). To evaluate whether FGF-1 and FGF-2 can alter the proportion of cells in endoderm and mesoderm lineages, the expression endoderm marker Sox17 and Foxa2 as well as mesoderm marker Brachyary were evaluated at the end of day 14 differentiation (2.3.2.4). Furthermore, to understand whether TTF-1, a transcriptional factor that were expressed at the onset of lung development and by thyroid, immunostaining of TTF-1 and counting positive cells using flow cytometry were performed at the end of day 14 differentiation (Chapter 2.3.4). The expression of SPC, SPD, CC10, Aquaporin 5 and GAPDH were analyzed using RT-PCR at day 28 (Chapter 2.3.2.3). Furthermore, in order to know whether the addition of FGF-1 or FGF-2 could guide more endodermal cells into hepatic lineage, hepatic marker albumin was also investigated at day 28.

To investigate the effects of FGF10 on the differentiation of mESCs into pulmonary progenitors, FGF10 at various concentrations were added to mESCs differentiated on gelatin-coated TCP from day 11 to day 14 or from day 15 to day 18. Conventional PCR and realtime PCR of SPC expression were performed at day 28. A further experiment was performed by supplementing FGF-10 to the mESCs differentiating on Matrigel-coated TCP to understand whether there is a synergistic effect between ECM and FGF-10. Effects of FGF-7 were investigated preliminarily in a way similar to the methodology used to investigate FGF-10, but at fixed concentration of 10 ng/ml of FGF-7 was used.
4.3. Results

4.3.1. Conventional and realtime PCR

The endoderm markers, sox17 and foxa2 RNAs, and the mesoderm marker, brachyury RNA, were recorded at the end of day 14 of the differentiation culture, after addition of FGF-1 and FGF-2 at high concentrations for 4 days. In general, expression levels of sox17, foxa2 and brachyury RNAs were found to be either higher than control (no addition of any growth factors) or unchanged for all FGF-1 and/or FGF-2 treated samples, except the expression of sox17 RNA which was slightly lower in the culture that had received 500 ng/ml FGF-1 (Figure 4-1).

Figure 4-1 Expression of mRNAs of the endoderm markers, SOX17 and Foxa2, as well as the mesoderm marker, Brachyury, of differentiating mESCs at the end of day 14. Sample A was supplemented with 500 ng/ml FGF-1, B with 1000 ng/ml FGF-1, C 100 ng/ml FGF-2, D 500 ng/ml FGF-2 and E 500 ng/ml FGF-1+100 ng/ml FGF-2. All of the FGFs were added between days 11-14. 4 independent samples in each condition were evaluated. The quantitative PCR measurements were repeated twice.

SPC is a specific marker for type II pneumocytes and is not present in any other cell types. Increased SPC mRNA expression was observed in samples treated with 500 ng/ml FGF-1 and 1000 ng/ml FGF-1 (Figure 4-2). SPC RNA expression levels similar to those of the control were seen for cultures that had been supplemented with FGF-2. Supplementation with a combination of FGF-1 and FGF-2 did not yield SPC RNA expression above that of control cultures. To quantify the expression of SPC RNA more precisely, realtime PCR was performed on control, 500 ng/ml and 1000 ng/ml FGF-1 treated samples. Supplementation of the culture medium with 1000 ng/ml FGF-1 resulted in SPC RNA expression that was 2.6 times higher than that of the control (P < 0.05) (Figure 4-2). Samples supplemented with 500 ng/ml FGF-1
presented higher average SPC RNA expression, however, the difference did not reach statistical significance due to the large standard deviation. In addition to SPC RNA, samples supplemented with 1000 ng/ml FGF-1 also contained higher levels of CC10, suggesting the presence of bronchiolar phenotype/s in the cultures. In contrast with the FGF-1 treated samples, FGF-2 treated samples, including those treated with both 500 ng/ml FGF-1 and 100 ng/ml FGF-2, expressed levels of SPD, CC10 and aquaporin 5 that were lower than those of the control.

Figure 4-2 (A) Results of conventional PCR of cells in group 1 and (B) quantitative PCR of SPC mRNA of sample 1-1 (control), 1-2 (500 ng/ml FGF-1) and 1-3 (1000 ng/ml FGF-1) at the end of day 28. * Indicates significant difference from control values (P<0.05). More than 3 independent samples in each condition were evaluated. The quantitative PCR measurements were repeated 3 times.

To assess the differentiation into another endoderm lineage during early adherent culture stage, PCR was performed to detect albumin RNA, present in hepatocytes. No increase in albumin RNA expression was seen in samples treated with FGFs. It was observed that a slight decrease in albumin RNA occurred in samples treated with
FGF-2 and in samples treated with both FGF-1 and FGF-2 compared with the control.

In addition to FGF-1 and FGF-2, the effects of FGF-7 and FGF-10 on SPC RNA expression were also evaluated. Low dose FGF-7 (10 ng/ml) and varying doses of FGF-10 (20 ng/ml to 200 ng/ml) were added to cells during periods A and B. Surprisingly, conventional PCR results indicated that FGF-10 at 20-200 ng/ml had no effect on the differentiating cells cultured on gelatin during day 11-18 (Figure 4-3A). In order to confirm this result, realtime PCR for SPC RNA was performed and similar negative results were obtained (Figure 4-3B). As FGF-10 did not increase SPC RNA expression in cells cultured on gelatin, it was interesting to see whether FGF-10 required additive synergy from an extracellular matrix. Previously, the positive effect was reported of extracellular matrix coating, namely laminin 332 and Matrigel, on pneumocyte differentiation [252]. In this report, 60 ng/ml of FGF-10 was added to cells growing on Matrigel-coated tissue culture plates and the expression of SPC and aquaporin 5 RNAs was evaluated. In contrast with cells differentiated on gelatin, cells differentiated on Matrigel demonstrated higher levels of SPC RNA when 60 ng/ml of FGF-10 was added to the culture during either days 11-14 or days 15-18 (Figure 4-4). Slightly higher levels of aquaporin 5 RNA were found in cells grown under the latter conditions (Figure 4-4). In contrast to FGF-10, FGF-7 did not induce significantly higher SPC expression than FGF-10 in combination with Matrigel coating (Figure 4-4).
Figure 4-3 (A) SPC mRNA expression of FGF-10 treated cells measured by conventional PCR using GAPDH as the housekeeping gene. (B) Relative SPC mRNA expression of the same cells measured by real-time PCR. Both measurements were performed at the end of day 28 differentiation. FGF-10 at 20 ng/ml to 200 ng/ml was added to the culture medium during either days 11-14 or days 15-18. No treated samples demonstrated a SPC expression level different from that of the control. 4 independent samples in each condition were evaluated. Both conventional PCR and quantitative PCR measurements were repeated twice.
Figure 4-4 (A) Conventional PCR measurement of mRNAs in FGF-10 treated cells cultured on gelatin-coated or Matrigel-coated tissue culture plates (B) SPC mRNA expression of the FGF-10 treated cells cultured on gelatin coating measured by realtime PCR (C) SPC expression of the FGF-10 treated cells cultured on Matrigel coating measured by realtime PCR (D) Relative SPC expression of the FGF-7 treated cells cultured on Matrigel coating measured by realtime PCR. All measurements were performed at the end of day 28 differentiation. In (B), (C) and (D), 4 independent samples in each condition were evaluated. The quantitative PCR measurements were repeated 3 times.

4.3.2. TTF-1-positive cells and SPC-GFP-expressing cells

Immunostaining of TTF-1 was performed on attached EBs at the end of day 14 of the differentiation culture. Figure 4-5A demonstrates a representative colony from the day 14 differentiation culture. TTF-1-positive cells were found to form clusters in most of the samples, suggesting the necessity of cell-cell interaction during their differentiation. TTF-1-positive cells were mainly present at the borders of the attached colonies. The frequency of TTF-1-positive cells in each colony appeared to vary. As with
TTF-1-immunoreactive cells, SPC-eGFP-positive cells formed clusters (Figure 4-5C-E) and some showed thin, neuron-alike cytoplasmic processes (Figure 4-5C-E). A similar morphology of SPC-eGFP positive cells were reported recently by another group [253].
Figure 4-5 (A1) TTF-1-immunoreactive cells (green) at the end of day 14 differentiation culture. (A2) The same field showing the DAPI-stained cell nuclei. The overlay of (A1) and (A2) was shown in (A3).
(B) The negative control of TTF-1 staining. (C1) SPC-eGFP positive cells of day 21 differentiation culture. (C2) The same field as C1 combined with light microscopy image. (D) SPC-eGFP-positive cells of adherent EBs prepared by cryosection at the end of day 21. (E) Confocal laser microscopy image of SPC-eGFP cells at the end day 21.

Due to the variability of each colony, the frequencies of TTF-1 positive cells in each condition were further evaluated using flow cytometry (Figure 4-6). A single peak, with only 0.76% of cells scored TTF-1 positive, was observed in the experimental control, in which no FGF-1 and FGF-2 was added during differentiation (Figure 4-6C). Because mESCs had already differentiated into various cell types when the flow cytometry was performed at the end of the day 14, one has to notice that the single peak observed in Figure 4-6C is the addition of many peaks induced by various cell types, with barely any TTF-1 positive cells in it. When TTF-1 positive cells were presented in the culture, as illustrated in Figure 4-6G-H, we observed a “peak shift”, rather than a separate peak. The peak shift suggested that the added FGF-2 altered the differentiation profile of the mESCs. Cells that had green fluorescence intensity above the threshold in FL1-channel were regarded as TTF-1 positive cells. Cells treated with 500 μg/ml of FGF-2 had highest TTF-1 positive frequency; cells treated with both 500 μg/ml of FGF-1 and 100 μg/ml of FGF-2 had second highest frequency of TTF-1 positive cells. In contrast, 100 μg/ml FGF-2-treated cells had a frequency of TTF-1 positive cell similar that of the cells with no FGF treatment.
Figure 4-6 Flow cytometry of TTF-1-immunoreactive cells performed at the end of day 14. Figures (A) and (B) showed negative controls for flow cytometry (experimental control stained without 1st layer antibodies). In (A), a size gate was applied to exclude cell debris. (B) A threshold was defined to score 0.1% of cells positive. The gating of both (A) and (B) were applied to all other samples. Figures (C) to (H) represented samples stained with TTF-1 antibodies. (C) Sample 1-1, the experimental control (no FGF supplement was added during differentiation). The outline of the histogram of the experimental control, as demonstrated in (C), was illustrated in (D-H) for better understanding of the peak shift. (D) Sample 1-2 (500 ng/ml FGF-1) (E) sample 1-3 (1000 ng/ml FGF-1) (F) sample 1-4 (100 ng/ml FGF-2) (G) sample 1-5 (500 ng/ml FGF-2) (H) sample 1-6 (500 ng/ml FGF-1+100 ng/ml FGF-2). Four independent samples in each condition were evaluated.
4.4. Discussion

The protocol described in this study was reported to generate differentiated cultures that comprised cells expressing the transgene and mRNAs for SPA, SPB, SPC, SPD, CC10 and aquaporin 5 from SPC-eGFP-transfected mESCs [201]. In the same report, the negative staining of SPC and immature development of the lamellar bodies suggested that the SPC-GFP positive cells could be progenitor cells for type II pneumocytes [203]. Furthermore, only a low percentage of cells could be directed to enter the pulmonary lineage using the protocol. In order to improve the yield, FGF-1, 2, 7 and 10 were added into the differentiation culture during the cell adhesion stage. The concentrations of FGF used for each group were selected on the basis of data obtained from in vitro organogenesis or morphogenesis models reported previously [83, 85, 92, 254].

In vivo, ventral foregut endoderm demonstrates multipotent capacity. It can be directed to generate pancreas [255], liver [256] and lung [85] by controlling the addition of FGF. The expression of TTF-1 in the ventral foregut endoderm was found to play a crucial role in early lung development [246, 257, 258]. It has been reported that direct contact of the cardiac mesoderm with ventral foregut endoderm is required for the initiation of TTF-1 expression in vitro [92]. Initiation of TTF-1 expression can also be observed following supplementation with exogenous FGF-1 and FGF-2 in the absence of cardiac endoderm [85, 92]. These in vitro embryonic explant models provide information on the control of the downstream lineage from endoderm but recapitulation of the events in these models using ESCs is complicated because of the high degree of variance during the differentiation process. Furthermore, little attention had been paid to the effects of high doses of FGF-1 and FGF-2 on the differentiation of the downstream endoderm lineage from ESCs. Only low doses of FGF-1, reported to induce hepatocyte formation, has ever been investigated [259, 260].

Growth factor addition does not direct ESC differentiation exclusively into a specific cell type, rather it alters the relative proportions of the various phenotypes that emerge [142]. In the experiments, we added FGF-1 and FGF-2 to the differentiating mESCs on the gelatin-coated TCP, which did not contain any trace amounts of growth factors like Matrigel coating. It was found that higher expression of SPC mRNA occurred in
samples which received 1000 ng/ml of FGF-1 compared with 500 ng/ml of FGF-1, suggesting a dose-dependent effect. FGF-1 has previously been observed to elicit immediate endodermal budding of embryonic lung tissue [83] and alter surfactant protein gene expression [69]. Consistent with these observations, increased SPC RNA expression was observed in FGF1-treated differentiating cultures.

Despite the elevated expression of both endodermal and mesodermal markers induced by high doses of FGF-1 and FGF-2, neither type I nor type II pneumocyte markers were enriched by FGF-2 or by a combination of FGF-1 and FGF-2, suggesting that high doses of FGF-2 were not able to upregulate the pulmonary lineage differentiation. FGF-2 has been shown to be a more effective inducer than FGF-1 of surfactant protein expression by pulmonary epithelium [86]. Additionally, FGF-2 is abundantly expressed and localized in epithelial basement membranes at the late stage of fetal lung development, during which time lung epithelial cells undergo major morphological changes and cytodifferentiation [86]. Consequently, the ability of the “early” adherent culture to be programmed by FGF-2 signalling may be restricted. FGF-1, however, appears to act earlier in lung development and increases in SPC mRNA expression observed in this experiment may be attributed to the role of FGF-1 in eliciting lung bud formation necessary for branching morphogenesis [83].

In contrast to the reports that high dosage of FGF-2 can enhance pulmonary lineage formation in embryo explants [85], the negative effect of FGF-2 against the differentiation into pulmonary lineage, as observed in the current experiments, gave rise to a question: does addition of FGF-2 guide the endoderm cells into other derivatives, such as the hepatic lineage? In order to answer this question, the presence of albumin, a marker of hepatocyte, was assessed. No increase was noted in albumin expression following medium supplementation with either FGF-2 or FGF-2 plus FGF-1, suggesting that FGF-2 can also not lead the differentiation into hepatic lineage under our experimental conditions.

During development, TTF-1 is expressed by distal lung progenitors and thyroid follicular cells, another endoderm derivative. It is therefore not specific to lung epithelium. However, TTF-1 is a transcriptional factor that serves as a master gene to integrate genetic developmental lung morphogenesis and cell lineage determination
[246, 251, 258, 261]. In particular, it has also been shown to modulate critically SPC expression by type II pneumocytes in conjunction with Erm, a transcription factor activated by FGF [262]. Unfortunately, TTF-1 RNA could not be detected using conventional PCR at the end of day 14 of the differentiation culture, despite more than 6 different pairs of TTF-1 primers were tested in the preliminary experiments for this study. Previous publication by our group also showed that even realtime PCR was employed, only very weak expression of TTF-1 was detected at the end of day 14 differentiation [203]. Due to the limitation in time, we did not solve this technical issue or further evaluate TTF-1 using realtime PCR, instead, we immunostained TTF-1 protein using a highly sensitive TTF-1 antibody supplied by Novacastra (New castle upon Tyne, UK). The immunostaining demonstrated clusters of TTF-1-positive cells located at the borders of the adherent EBs. The TTF-1-positive cells made up a large percentage of cells in some colonies. In a small number of colonies, TTF-1-positive cells were not detectable. Flow cytometry presented a more complete picture of frequency of TTF-1 cells. Higher frequency of TTF-1 positive cells were found in sample 1-5 (500 μg/ml FGF-2 supplement, see Table 2) and 1-6 (500 μg/ml FGF-1 + 100 μg/ml FGF-2 supplement, see Table 2) at the end of day 14. In contrast, SPC expression was found higher in sample 1-2 (500 μg/ml FGF-1 supplement, see Table 2) and sample 1-3 (1000 μg/ml FGF-1 supplement, see Table 2) on day 28, suggesting that a major proportion of TTF-1-positive cells found on day 14 did not turn into pulmonary progenitor cells subsequently during day 15 to day 28.

In addition to FGF-1 and FGF-2, FGF-7 and FGF-10, particularly FGF-10, are the most studied growth factors in lung morphogenesis [83, 254, 263, 264]. FGF-10 was initially added at various concentrations to the differentiating cells cultured on gelatin-coated tissue culture plates, but no change in SPC RNA expression could be observed. When the gelatin coating was replaced with Matrigel subsequently, cells supplemented with FGF-10 on day 11-14 and day 15-18 demonstrated enhanced SPC RNA expression. The necessity of the synergistic effect of FGF-10 and Matrigel in in vitro lung explant models was also supported by a previous report [83], which demonstrated that FGF-10 did not support endoderm budding on collagen gels, though budding occurred on Matrigel. The same report showed that FGF produced no response in any tested tissue cultured on collagen. This discrepancy between the synergistic effects of Matrigel and collagen may be because the former contains
heparin sulphate proteoglycan, which has been shown to potentiate the biological activity of FGFs [263, 265]. Matrigel did not work only in the presence of FGF-10, it can enhance the expression of pneumocyte markers and increase the number of SPC-eGFP positive cells in differentiating mESCs without the help of any growth factors (Chapter 3). However, it has to be noticed that even the growth factor-reduced Matrigel, as used in the experiments, also contain trace amounts of growth factors, including 0-0.1 pg/ml of bFGF, <0.5 ng/ml EGF, 5 ng/ml IGF-1, <5 pg/ml PDGF, <0.2 ng/ml NGF, 1.7 ng/ml TGF-beta. Despite these growth factors were at very low concentrations, more experiments should be performed to rule out the possibility that the observed synergistic effect were actually induced by the FGF-10 and the trace amounts of growth factors in the Matrigel coating.

As for FGF-7, 10 ng/ml was chosen as a fact that it is the optimal concentration to enhance the type II pneumocyte maturation [89, 90]. 10 ng/ml of FGF-7 was added to cells cultured on Matrigel. It did not induce any effect when added on day 11-14. When it was added on day 15-18, realtime PCR demonstrated higher average SPC RNA expression with large variation precluding statistical significance.

As no single growth factor can recapitulate the complex natural repertoire of molecular developmental signalling, only a small part of the natural signalling cascade involved in embryonic lung development has been highlighted in this study. The overlapping functions and dynamic interactions of the many growth factors present during in vivo lung development means that further studies, perhaps combining more FGF supplementation on Matrigel-coated tissue culture plates, are required to increase sufficiently the differentiation of distal lung epithelial cells from ESCs for implantation therapy. The use of FGF can also be applied to hESCs because of the similarity of human and murine in terms of development mechanisms.

4.5. Conclusion

It was demonstrated that fibroblast growth factors play roles in the three-step differentiation protocol which we applied to differentiate mESCs toward pulmonary progenitors. High concentration of FGF-1 enhanced the expression of the SPC mRNA of the differentiated pulmonary progenitors, whereas high concentration of FGF-2 did
not show the same effect. A synergistic effect between FGF-10 and the growth factor-reduced Matrigel to enhance the SPC expression of the differentiated pulmonary progenitors was observed. However, more experiments should be performed to investigate whether the observed synergistic effect was caused by the trace amounts of growth factor in the Matrigel.
Chapter 5. Peptide-grafted poly(D,L-lactide) and its application in A549 cell culture and differentiation of murine embryonic stem cells into pulmonary epithelial progenitor cells

5.1. Introduction

Poly(D,L-lactide) (PDLLA) has been used safely in medical applications with FDA approval [266] for decades and can be easily processed into various shapes. Thus, it is a promising scaffold material for tissue engineering applications. As polylactide is a hydrophobic material, efforts have been made to render it more hydrophilic and suitable for the maintenance and growth of various cell types [41, 164, 165, 267], although few publications can be found for lung tissue applications [22, 195-198].

The simplest way to enhance cellular responses to a material is to introduce bioactive molecules to its surface by direct application of protein solutions. For lung tissue, coating with proteins such as laminin was shown to enhance the expression of surfactant protein C, a phenotypic marker of type II pneumocyte, by mature type II pneumocytes [50, 120, 268]. In addition, laminin can upregulate the differentiation of murine embryonic stem cells (mESCs) into pulmonary progenitors [252]. However, during the coating process, where laminin adsorption occurs, is it difficult to evaluate in which configuration the protein adsorbed and which exposed peptide domain confers bioactivity after protein adsorption. In addition, the use of protein coating of scaffold surfaces is prohibitively expensive; furthermore, some commonly-used proteins to coat biomaterials have been derived from tumour cells, making their clinical applications questionable. Small bioactive peptides that can mimic extracellular matrix protein functions and be synthesised using automated synthesis equipment in large scale, such as RGD (Figure 5-1) and YIGSR (Figure 5-1), and therefore, have attracted much attention. RGD and YIGSR have been widely used over the past two decades for the modification of biomaterial surfaces [25, 26, 269].
When cells are seeded onto a solid support, they adhere to the underlying substrate via integrin and non-integrin receptors. RGD is the minimum peptide sequence of fibronectin that mediates integrin-mediated cell binding. As for non-integrin cell adhesion, 67LR, one of the major non-integrin cell adhesion receptors in the plasma membrane, can bind to YIGSR and VAPG sequences of elastin [270]. YIGSR is the minimum sequence of laminin necessary for cell adhesion and 67LR receptor binding of epithelial cells [271]. The YIGSR sequence has been reported to be able to replace the function of laminin coating in primary pneumocyte culture [272].

Here peptide mixtures containing various percentages of RGD and YIGSR were grafted to the surface of PDLLA films using succinic acid as a spacer using the well-established EDC coupling reaction. The prepared peptide-grafted PDLLA films were characterised by water contact angle measurements and AFM. The potential use of peptide-grafted surfaces for the engineering of pulmonary epithelial tissue was investigated in this study using two approaches: firstly, the effects were evaluated of peptide-grafted PDLLA surfaces on the attachment, proliferation and the cytoskeleton of A549 cells, a widely-used line of distal pulmonary epithelial cells, type II pneumocytes. A549 culture was also performed on tissue culture plastic (TCP) and unmodified PDLLA films for comparison with the modified PDLLA films. Secondly, the effects were investigated of the modified surfaces on the differentiation of mESCs into type II pneumocytes and their progenitors.
5.2. Methodology

RGD and YIGSR were dissolved in DD-water at five proportions. The five “cocktails” of the peptide solution were grafted to the 4% ethylenediamine-treated PDLLA films (Chapter 2.5.2 and 2.5.4). Water contact angle and surface roughness using AFM were performed to characterize the peptide-grafted PDLLA films (Chapter 2.6.2 and 2.6.5).

Two independent cell culture experiments were performed on the peptide-grafted surfaces. In the first one, A549 cells were seeded on the peptide-grafted PDLLA films and cultured for 4 days. Cell attachment was evaluated 2h after A549 seeding using Hoechst dye (Chapter 2.7.3). Formation of focal adhesion points and cytoskeleton of A549 cells was determined 24h after cell seeding using immunostaining and phalloidin, respectively (Chapter 2.3.3). A549 population and A549 metabolic activity was evaluated at day 2 and day 4 using hoechst dye and WST-1 reagent (Chapter 2.7.4).

The second experiment was to differentiate mESC into pulmonary progenitors on the peptide-grafted PDLLA films using the 3-step differentiation protocol (Chapter 2.2.2.3.3), similar to the experiments described in Chapter 3. The only difference is that the protein-coated PDLLA films were replaced by peptide-grafted PDLLA films. This experiment is pretty preliminary; therefore only SPC expression of the differentiating cells at day 28 was performed using realtime PCR (Chapter 2.3.2.4). The SPC-eGFP positive cells were recorded using fluorescence microscopy on day 21 (Chapter 2.3.1).

5.3. Results

5.3.1. Surface characterization of peptide-grafted PDLLA

The water contact angles and surface roughness of the peptide-grafted surfaces were evaluated. All peptide-grafted surfaces had lower advancing and receding contact angles than unmodified PDLLA (Figure 5-2). The receding contact angles of 50RGD-50YIGSR, 25RGD-75YIGSR and 0RGD-100YIGSR were even lower than those measured on TCP, which is normally treated by the manufacturer in oxygen.
plasma to enhance the cell responses. A trend of decreasing contact angles could be observed as the YIGSR proportion increased. The contact angle hysteresis, the difference between the advancing contact angle and receding contact angle, was smallest on unmodified PDLLA. Contact angle hysteresis is an index of surface heterogeneity of hydrophilic and hydrophobic domains, but can also be affected by the surface roughness. The contact angle hysteresis was largest for 50RGD-50YIGSR, followed by 25RGD-75YIGSR and 0RGD-100YIGSR. As the roughness parameter $R_a$ of all modified PDLLA was smaller than 1 nm, which is far smaller than the roughness scale that can affect contact angles [27, 200, 273, 274], the contact angle hysteresis of 50RGD-50YIGSR can only be the result of the inhomogeneous distribution of hydrophilic and hydrophobic domains on the surface of modified PDLLA films. It was also found that $R_a$ decreased as the YIGSR percentage increased (Table 5-1). This finding is interesting because the size of YIGSR is larger than the size of RGD. It can be explained by the lower coupling yield of YIGSR as compared to the RGD peptide [26] and will be explained in detail in the discussion.
Figure 5-2 Dynamic (advancing and receding) captive bubble water contact angles on PDLLA, TCP and surface-treated PDLLA films (n=6). The advancing and receding contact angles of all surface-modified PDLLA samples were significantly lower than PDLLA (P<0.005). + and ++ stand for significant difference between peptide-grafted surfaces and TCP (+ for P<0.05; ++ for P<0.005). The water contact angle measurements were repeated 3 times.
Table 5-1 Roughness parameter ($R_a$) of PDLLA and surface-modified samples. All samples have $R_a$ larger than PDLLA with P<0.005. 4 independent samples in each condition were probed. The AFM probing was repeated 3 times.

<table>
<thead>
<tr>
<th>Peptide-grafted surface</th>
<th>$R_a$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDLLA</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>4% ethylenediamine- treated PDLLA</td>
<td>0.25 ± 0.01</td>
</tr>
<tr>
<td>RGD100-YIGSR0</td>
<td>0.51 ± 0.01</td>
</tr>
<tr>
<td>RGD75-YIGSR25</td>
<td>0.47 ± 0.00</td>
</tr>
<tr>
<td>RGD50-YIGSR50</td>
<td>0.43 ± 0.05</td>
</tr>
<tr>
<td>RGD25-YIGSR75</td>
<td>0.41 ± 0.07</td>
</tr>
<tr>
<td>RGD0-YIGSR100</td>
<td>0.29 ± 0.03</td>
</tr>
</tbody>
</table>

5.3.2. Attachment of A549 cells to peptide-grafted PDLLA

Cell attachment can be evaluated by determining the DNA content of the cells attached to the surface 2 h after cell seeding using Hoechst 33258 dye. All peptide-grafted surfaces except 25RGD-100YIGSR and 0RGD-100YIGSR demonstrated higher cell attachment than the unmodified PDLLA (Figure 5-3). However, A549 attachment on all samples were still significantly lower than TCP. Cell adhesion was found to be higher on peptide-grafted PDLLA films with more RGD as compared to those containing more YIGSR.
5.3.3. Cell activity and population of A549 cells on days 2 and 4

WST-1 and Hoechst assays were performed on both days 2 and 4 of the cell culture. The WST-1 assay measured the overall mitochondrial activity of the whole cell population and the Hoechst assay measured the total DNA concentration. After day 4, the A549 cells reached 100% confluence and died quickly. On day 2, no difference in A549 activity was found between all peptide-grafted surfaces and unmodified PDLLA (Figure 5-4). A549 cultured on TCP, however, demonstrated higher activity than the cells cultured on unmodified PDLLA on day 2. On day 4, TCP, RGD100-YIGSR0, RGD75-YIGSR25 and RGD0-YIGSR100 induced higher cell activity levels than unmodified PDLLA. On day 4, the A549 activities on all peptide-grafted PDLLA films except 75RGD-25YIGSR were significantly lowered than TCP.
More A549 cells were found on TCP than on unmodified PDLLA on both day 2 and day 4. On day 2 there were fewer A549 cells on all peptide-grafted PDLLA films than on unmodified PDLLA films, except 50RGD-50YIGSR (Figure 5-5). On day 4 there were more A549 cells on RGD0-YIGSR100 than on unmodified PDLLA. No difference in A549 cell population can be found between the other peptide-grafted samples and unmodified PDLLA. Comparing to TCP, cell numbers on all peptide-grafted surface were significantly lower on both day 2 and day 4.
Figure 5-5 Population of A549 cells at day 2 and day 4 was evaluated by the total DNA content using Hoechst dye ($n = 4$–$5$ for all samples). * and ** stand for significant difference between peptide-grafted surfaces and PDLLA (* for $P<0.05$; ** for $P<0.005$). + and ++ stand for significant difference between peptide-grafted surfaces and TCP (+ for $P<0.05$; ++ for $P<0.005$). The evaluation of cell population using Hoechst dye was repeated 3 times.

5.3.4. Visualization of cell focal adhesion points and cytoskeleton

The localization of F-actin, a cytoskeletal protein, and vinculin, a focal adhesion protein, were visualized 24 h after A549 cells were seeded onto TCP, unmodified PDLLA and peptide-grafted PDLLA films. A549 cells seeded on TCP and PDLLA demonstrated weak cytoskeleton formation (Figure 5-6A, B). Local distributions of the immunoreactive vinculin could be found, nevertheless, no spindle-like structures immunoreactive for vinculin, which is indicative of mature focal adhesion point
formation, were observed at the junction of F-actin-stained and cytoplasmic processes. This pattern suggests immature formation of focal adhesion points. In contrast to cells cultured on unmodified PDLLA and TCP, numerous spindle-like vinculin-immunoreactive adhesion points and distinct cytoskeleton were observed in A549 cells cultured on 100RGD-0YIGSR (Figure 5-6D). Cytoskeleton and adhesion point formation decreased with the decreasing percentage of RGD tripeptides. Spindle-like vinculin-immunoreactive structures were found not just at the cytoplasmic processes of the cells, but also underneath the cytoplasm in 100RGD-0YIGSR, 75RGD-25YIGSR and 50RGD-50YIGSR (Figure 5-6D-F). For cells cultured on 25RGD-75YIGSR and 0RGD-100YIGSR, vinculin immunoreactivity could barely be seen (Figure 5-6G, H).
Figure 5-6 Immunostaining of vinculin and labelling of F-actin in A549 cells cultured on TCP, PDLLA and peptide-grafted surfaces for 24 h. The left panel shows vinculin, and the right column shows F-actin. In the left panel, arrows indicate vinculin with “spindle” shape. In the right panel, arrows indicated strong F-actin within the cytoplasm. Image (A) to (H) stood for: (A) TCP, (B) PDLLA, (C) Negative control for vinculin staining, (D) 100RGD-0YIGSR, (E) 75RGD-25YIGSR, (F) 50RGD-50YIGSR, (G) 25RGD-75YIGSR, (H) 0RGD-100YIGSR.
5.3.5. Differentiation of pulmonary epithelial progenitors from murine embryonic stem cells on peptide-grafted PDLLA

mESCs were differentiated into pulmonary epithelial progenitors on the peptide-grafted PDLLA films and the experimental control, gelatin-coated PDLLA films. The pulmonary progenitors were SPC-eGFP positive and could be observed under the microscope (Figure 5-7A-D). The pulmonary progenitors differentiated on all samples possessed the same morphology, i.e. they were spindle-like and formed clusters within the attached EBs. The number of SPC-eGFP cells in different EBs varied significantly; therefore, a more appropriate characterization of the pulmonary progenitors, expression of SPC mRNA, was performed using quantitative PCR. All peptide-grafted surfaces were found to enhance the differentiation of the mESCs into pulmonary epithelial progenitors (Figure 5-7E) as compared to the control gelatin-coated PDLLA film. Among all the surfaces, RGD0-YIGSR100 enhanced SPC mRNA expression 4 fold more than the gelatin-coated PDLLA. The expression of SPC mRNA was also found to increase as the percentage of the YIGSR peptide increased.
Figure 5-7  SPC-eGFP-positive pulmonary epithelial progenitors were differentiated from mESCs for 28 days on (A) gelatin-coated PDLLA film, (B) 100RGD-0YIGSR, (C) 50RGD-100YIGSR and (D) 0RGD-100YIGSR. (E) The relative levels of SPC mRNA expression of the mESCs differentiated on peptide-grafted PDLLA films compared with the SPC mRNA expression of cells differentiated on the
experimental control, gelatin-coated PDLLA films (* P<0.05; ** P<0.005). 4 independent samples in each condition were evaluated. The quantitative PCR were repeated twice due to the time limit.

5.4. Discussion

The development of a synthetic material suitable for pneumocyte culture is still an underexplored area of research. In the present context, the water wettability and surface roughness of peptide-grafted PDLLA films were investigated and the effect of the composition of the grafted peptide layer on the responses of A549 cells and the differentiation of mESCs cells evaluated. The results suggested that pulmonary epithelial cell behaviour, including cell attachment, focal adhesion point formation, activity and population size, is dependent on the type and composition of the grafted peptide layer on the PDLLA surface. In addition, this report also showed that modification of PDLLA films by peptide-grafting can affect the long-term differentiation of mESCs into pulmonary progenitors.

Bioactive peptides were grafted to PDLLA films pretreated with 4% ethylenediamine, using a process termed aminolysis where ethylenediamine breaks down the backbone of the polyester chains and introduces three types of hydrophilic species to the surface; primary amines, alcohols and amides. These hydrophilic species lower the water contact angles of the modified polymer surface as demonstrated in the literature [33, 34, 40]. The introduced primary amines offer sites for peptide grafting using EDC coupling reaction, which can couple carboxylic acids and amines in two steps [275, 276]. Grafting RGD and YIGSR using EDC to surfaces has been described in the literature [166] and was achieved by the formation of amide bonds on arginine and tyrosine, respectively. The addition of NHS to EDC in the grafting reaction generates a semi-stable amine-reactive intermediate. The amine-reactive intermediate has a long half-life for up to several hours at pH 8, and thus improves the grafting efficiency [276]. The guanidino groups of arginines on both peptides are non-reactive to the amine-reactive intermediates because of the eliminated nucleophilicity due to the protonation in aqueous solution [26] and the delocalization of the electrons into the Y framework of the guanidine group. During the grafting of YIGSR, the grafting reaction occurred at the amine of the tyrosine of YIGSR, however, the steric hindrance by the hydroxyphenyl groups of tyrosine was reported to give a lower grafting yield as compared with RGD [26]. The grafted-peptide layer of
75RGD-25YIGSR, 50RGD-50YIGSR and 25RGD-75YIGSR, therefore, comprised of less than 25%, 50% and 75% of YIGSR, respectively. The grafted-peptide layer of 0RGD-100YIGSR is still consisted of 100% of YIGSR, however the number of the YIGSR peptides should be less than the number of the RGD peptides of 100RGD-0YIGSR. This can explain why the roughness parameter \( R_a \) of the peptide-grafted PDLLA films decreased as the YIGSR percentage increased (Table 5-1).

Many factors determine (water) contact angles, including surface chemistry and roughness of the solid surface. AFM demonstrated that all peptide-grafted samples had a \( R_a < 1 \) nm. The roughness of all modified films is far smaller than the scale which can affect the contact angles as described previously [27, 200, 273, 274]. Therefore, the reduced water contact angle is solely the result of functional surface groups created by aminolysis and grafted peptides. Despite the fact that the surface roughness did not affect the surface wettability, another question arose as to whether it affects cell adhesion. Peptides are normally grafted to the material surface using spacers [24, 30, 269]. An optimal range for spacer length of 11 - 32 Å has been reported, which allows the grafted peptides to reach the integrin or non-integrin cell adhesion receptors [269], although spacers have not always been used [30]. It is thought that spacers are needed in some cases in order to compensate for the surface roughness of the materials [26]. The substrate material used for peptide grafting, 4% ethylenediamine-treated PDLLA films, had \( R_a=0.25 \) nm, and the spacer, succinic acid, had a maximal dimension of 7.8 Å, which could be determined by calculating the maximal dimension of all configurations of succinic acid. Despite 7.8 Å being slightly smaller than the lower limit of the “optimal spacer range” stated above, 7.8 Å is still more than enough to compensate the roughness of the 4% ethylenediamine-treated PDLLA films. The results of the vinculin and cytoskeletal staining also confirmed that the grafted RGD or YIGSR can reach the A549 cells and, as a result, did affect A549 cells.

As mentioned, YIGSR was the minimum sequence of laminin necessary for efficient cell adhesion and receptor binding of epithelial cells [272]. Its inductive effect on cell migration and focal adhesion point formation was reported to be less than RGD for fibroblasts [24]. The results showed that higher levels of cell attachment can be
observed on RGD-dominant samples despite their higher water contact angles. This result is in agreement with a previous report, which suggested that cells have a higher affinity to RGD than YIGSR [24]. In addition to cell attachment, the overall activity and populations of A549 cells on modified PDLLA films were also evaluated. Both WST-1 and Hoechst assays were performed on days 2 and 4 after A549 cell seeding. Among five peptide-grafted PDLLA films, only 0RGD-100YIGSR can induce both higher A549 activity and more A549 cells than unmodified PDLLA film on day 4. On day 2 this positive effect could not be observed, suggesting a time-dependent effect of the YIGSR peptide to the A549 cells. The time-dependent effects of the grafted peptide layer to the A549 cells were supported by the finding that there were fewer A549 cells on all peptide-grafted PDLLA films except 50RGD-50YIGSR than on unmodified PDLLA only on day 2 but not on day 4.

A protocol has previously been established by colleagues for the differentiation of mESCs into progenitors of distal airway epithelium, specifically type II pneumocytes [203, 220]. The pulmonary progenitors obtained in this study had the same morphology as the cells obtained in the previous study [203, 220], suggesting that peptide-grafted PDLLA did not reroute the differentiation pathway into other lineages. Instead, the peptide-grafted PDLLA enhanced the expression of mRNA of surfactant protein C (SPC). SPC is the sole marker that is ONLY expressed by type II pneumocytes, and thus, SPC mRNA was used in this study to evaluate the effect of the peptide-grafted PDLLA films on stem cell differentiation. The fact that SPC mRNA expression changed in parallel with the proportion of YIGSR in the grafted peptide layer implied a role of surface composition on the differentiation of stem cells. This may be explained by the fact that YIGSR peptides can mimic the function of laminin whereas RGD mainly mimics the fibronectin function. These results are in agreement with those of a previous study, which demonstrated that a laminin coating on TCP can enhance the SPC expression several folds more than fibronectin coating [252]. Furthermore, the literature also indicated that the phenotype of type II pneumocytes can be maintained better on laminin than on fibronectin [91].
5.5. Conclusion

The grafted peptides on PDLLA films result in a reduction of the water contact angles, i.e. resulting in an increased hydrophilicity of the modified PDLLA surfaces and increase the surface roughness of the PDLLA films. They also influence the response of the cultured pulmonary epithelial cell A549, such as attachment, the formation of focal adhesion points, cell activities and populations. In another preliminary experiment, it appeared that YIGSR-grafted PDLLA films may have the potential to serve as the differentiation environment for the differentiation of mESCs towards pulmonary epithelial progenitors. However, more investigations should be performed to confirm this finding.
Chapter 6. Functionalized Poly(D,L-lactide) for pulmonary epithelial cell culture

6.1. Introduction

Pulmonary diseases have long been one of the major causes of death in the world. One of the pulmonary disease, pulmonary fibrosis, affects millions of people worldwide every year. Pulmonary fibrosis is irreversible and no effective treatment is currently available [277]. A promising strategy to conventional medical treatment against pulmonary fibrosis, tissue engineering of pulmonary tissue, attracted much attention in the last 5 years. The basic principal of tissue engineering is to restore the function of a target tissue by implanting cells cultured on appropriate scaffolds to the impaired tissue [9, 14]. To restore lung function, type II pulmonary epithelial cells is the choice of cells because they were found to proliferate and transdifferentiate into type I pulmonary epithelial cells, which are responsible for gas-exchange, when the pulmonary epithelium is injured.

The choice of the scaffold material is also important for the culture of pulmonary epithelial cells. Poly(D,L-lactide) (PDLLA) has been used safely in medical applications for decades and can be processed easily into various structures. It was proven biocompatible for the culture of pulmonary epithelial cells [198]. It is degraded by hydrolysis of the polyester backbone without the need of enzymes. The hydrolysis rate as well as the mechanical properties of the PDLLA can be easily modified by changing the ratio of D- and L- isomers of PDLLA. The bulk PDLLA is hard, however, when it is processed into scaffolds, the mechanical properties of the scaffolds can range from soft to very hard, depending on the preparation conditions of the scaffolds [20, 181]. The flexibility in the properties of PDLLA makes it a suitable scaffold material for tissue engineering applications. As polylactide is a hydrophobic material containing no reactive functional groups in the side chains, efforts have been made to modify its surface character in order to make it suitable for the maintenance and growth of various cell types [41, 164]. Although publications described surface modification of PDLLA scaffolds for cell culture applications [36-39, 146, 165,
[278-281], none explored pulmonary epithelial cells. In this study, the growth of A549 cells, a widely-used pulmonary cell line [61], on the functionalized PDLLA films was assessed.

The strategy used to modify the surface of PDLLA scaffolds without using costly bioactive molecules, such as extracellular matrix proteins, is to introduce functional groups into the surface of PDLLA scaffolds [36, 165]. Functional groups such as amines and carboxylic acids have been introduced into PDLLA surface to enhance the attachment of osteoblasts [282, 283] and both the density and the distribution of the functional groups have been shown to affect osteoblast responses [171, 284, 285].

In order to understand the effect of functional groups on pulmonary cell culture, the modification of PDLLA films was investigated using two simple techniques. Amines were introduced into the surface of PDLLA films by aminolysis (Figure 6-1), a transesterification reaction, using different concentrations of ethylenediamine in isopropanol. Furthermore, amine-terminated and carboxylic acids-terminated tree-like branched architectures were introduced into the surface of PDLLA films. Thus, it is called branching modification throughout this study. Because PDLLA lacks reactive functional groups, PDLLA films were treated by aminolysis first to obtain free surface amines, then branched architectures were prepared by repetitively grafting spacers and “trifunctional molecules” to surface amines, generating structures similar to tree branching [31]. The “trifunctional molecule” has three functional groups, one of which reacted with the spacer of the same generation, and the remaining two functional groups are reacted with two spacers of the next generation. This approach can enhance the density of local functional groups and alter their distribution in 2D environments and 3D scaffolds. Tissue culture plastic, unmodified PDLLA films, PDLLA films treated with aminolysis and branch modification were characterized with respect to their surface properties. The attachment, proliferation, population and focal adhesion point formation of A549 cells were assessed on the all polymer films.
6.2. Methodology

Functional groups were introduced to the surface of PDLLA films using aminolysis and branching modification (Chapter 2.5.2 and 2.5.3). For aminolysis, 1, 2, 4, 8, 15% of ethylenediamine in isopropanol were used to treat PDLLA films to generate five different kinds of PDLLA films containing amines on the surface (Chapter 2.5.2). Branching modification can generate PDLLA containing 1, 2 or 3 generations of amine-terminated branched architectures or 1, 2 or 3 generations of carboxylic acid-terminated branched architectures on the surface (Chapter 2.5.3). A total of six different samples were prepared using branching modification.

The primary amines on the surface of the aminolyzed PDLLA films were measured using fluorescamine assay (Chapter 2.6.4). To evaluate whether the “branching modification“ worked, the surface compositions of the PDLLA films treated with branching-modification were determined using XPS (Chapter 2.6.6). Their surface chemistry was also evaluated using ζ potential measurements (Chapter 2.6.1). Surface characterizations, including water contact angle and surface roughness, were performed on all functionalized PDLLA films (Chapter 2.6.2 and 2.6.5).

To evaluate the effects of surface functional groups on A549 responses, A549 cells were seeded on the functionalized PDLLA films and cultured for 4 days. Cell attachments were evaluated 2h after A549 seeding using Hoechst dye (Chapter 2.7.3). Formation of focal adhesion points and cytoskeleton of A549 cells was determined 24h after cell seeding using immunostaining and phalloidin, respectively (Chapter 2.3.3). A549 population and A549 metabolic activity was evaluated at day 2 and day 4 using Hoechst dye and WST-1 reagent (Chapter 2.7.3 and 2.7.4).
6.3. Results

The surfaces of PDLLA films modified by aminolysis and branching modification were characterized with respect to their surface chemistry, topography and wettability. The attachment, focal adhesion point formation, population and activity of pulmonary epithelial cells (A549 line) cultured on these functionalized PDLLA films were further assessed.

6.3.1. Quantification of primary amine functional groups using fluorescamine assay

The simple fluorescence-based method, fluorescamine, was used to quantify the bulk concentration of primary amines of the aminolyzed PDLLA films. The higher the concentration of ethylenediamine solution, the higher the concentration of primary amines introduced to PDLLA films (Figure 6-2). A sharp increase in primary amines was found to be induced by ethylenediamine in isopropanol at ethylenediamine concentrations ranging from 6% to 10%. Above 15 % of ethylenediamine, a plateau of amine functionalization was reached.

![Figure 6-2](image.png)

Figure 6-2 Effect of ethylenediamine concentration in isopropanol on fluorescence intensity and the corresponding quantity of primary amine introduced to PDLLA films. 4 independent samples in each condition were evaluated. The fluorescamine assay was repeated 3 times.
6.3.2. Surface composition of modified PDLLA: X-ray photoelectron spectroscopy (XPS)

XPS was used to determine the surface composition of unmodified PDLLA and PDLLA films modified by branching modification. As nitrogen is not present in PDLLA, it is considered the best candidate to evaluate the surface modification. The nitrogen content is expected to increase with each degree of branching. A trend of increasing nitrogen and decreasing oxygen content could be observed for all generations of bADA and between the 1st and 2nd generations of the bTREN by XPS (Table 6-1). There was not significant difference in nitrogen and oxygen content between 2nd and 3rd generations of bTREN.

Table 6-1 Surface composition of modified PDLLA as determined by XPS. 5 independent samples in each condition were evaluated. The XPS probing was performed only once due to the budget limit.

<table>
<thead>
<tr>
<th>Samples</th>
<th>C (%)</th>
<th>N (%)</th>
<th>O (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDLLA</td>
<td>57.90 ± 0.52</td>
<td>0</td>
<td>42.10 ± 0.52</td>
</tr>
<tr>
<td>bADA1</td>
<td>58.85 ± 0.41</td>
<td>2.49 ± 0.02</td>
<td>37.80 ± 0.54</td>
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<tr>
<td>bADA2</td>
<td>61.27 ± 3.60</td>
<td>2.89 ± 0.12</td>
<td>34.00 ± 5.91</td>
</tr>
<tr>
<td>bADA3</td>
<td>60.05 ± 0.21</td>
<td>4.19 ± 0.63</td>
<td>33.00 ± 2.16</td>
</tr>
<tr>
<td>bTREN1</td>
<td>59.57 ± 0.04</td>
<td>2.45 ± 0.20</td>
<td>37.68 ± 0.25</td>
</tr>
<tr>
<td>bTREN2</td>
<td>61.53 ± 0.68</td>
<td>3.18 ± 0.31</td>
<td>35.11 ± 0.58</td>
</tr>
<tr>
<td>bTREN3</td>
<td>60.06 ± 0.63</td>
<td>3.02 ± 0.34</td>
<td>36.36 ± 1.23</td>
</tr>
</tbody>
</table>

6.3.3. Wettability of wet PDLLA surfaces: dynamic contact angles

All surface-modified PDLLA films had lower advancing and receding contact angles than unmodified PDLLA (Figure 6-3). 15%EDiamine, bTREN1, bTREN2, bADA2 and bADA3 had lower advancing and receding contact angles than TCP. PDLLA films treated with more concentrated ethylenediamine had lower contact angles, particularly 8%EDiamine and 15%EDiamine, than PDLLA films treated with a less concentrated ethylenediamine solution. In the bTREN group, it was notable that bTREN3 had significantly higher advancing contact angles (P<0.005) than bTREN1 and bTREN2. In the bADA group, as expected, contact angles decreased as the number of branching generations increased.
Figure 6-3 Dynamic water contact angles on PDLLA, TCP and modified PDLLA films measured using the captive bubble technique. The advancing and receding contact angles of all modified PDLLA were significantly lower than PDLLA (P<0.005, n = 4). + and ++ stand for significant difference between functionalized surfaces and TCP (+ for P<0.05; ++ for P<0.005). The water contact angle measurements were repeated 3 times.

**6.3.4. Surface topography and roughness of modified PDLLA:**

**Atomic Force Microscopy (AFM)**

Unmodified PDLLA, 1%EDiamine, 2%EDiamine, 4%EDiamine and 8%EDiamine had homogeneous surfaces containing no distinct surface features. 15%EDiamine, in contrast, had an irregular surface topography with $R_a = 1.14$ nm. Furthermore, $R_a$ was found to increase as the concentration of ethylenediamine increased (Figure 6-4 and Table 6-2). For the samples modified with branched architectures, the formation of droplet-like structures on the surface of both bTREN and bADA was observed. The size of these structures increased as the branching generation increased (Figure 6-4 and Table 6-2). Compared with bTREN with the same number of generations, bADA had higher $R_a$, i.e. $R_a$ of TREN1 was 0.75 and $R_a$ of ADA1 1.58, $R_a$ of TREN2 0.97 and $R_a$ of ADA2 3.13, $R_a$ of TREN3 1.38 and $R_a$ of ADA3 3.48.
Figure 6-4 AFM scans of unmodified and modified PDLLA films. An area of 1μm x 1μm was imaged. (A) unmodified PDLLA film and aminolyzed PDLLA films, (B) PDLLA films grafted with branched architectures.
Table 6-2 Roughness parameter (Rₐ) of original PDLLA and modified samples (units: nm). 5 independent samples in each condition were evaluated. The AFM probing was repeated twice.

<table>
<thead>
<tr>
<th>PDLLA and aminolyzed surface</th>
<th>Rₐ Mean</th>
<th>Rₐ SD</th>
<th>Branching modification</th>
<th>Rₐ Mean</th>
<th>Rₛ SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDLLA</td>
<td>0.14</td>
<td>0.06</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1% TREN1</td>
<td>0.16</td>
<td>0.02</td>
<td></td>
<td>0.75**</td>
<td>0.03</td>
</tr>
<tr>
<td>2% TREN2</td>
<td>0.21</td>
<td>0.04</td>
<td></td>
<td>0.97**</td>
<td>0.08</td>
</tr>
<tr>
<td>4% TREN2</td>
<td>0.25</td>
<td>0.04</td>
<td></td>
<td>1.38**</td>
<td>0.15</td>
</tr>
<tr>
<td>8% ADA1</td>
<td>0.28</td>
<td>0.10</td>
<td></td>
<td>1.58**</td>
<td>0.12</td>
</tr>
<tr>
<td>15% ADA3</td>
<td>1.14**</td>
<td>0.15</td>
<td></td>
<td>3.13**</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.48**</td>
<td>0.34</td>
</tr>
</tbody>
</table>

*P<0.05; **P<0.005; SD = standard deviation of the mean.

6.3.5. Surface character of modified PDLLA: ζ-potential measurements

Figure 6-5 shows the ζ-potential of PDLLA, 4%EDiamine, bTREN3 and bADA3 as function of pH (ζ=f(pH)). PDLLA contains mainly Bronsted acidic functional groups, which resulted in an IEP, where ζ=0, of pH 3.5 and a plateau (ζ_plateau) of -29 mV in the alkaline region [214]. PDLLA is made by ring-opening polymerization of lactide and, therefore, it contains carboxylic acid functional groups at one end of each polymer chain. Both 4%EDiamine and bADA had IEP values at pH of 3.7, close to the IEP of PDLLA. In contrast, bTREN3 has an IEP of 4.5, much higher than the other three samples. Among the four samples tested, bTREN3 and bADA3 showed highest and lowest ζ_plateau-value at -25.7 mV and -38.4 mV, respectively, whereas PDLLA and 4%EDiamne showed no statistically significant difference.
Figure 6-5 \( \xi \)-potential of PDLLA films, 4%EDdiamine, bTREN3 and bADA3 films measured as a function of pH in a 1mM KCl supporting electrolyte. 4 independent samples in each condition were evaluated. The \( \xi \)-potential measurements were repeated 3 times.

6.3.6. Determination of cell attachment using the Hoechst 33258 dye

Cell attachment can be evaluated by determining the DNA content of the cells attached to the surface 2 h after cell seeding. Among all the experimental samples, 4%EDdiamine, 8%EDdiamine and 15%EDdiamine induced significantly higher cell attachment than the unmodified PDLLA (Figure 6-6). Also both bTREN and bADA PDLLA films, which possessed two or three generations, exhibited higher cell attachment than unmodified PDLLA. The A549 cell attachment on PDLLA with three modified branched architectures was in the following order: bTREN3 > bTREN2 = bTREN1 and bADA3 = bADA2 > bADA1. Furthermore, bTREN3 and bADA3 resulted in cell attachments at comparable level to those on TCP.
6.3.7. Visualization of cell focal adhesion and cytoskeleton

F-actin, a cytoskeletal protein, and vinculin, a focal adhesion protein, in A549 cells were stained 24 h after seeding. A549 cells seeded on TCP, PDLLA and aminolysis-treated PDLLA did not demonstrate distinct F-actin formation (Figure 6-7A-H). Spindle-like structures immunoreactive for vinculin were not observed at the junction of the F-actin and the cytoplasmic processes (Figure 6-7A-H). In the group of branched architectures, weak spindle-like vinculin-positive structures could be found in cells grown on bTREN (Figure 6-8B-D), and spindle-like vinculin-positive structures were barely seen in cells on bADA (Figure 6-8E-G).
Figure 6-7 Immunostaining of vinculin (left panel) and F-actin (right panel) in A549 cells cultured on PDLLA, TCP and aminolyzed PDLLA films after 24 h in culture. Four independent samples of each condition were recorded. Vinculin immunoreactivity can be seen at the end of some cell processes, but no immunoreactive “spindle-like” structures were seen. F-actins stained by phalloidin were not distinct in the most cells cultured on these surfaces for 24h.
Figure 6-8  Immunostaining of vinculin (left panel) and F-actin (right panel) in A549 cells cultured on PDLLA films with branched architectures after 24 h in culture. In the left panel, arrows indicate vinculin-immunostained structures with spindle shape. Weak vinculin-immunoreactive spindle-like structures were seen in the cells cultured on bTREN samples.

6.3.8. **A549 cell activity and number on days 2 and 4**

WST-1 assay (Figure 6-9) and Hoechst assay for DNA quantification (Figure 6-10) were performed only on days 2 and 4 because after day 4, A549 cells reached 100% confluence and died quickly. Unmodified PDLLA was found to induce lower cell activity than TCP on both day 2 and day 4. All aminolyzed films were found to induce similar levels of A549 activity as PDLLA on both days. But no difference was found between each condition of the aminolysis group. The cell activity on bTREN was
found to be significantly lower than unmodified PDLLA on day 2, but higher than on unmodified PDLLA on day 4, suggesting a time-dependent metabolic cell response. On day 4, the A549 cell activities on bTREN were comparable to those on TCP. There was no significant difference in cell activity between any bADA and unmodified PDLLA on either day 2 or day 4.

As far as cell population size was concerned, aminolyzed PDLLA, except for 15%EDiamine, induced higher cell numbers than unmodified PDLLA on day 4. Cell numbers obtained on all branching modified PDLLA samples were significantly lower than those on unmodified PDLLA on day 2. On day 4, bTREN2 and bTREN 3 had significantly larger cell populations but all bADA samples had lower cell numbers than the unmodified PDLLA. Furthermore, the cell numbers on aminolyzed-PDLLA films except 15%EDiamine were comparable to those on TCP on day 4.

Figure 6-9 Activity of A549 cells on day 2 and day 4 as determined using WST-1 assay (n = 6 for all samples). * and ** stand for significant difference between functionalized surfaces and PDLLA (* for P<0.05; ** for P<0.005). + and ++ stand for significant difference between functionalized surfaces and TCP (+ for P<0.05; ++ for P<0.005). The WST-1 assay was repeated 3 times.
6.4. Discussion

In this study, the aim was to modify the surface of PDLLA in order to increase its capacity to support the growth of pulmonary epithelium. PDLLA films were modified using aminolysis and by attaching branched architectures to the PDLLA surface. Aminolysis introduced amines into the surface of PDLLA films. Branching modification enhanced the concentration of carboxylic acids and amines in the vicinity of the branched architectures. The results suggest that the concentration, type and distribution of surface functional groups affect the growth and activity of pulmonary epithelial cells on PDLLA.

Aminolysis has long been used in the textile industry to modify the surface of synthetic fibres, such PET [168]. Its application for the surface modification of biomaterials has only been explored in the past few years [33, 34, 40]. Here,
1%-15% ethylenediamine was used in isopropanol to modify the surface of PDLLA for 5 min. Aminolysis is a form of trans-esterification reaction where the carbonyl carbon is attacked by the nucleophilic nitrogen of the amine to generate amines and alcohols (Figure 6-1). Most aminolysis reactions are carried out in either a basic aqueous solution or in a solvent with high $\pi$-basicity, such as isopropanol. Isopropanol was used instead of water as solvent to make up ethylenediamine solutions because, in isopropanol, aminolysis is the sole reaction whereas, in water, concurrent hydrolysis of PDLLA occurs [33].

Zhu et al. [34] reported the difficulty of detecting nitrogen using XPS on PCL using a similar aminolysis modification. A more sensitive method using fluorescence microscopy to quantify rhodamine B isothiocyanate-labelled amines was employed instead [34]. Also, a fluorescence assay, fluorescamine, was used to quantify primary amines. Fluorescamine can react with primary amines to form a primary amine adduct which absorbs light at 390 nm and emits light at 475 nm. It is an extremely sensitive reagent, being able to detect amines at sub-picomole ($< 10^{-12}$ mole) levels [286]. A plateau of fluorescence intensity was observed when exceeding 15% of ethylenediamine concentration in isopropanol (Figure 6-2). Consequently, ethylenediamine concentrations up to 15% were used for subsequent experiments. Aminolysis of PDLLA using ethylenediamine generates three hydrophilic species, primary amines, alcohols and amides, all of which help to improve the wettability of the modified surfaces by water, i.e. result in lower contact angles as measured by the captive bubble method.

Surface modification using succinic acid and TREN to form branched architectures for fibroblast culture was first reported using solely EDC as the coupling reagent [31]. The drawback when using EDC alone is that the unstable intermediate has a very short lifetime of only a few minutes [276]. Therefore, the protocol was improved firstly by adding NHS to generate amine-reactive NHS esters with a longer lifetime and, secondly, by controlling the pH at 5 and 8 during the first and second steps of the coupling reactions to improve the coupling efficiency, respectively. EDC was used to couple carboxylic acids and amines in two steps of each reaction. In the first step, EDC reacted best with carboxylic acid at around pH 5 [275, 276], forming unstable amine-reactive O-acylisourea intermediates. NHS then replaced the role of EDC,
forming semi-stable, amine-reactive NHS-esters. In the second step, TREN or ADA, both containing amine groups, were added to the amine-reactive NHS-esters to generate amide bonds. The amine-reactive NHS-esters undergo faster hydrolysis when the pH increases, moreover, the reactivity of it to amines also increases with pH [213]. Therefore, a suitable pH should be determined to maximize the reaction product of the second step of the EDC coupling reaction. In the preliminary test, pH 8 was found to be a good balance between the reaction rate and the hydrolysis rate of the amine-reactive NHS-esters and thus used in the second step of all EDC coupling reactions. Despite, the hydrolysis of the amine-reactive NHS-esters still results in a coupling efficiency much less than 100% [275]. Consequently, it is impossible to reach 100% branching efficiency, and thus branched architectures with different generations might have existed on the same surfaces, e.g. bADA3 can have branched architectures with three, two or even only one generation.

During the preparation of branched architectures, unwanted reactions might also have occurred during reaction 1 and reaction 2 (Figure 2-12). Succinic acid has two carboxylic acids, both of which have the potential to react with amines on aminolysis-treated PDLLA (Figure 2-12, dashed circle A). Furthermore, TREN has 3 amino groups, all of which could react with acid groups introduced by succinic acid in reaction 1 (Figure 2-12, dashed circle B and C). Therefore, if more than one coupling reaction occurred between one molecule of succinic acid and TREN, the number of the available functional groups at the surface decreased rather than increased. As the number of the branching generations increased, it could be more likely to see unwanted coupling reactions due to the proximity between adjacent branched architectures. This might explain why the nitrogen content of bTREN3 was not significantly higher than bTREN2 (Table 6-1). The water contact angle of bTREN3 was also not significantly lower than bTREN2 (Figure 6-3). In contrast, when creating bADA the above mentioned problems for bTREN will not be encountered. Except for the first generation, bADA used GABA and ADA as the spacers and “trifunctional molecules”, respectively. Both GABA and ADA have only one primary amine, therefore, in the coupling reaction (Figure 2-12, reaction 3-5), no unwanted coupling reactions will occur. The advantage of the design was reflected most significantly in the increase of nitrogen content and the reduction of the measured water contact angles as the number of branching generations increased (Table 6-1, Figure 6-3), e.g.
bADA3 films have a higher nitrogen content and lower water contact angle than bADA2. Another problem with the branching modifications of both bTREN and bADA is that it was prepared in an aqueous solution. PDLLA is known to be susceptible to water [156], i.e. it degrades by hydrolysis. During the modification and subsequent washing of the samples, cleavage of the ester backbone by water is prone to occur, generating one carboxylic acid at one end of the broken polymer chains. Therefore, it is clear that hydrolysis-induced surface carboxylic acids actually participated in the branching reactions, especially in step 1 of reaction 2 to reaction 5 (Figure 2-12).

The limitation of XPS is that it only samples a relatively small area of the materials surface (approx. 0.04 mm²). To evaluate the entire modified surface, another measurement has to be applied. Accordingly, $\zeta$-potential measurements were performed on the whole modified surface over a wide pH range. The $\zeta$-potential is determined by the formation of the electrochemical double layer, i.e. the dissociation of functional groups according to their $pK_a$ and the adsorption of ions present the background electrolyte ($K^+$, $Cl^-$, $H_3O^+$ or $OH^-$). $\zeta=f(pH)$ (Figure 6-5) demonstrated much higher IEP and $\zeta_{plateau}$ of bTREN than bADA. The IEP is a measure of the surface acidity or basicity. Surfaces that are more acidic, i.e. contain more or stronger acidic groups, have lower IEP than a surface that is less acid, and vice versa. The presence of the $\zeta_{plateau}$ is attributed to the dissociation of the charge-determining surface groups and the adsorption of the water and ions at the corresponding pH [202]. In general, a surface that has more or stronger dissociating acid groups has higher $\zeta_{plateau}$ than a surface that has less or weaker dissociating acid groups. Thus, the experimental result that bADA3 had lower IEP and $\zeta_{plateau}$ than bTREN3 met our expectation because bADA3 possessed carboxylic acids at the end of the branched architectures while bTREN3 possessed amines at the end of the branched architectures.

All modifications used in this study reduced the water contact angle, i.e. improved wetting behaviour of the modified PDLLA films. Factors that determined the contact angle include the surface chemistry and surface roughness. AFM demonstrated that the samples in the aminolysis group had a $R_a$ smaller than 1.2 nm and samples in the branching modification group smaller than 3.5 nm. The roughness of all samples is far
smaller than a micrometre which is the scale commonly thought to affect contact angles [200]. Therefore, the reduced water contact angle is solely the result of introduced surface functional groups. Comparing the water contact angle with the level of cell attachment, a correlation can be found in the samples modified using the same modification technique, i.e. aminolysis, amine-terminated branched architectures and carboxylic acid-terminated branched architectures, such that substrates exhibiting better cell attachment correspond to lower water contact angles. The correlation was also observed by others before [287]. Despite the fact that the nanoscale surface roughness did not affect to the contact angle as described, it could be argued that the enhanced A549 attachment is attributed to surface roughness. A previous publication indicated that surfaces with $R_a > 10$ nm can enhance cell attachment [27]. The positive effect of surface roughness with $R_a < 10$ nm to osteoblast responses was recently demonstrated [288, 289]. However, the effect of nanoscale roughness on pulmonary epithelial cells has not yet been investigated. Accordingly, it is still not clear whether the enhanced cell attachment to the modified surface was solely due to the functional groups introduced.

Another question that arose from the cell attachment experiments was whether the cells were in direct contact with the functional groups. Some studies used serum-free medium in order to investigate the effect of surface functional groups on the cell culture to avoid a secondary surface modification by protein adsorption from the serum [290]. Since protein adsorption is always the first event occurring when a biomaterial comes in contact with body fluids, using serum-containing medium to evaluate the surface modification can be more representative of the in vivo condition. It has been suggested that long-term total protein adsorption on functional-group-enriched polylactide (PLA) films remain unchanged [291]. However, the same study also demonstrated that protein adsorption was faster for functional group-enriched polylactide than unmodified PLA and lasted for 800 min as determined using a quartz crystal microbalance. In the present case, the cell culture medium contained 10% FBS, thus the enhanced cell attachment induced by the samples that possessed more functional groups, such as 15%EDiamine, bTREN3 and bADA3, might be partly attributed to more serum protein adsorption because the cell attachment was measured just 2 h after cell seeding, which is far shorter than the 800 min period of the serum protein adsorption demonstrated in the literature [291].
A previous study reported better cell responses on surfaces possessing homogeneously-distributed amines than on surfaces possessing carboxylic acids [171]. In this study, bTREN and bADA contained amines and carboxylic acids, respectively. The main difference from previous reports is that our branched architectures altered the distribution of the functional groups, i.e. functional groups were more concentrated where the branched architectures are located. This difference in functional group distribution was reflected by the altered A549 cell behaviour: cells growing on bTREN exhibited some vinculin-inducing potential, but those on bADA did not. In addition to focal adhesion formation, differences in results obtained using WST-1 and Hoechst assays were observed. The WST-1 assay measured the overall mitochondrial activity of the whole cell population and the Hoechst assay measured total DNA concentration. The activity and population size of A549 cells were found to be time-dependent. For example, the level of cell activity on the bTREN group was slightly lower than that on bADA on day 2, but it was much higher than on bADA on day 4. Cell activity and population size can be discussed together to obtain a more complete overview of A549 responses. A good example is to compare the results obtained for bADA1 with those obtained for unmodified PDLLA. Both of them had a similar cell number on day 4, however, the activity of the cells on bADA1 was significantly higher than that of cells growing on unmodified PDLLA, which implied that cells on bADA1 had a higher average cell activity than those on unmodified PDLLA.

6.5. Conclusion

The development of a synthetic material suitable for pneumocyte cultures is still a very new area of research. In this present context, the effect was investigated of the amine density and branched architectures on the surface properties of PDLLA, a polymer previously shown to be a suitable support for pneumocyte cultures [198]. All surface modifications can reduce the water contact angle of PDLLA films significantly. PDLLA films modified with branching modifications demonstrated droplet-like surface topography. Amine-terminated branched architecture showed higher IEP and $\zeta_{\text{plateau}}$ than the carboxylic acid-terminated branched architectures. The pulmonary epithelial cell behaviour, such as cell attachment, focal adhesion formation,
short-term cell activity and population size, are dependent on the modifications of the PDLLA films. PDLLA films modified using 15% ethylenediamine and branched architectures with two and three generations can induce better A549 attachment. The latter can also induce better focal adhesion point formation, higher A549 activity and higher A549 population. Therefore, modification of PDLLA scaffolds with amine-terminated branched architectures for two or three generations was recommended as the surface modification for the future engineering of pulmonary epithelial tissue.
Chapter 7. Preparation and modification of Poly(D,L-lactide) scaffolds for pulmonary epithelial cell culture

7.1. Introduction

The aim of this study was to develop a 3D scaffold made of a synthetic material suitable for the engineering of pulmonary epithelial tissue. In this study, particular focus was placed on the effects of the preparation conditions of scaffolds on the resulting pore morphology of the prepared scaffolds and on the subsequent culture of pulmonary epithelial cells. The effects of the surface modification of the prepared 3D scaffolds on the cell responses were also studied.

The development of the field of tissue engineering has progressed rapidly in the past few years. Artificial tissues, such as bone [288, 292, 293] and cartilage [180], have been successfully developed in vitro using cells cultured on synthetic scaffolds. For the engineering of lung tissue, scaffolds need to be completely different from those designed for other tissues, mainly due to a special feature of lung: lung is porous. The only other porous tissue in the human body is the cancellous bone. The gas-exchange component of the lung is made up of more than three million functional units, called alveoli, having an average diameter of 100-200 μm. The total surface area of the alveoli is around 70-90 m² and they are lined by two types of endoderm-derived pulmonary epithelial cells. The engineering of lung tissue has progressed much slower than that of other tissues. This is due to the difficulty of culturing primary pulmonary epithelial cells [10, 78, 268] and also due to the limited number of scaffolds specifically designed for lung. In recent years, the lack of primary cells has been overcome by the generation of lung epithelial cells and their progenitors from various types of stem cells [17, 18, 203, 219, 220]. As for appropriate scaffolds, hydrogels made of extracellular matrix proteins, such collagen [196], GAG [196] or Matrigel [22] have been tested. Scaffolds made from proteins take advantage of their bioactive properties but can be very expensive. Furthermore, many proteins are derived from neoplastic tissues and thereby doubtful with respect to their long-term safety in the clinical applications.
Synthetic polymer scaffolds can be processed into a porous framework which facilitates gas and medium diffusion. The porous structures can be optimised to mimic the target organ and allow the in-growth of vascular supply and host tissue \textit{in vivo}. Furthermore, synthetic scaffolds can also be used for the \textit{in vitro} expansion of populating cells. Poly(D,L-lactide) (PDLLA), a degradable polyester, was used for the scaffold preparation in this study. PDLLA has various biomedical applications due to its biodegradability and biocompatibility \cite{146, 294}. The mechanical properties of the PDLLA can be tuned by adjusting the ratio of D- or L-isomer \cite{146, 150}. PDLLA can also be blended with other polymers for various applications \cite{18-21}. Recently, it was found that extracellular matrix protein-coated PDLLA is a suitable scaffold for the differentiation of pulmonary epithelial cells from embryonic stem cells \cite{252}.

Thermally Induced Phase Separation (TIPS) is one of many methods to fabricate porous scaffolds. This technique was long used in industry to generate porous membranes for separation applications \cite{295-297}. The principle of TIPS is based on the change in the thermodynamic state of a polymer solution as a function of temperature ranges. A polymer solution phase-separates into a polymer-rich phase and a polymer-poor phase when the polymer solution is quenched to the temperature below the binodal curve of a temperature-composition phase diagram of the polymer solution \cite{184}. Interconnectivity of the same phase (polymer-poor or polymer-rich) only occurs when the polymer solution is further brought to the unstable zone below the spinodal demixing curve. Scaffolds can be obtained by removing the solvent from the quenched phase-separated polymer solution using freeze-drying. Factors such as solution quenching rate, quenching temperature, solution composition and coarsening time and temperature can alter the final morphology of the scaffolds. Among all these factors, coarsening, a phenomenon that involves the time-dependent reduction of interfacial energy by reducing the interfacial area between the polymer-rich and polymer-poor phase, was studied recently for the fabrication of PLLA scaffolds \cite{185, 189, 190}. During coarsening the tendency of reducing the interfacial energy results in the growth of phase-separated droplets over time. These droplets may further coalesce to form larger droplets.

When subjected to temperature change, liquid-liquid phase separation occurring in a PDLLA-water-dioxane ternary system can generate scaffolds with round porous
structures [298, 299]. In this study, the pore size (distribution) of scaffolds created this way was manipulated by playing with coarsening temperatures and coarsening time periods in order to generate scaffolds with pore sizes similar to the dimension of lung alveoli.

In addition to liquid-liquid phase separation, scaffolds were also prepared using solid-liquid phase separation. Solid-liquid phase separation using PDLLA-dimethyl-carbonate (DMC) generates scaffolds with anisotropic ladder-like tubular pores [181, 198]. The pore size of scaffolds prepared in this way is determined by the size of DMC crystals [181] The size of DMC crystals can be tuned by controlling the temperature at which the PDLLA-DMC solution was frozen.

In order to understand whether the scaffolds prepared by liquid-liquid and/or solid-liquid phase separation are suitable culture environments for pulmonary epithelial cells, cell culture on manufactured scaffolds was performed using A549 cells. A549 cells are human pneumocyte-like cells originally derived from an alveolar cell carcinoma. A549 has been widely used as a model to study pulmonary epithelial cell-biomaterial interactions [195, 300-303]. The metabolic activity of A549 cells and their migration into the prepared scaffolds were recorded using WST-1 reagents and microscope, respectively.

Not only the porous morphology but also the surface properties of the scaffolds can affect the activity of A549 cells cultured on the scaffolds. As PDLLA is known for its hydrophobicity, efforts to adjust its surface properties, i.e. make it more hydrophilic, have been made [165, 267]. The introduction of functional groups into the surface of PDLLA films using aminolysis was recently reported [304], which introduces amines to the surface of the PDLLA films. Aminolysis was followed by another treatment called branching modification, a technique that can generate amine- or carboxylic acid-terminated tree-like branched architectures on the material surface [304]. In this study, both surface modifications were applied to 3D scaffolds in order to create scaffolds possessing functional groups on the surface. A549 cells were seeded on the modified scaffolds and their activity was further examined using WST-1 reagent.
7.2. Methodology

The objective of this chapter is to prepare scaffolds suitable for the culture of pulmonary epithelial cells using liquid-liquid phase separation and solid-liquid phase separation. The “coarsening” effects on the pore size and porous structure were studied using the liquid-liquid phase separation system. The coarsening periods of 10 min, 20 min, 30 min and 60 min as well as coarsening temperatures from 12 to 27°C were used (Chapter 2.4.2.1). Two more scaffolds using solid-liquid phase separation were also prepared for comparison (Chapter 2.4.2.2). The pore sizes of the scaffolds showing on the SEM microimages were analyzed using image analysis software (Chapter 2.6.3).

To study whether the prepared scaffolds are suitable for the culture of pulmonary epithelial cells, A549 were seeded onto some scaffolds and cultured for 16 days (Chapter 2.7.1). The activities of the cultured A549 cells were evaluated using WST-1 reagent (Chapter 2.7.4). The penetrations of the A549 into the middle of the scaffolds were recorded on day 16 using SEM and fluorescence microscopy (Chapter 2.7.2).

We further modify the surface of some scaffolds using aminolysis and branching modification (Chapter 2.5.3), as described in Chapter 6. Similarly, A549 cells were seeded into scaffolds, and their metabolic activities were measured using WST-1 reagent (Chapter 2.7.4).

7.3. Results

7.3.1. Morphological analysis of the scaffolds

The pore size of the scaffolds prepared by liquid-liquid phase separation at a coarsening temperature of 24°C was found to increase with the coarsening time (Figure 7-1, Figure 7-2). After 60 min coarsening, a solvent-rich layer formed on top of the polymer-rich layer, which could be observed on top of the prepared scaffold (Figure 7-3). Well-defined macroporous structures were observed for the scaffolds LL10M, LL20M and LL30M, however, it is worth noting that many pores were poorly-connected or only connected to each other via small (10 - 20 μm) pore throats (indicated by arrows in Figure 7-4A, B). These pore throats were smaller than the diameter of A549 cells, thereby preventing cell migration into the scaffold from one
pore to the next. Pore throats at hundred micrometre scales were also observed (Figure 7-4A1, A2), however, their number was much smaller than the number of pore throats with diameters ranging from 10 to 20 \( \mu m \).

Figure 7-1 Scanning electron micrographs of PDLLA scaffolds prepared by liquid-liquid phase
separation using a coarsening temperature of 24°C and increasing coarsening times (group1): (A) no coarsening was performed (LL0M); (B) coarsening for 10 min (LL10M); (C) coarsening for 20 min (LL20M); (D) coarsening for 30 min (LL30M); (E) coarsening for 60 min (LL60M). All figures are at 50x magnification.

Figure 7-2 The effect of coarsening time on the mean pore size. The error bar indicates the standard deviation. The pore sizes of scaffolds prepared using different coarsening time are significantly different to each other (In each condition, 6 independent samples were imaged. More than 80 pores in each image were measured. SEM probing was repeated twice).

Figure 7-3 Scaffolds prepared by liquid-liquid phase separation using a coarsening temperature of 24°C and increasing coarsening times (0 min, 10 min, 20 min and 30 min). From left to right: LL0M, LL10M, LL20M, LL30M, LL60M.
Figure 7-4 Scanning electron micrographs of scaffolds prepared by liquid-liquid phase separation using a temperature of 24°C. A1: LL20M at x200 magnification. A2 is an enlargement of the white square shown in A1 at x500 magnification. A3 is an enlargement of the white square in A2 at x1000 magnification. B: LL30M at x200 magnification. The arrows indicate small pore throats at 10 μm to 20 μm scale. The dotted line indicates a large pore throat more than 50 μm in diameter.

Seven different scaffolds (Group 2) were prepared using coarsening temperatures varying between 12°C and 27°C with 30 min coarsening time. Four of the scaffolds prepared, LL21C, LL18C, LL15C and LL12C, had average pore sizes exceeding 200 μm. The pore size of the scaffolds increased from 60 μm to 270 μm with decreasing coarsening temperature from 27 °C to 18 °C. The pore size of the scaffolds slightly decreased from 270 μm to 220μm when the coarsening temperature was further decreased from 18 °C to 12 °C (Figure 7-5, Figure 7-6). The pore walls of the scaffolds were getting rougher when the coarsening temperature was reduced from 27 °C to 15 °C (Figure 7-5 A-E). When the coarsening temperature was reduced to 12 °C,
scaffolds with smooth pore walls were again observed (Figure 7-5F). Unfortunately, the scaffolds LL18C (Figure 7-5 D) and LL12C (Figure 7-5 F) possessed many but poorly interconnected pores. The pores are connected to each other only via small pore throats.
Figure 7-5 Scanning electron micrographs of scaffolds prepared by liquid-liquid phase separation using different coarsening temperatures (group 2): (A) coarsening at 27 °C for 30 min; (B) coarsening at 24 °C for 30 min; (C) coarsening at 21 °C for 30 min; (D) coarsening at 18 °C for 30 min (E); coarsening at 15 °C for 30 min; (F) coarsening at 12 °C for 30 min..
In contrast to the isotropic porous architecture of scaffolds prepared by liquid-liquid phase separation, scaffolds made by solid-liquid phase separation had an anisotropic and ladder-like laminated porous structure (Figure 7-7), similar to that described in the literature [38, 181, 305]. The pore dimension of scaffold SL-25 ranged from 150 to 250 μm (Figure 7-7A, B) and that of scaffold SL-196 ranged from 40 to 50 μm (Figure 7-7C, D). The pores of both scaffolds SL-25C and SL-196C had a smoother surface than the pores of the scaffolds prepared by liquid-liquid phase separation.
Figure 7-7 Scanning electron micrographs of scaffolds prepared by solid-liquid phase separation (Group 3): 5% (w/v) PDLLA was dissolved in DMC and frozen at -25°C (SL-25C) or snap-frozen at -196°C (SL-196C) for 2 h (A) scaffold SL-25C sectioned along the direction of the laminated pores (B) scaffold SL-25C sectioned transverse to the direction of the laminated pores, (C) scaffold SL-196C, sectioned along the direction of the laminated pores, (D) scaffold SL-196C sectioned transverse to the direction of the laminated pores.

7.3.2. Penetration of A549 cells cultured on unmodified PDLLA scaffolds evaluated using fluorescence microscopy

Using fluorescence microscopy, not only the cells populating the outermost layer of the scaffolds but also those in pores close to the outermost layer could be observed. On the last day of culture, day 16, A549 cells transfected with pEGFP-N1 covered the outer surface of all scaffolds (Figure 7-8). Cells were seen to penetrate into the internal structure of all samples except LL24C, LL27C and SL-196C. It can be
observed clearly that in the areas where cells were not populated, scaffolds did not emit detectable autofluorescence (Figure 7-8A, B and H). Figure 7-8I showed a LL27C scaffold that were not seeded with cells. Similarly, no detectable autofluorescence was observed.

Figure 7-8 Fluorescent microscope images of A549 cells cultured on scaffolds prepared using liquid-liquid phase separation with coarsening temperature from 27°C to 12°C for 30 min (A-F) and solid-liquid phase separation with freezing temperature of -196°C (G) and -25°C (H), including (A) LL27C, (B) LL24C, (C) LL21C, (D) LL18C, (E) LL15C, (F) LL12C, (G) SL-196C and (H) SL-25C, after 16 d of culture. The scaffolds were sectioned along the direction of A549 seeding. (A), (B) and (H) showed the border of sectioned scaffolds, because cells were not found in the middle area of the sections. (C-G) showed the middle area of the sectioned scaffolds. (I) The LL27C without cells did not emit detectable fluorescence, but they scattered the fluorescence emitted by the A549 cells as shown in (C-G), resulting the obscurity of the some images.

7.3.3. Penetration of A549 cells cultured on unmodified PDLLA
scaffolds evaluated using scanning electron microscopy (SEM)

A549 cell migration into the middle of the scaffolds was investigated using SEM. SEM images indicated that A549 cells could indeed be observed in the centre of scaffolds LL12C, LL15C, LL18C, LL21C and SL-25C (Figure 7-9), confirming penetration of the A549 cells. A549 cells were not observed at the centre of scaffolds LL24C, LL27C and SL-196C. This finding was in agreement with those obtained from fluorescence microscopy (Figure 7-9).


7.3.4. A549 cell activity on the unmodified PDLLA scaffolds

The WST-1 cell viability assay was performed on days 4, 8, 12 and 16 to measure
A549 cell activity on the various scaffolds (Figure 7-10). Cells cultured on scaffold SL-25C demonstrated the highest overall levels of A549 cell activity as compared to cells cultured on all other scaffolds. In general, scaffolds which possessed larger porous structures induced A549 cell activity, except for scaffold LL18C one of the samples with the largest pores in the liquid-liquid phase separation group. The overall activity of A549 cells cultured on scaffold LL18C, however, was statistically lower to that of scaffolds LL12C, LL15C and LL21C. In additional to the A549 culture on PDLLA scaffolds, A549 cells were cultured on TCP and PDLLA films in parallel. The results were not shown here because it was found that A549 died quickly when they reached 100% confluence. It is due to the limited surface area in each well of a 24-well plate.

Figure 7-10 Activity of A549 cells cultured on scaffolds prepared by liquid-liquid and solid-liquid phase separation at day 4, 8, 12, 16. Statistical analysis of A549 cell activity on different scaffolds on day 16 was performed (n = 6). The WST-1 assay was repeated 3 times. The absorbance of the WST-1 reagent presented in this figure was the absorbance of each sample minus the background absorbance (WST-1 reagent added to empty wells containing no cells).
7.3.5. A549 activity on the modified PDLLA scaffolds

To study the influences of the surface modification of scaffolds on the pulmonary epithelial cells, A549 cells were also cultured on scaffold SL-25C modified using aminolysis (4%EDiamine) and by amine-terminated (bTREN3) and carboxylic acid-terminated (bADA) branching modifications. Cells grown on 4%EDiamine and bTREN3 over 16 days demonstrated higher overall activity than those on the unmodified PDLLA scaffolds (Figure 7-11).

Figure 7-11 Activities of A549 cells cultured on surface-modified scaffold SL-25C at day 4, 8, 12 and 16. Asterisks * indicates for P<0.05 and ** for P<0.005 (n = 6). The WST-1 assay was repeated 3 times.

7.4. Discussion

Most previous reports have used the PLLA-dioxane-water tertiary system as a model to study the effect of coarsening conditions on the pore structure of the scaffolds [20, 185, 189, 190, 306]. Instead of PLLA, PDLLA was used in the current study. The major difference between the two polymers is that PDLLA is an amorphous polymer whereas PLLA is semicrystalline. In the tertiary system using PLLA, a gelation temperature can be observed as the result of the partial crystallization in the polymer-rich phase [185]. The phenomenon was not observed in the tertiary system in
this study using amorphous PDLLA. Scaffolds prepared by liquid-liquid phase separation of 5% PDLLA-dioxane solution were reported to have different mechanical properties and pore morphology from those made from 5% PLLA-dioxane solution [20]. The percentage of water in the tertiary system also significantly affected the cloud point (temperature) of PDLLA in solution. A small increase of the amount of water, from 13% to 15.5%, added to the system can raise the cloud point from of a 5% PLLA-dioxane-water solution 40°C to 60°C [185]. Rather than a single cloud point, a temperature range in which the clouding of the polymer solution occurred was observed for PDLLA between 28°C-32°C in the preliminary test. This temperature range is due to the polydispersity of PDLLA.

The PDLLA-dioxane-water tertiary system has an upper critical solution temperature type phase separation behaviour, thus, when the polymer of the solution is brought down below the cloud temperature, the thermodynamic tendency of the phase separating polymer solution is to reduce the interfacial energy of polymer-rich and solvent-rich phases which causes the growth and subsequent coalescence of the phase-separated regions [189]. When the coalescence progresses the increasing polymer concentration in the polymer-rich phase, leads to an increased viscosity of the polymer-rich phase and a decreased number of solvent-rich drops subsequently results in a decrease of interconnectivity of the final scaffolds. During coarsening, the density, viscosity and solvent flux of both phases create a slow-changing balance of solvent-rich droplets distributed within a matrix of polymer-rich phase. When the solvent-rich phase grows to the extent that this balance can no longer be maintained, the polymer-rich phase sediments and both phases eventually separates so that the solvent-rich phase “floats” on top of a polymer-rich phase. This phenomenon was observed for scaffold LL60M. Among all other samples in group 1, scaffold LL30M possessed the largest pores because of the longest coarsening period. One problem with scaffold LL30M was that a large percentage of pores of were only connected to other pores only via very small pore throats, far too small for A549 cells to migrate into the scaffold. The poor interconnectivity of the scaffold LL30W was the result of the coalescence of the solvent-rich droplets and the concurrent increase of the distance between coalescing droplets of the phase-separated polymer solution during coarsening as described.
In an attempt to address the issue of pore interconnectivity, the porous structure of scaffolds prepared using liquid-liquid phase separation with coarsening period of 30 min was controlled using different coarsening temperatures ranging from 27°C to 12°C. The wide coarsening temperature range is accessible because of the lack of a gelation temperature of the tertiary system used due to the amorphous nature of PDLLA. The scaffold framework was getting much more irregular when the coarsening temperature was decreased from 27°C to 15°C, which is due to the shift of the polymer solution from the poorly-connected metastable region to the unstable region of the temperature-composition phase diagram of the PDLLA solution. When the coarsening temperature was further reduced to 12°C, sedimentation of polymer-rich phase occurred. In the sedimentated polymer-rich phase, phase separation still progressed to form both polymer-rich and polymer-poor phases. Smaller pores formed in between the already-formed large pores due to a progressing phase separation after sedimentation. Scaffold LL18C possessed the largest average pore size, 270 μm, among all samples prepared meanwhile scaffold LL18C had many poorly-connected pores that were connected to other pores only via small pore throats. This is because of the coalescence of the solvent-rich phase, the concurrent decrease of the number of solvent-rich droplets and the subsequent reduction in pore interconnectivity as described previously. The high number of small pore throats restricts the cell migration and nutrition diffusion when the scaffolds were cultured with A549 cells, which resulted in the lowest A549 activity among the four samples with pore sizes larger than 200 μm. After coarsening, the coarsened-polymer solutions were quenched in liquid nitrogen to snap-freeze the coarsened polymer solutions to minimize any further phase separation before the solvent was frozen and eventually removed by freeze-drying.

When the scaffolds were prepared by solid-liquid phase separation from 5% PDLLA-DMC solution, the polymer solution started to solidify before the occurrence of liquid-liquid phase separation [181]. In the freezing polymer solution, the PDLLA is rejected by the front of the growing DMC crystals. The porous structure of the scaffolds is therefore mainly determined by the formation DMC crystals [181]. When the polymer solution is frozen at -25°C in a freezer, the slower cooling rate allows the
growth of DMC crystals to continue, which results in larger pores. In contrast, when 5% PDLLA-DMC solution was quenched in liquid nitrogen, the fast cooling rate did not allow the solvent crystals to grow sufficiently large, which resulted in scaffolds with small pores.

To elucidate whether scaffolds prepared by liquid-liquid phase separation and solid-liquid phase separation are suitable culture environments for pulmonary epithelial cells, A549 cells were cultured on these scaffolds. The activity of A549 cells was measured using WST-1 reagent, a colorimetric assay similar to the commonly-used MTT [218] assay. WST-1 is a terazolium salt, when it is added to the cell containing cell culture medium, it is cleaved into formazan dye by succinate-tetrazolium reductase in the mitochondria of only metabolically active cells. The formazan dye can be quantified by measuring its absorbance using an ELISA reader. The measured absorbance is proportional to the metabolic activity of the cultured cells. Scaffold SL-25C induced the highest overall A549 activity of all prepared scaffolds. The high A549 activity induced by scaffold SL-25C was most likely due to the fact that its larger ladder-like pores, which were 150 - 250 μm in diameter, allow easy access of cells and exchange of nutrients and waste. In contrast to scaffold SL-25C, cells did not migrate into the middle of scaffold SL-196C due to its small pores, as demonstrated by SEM and the fluorescence microscope images. The overall A549 cell activity on scaffold SL-196C was also much lower than on scaffold SL-25C. SEM images demonstrated that A549 cells were not able to migrate into the middle of scaffolds LL27C and LL24C prepared by liquid-liquid phase separation. The former was prepared at a coarsening temperature of 27°C, which is only 1°C lower than the lower margin of the cloud point, so the state of the PDLLA solution at 27°C was located in the metastable region and close to the bimodal curve of the temperature-composition phase diagram, where the initiation of nucleation and growth mechanism generated small droplet-like solvent-rich phase dispersed in the polymer-rich phase. Even after 30 min coarsening at 27°C, the pores of scaffold LL27C were still too small to allow cell migration into its centre. Scaffold LL24C had much larger pores than scaffold LL27C, but the pore interconnectivity was poor. Other scaffolds, including LL12C, LL15C and LL21C, induced similar levels of cell activities over the whole culture period. Scaffold LL18C induced lower cell activities.
than scaffolds LL12C, LL15C and LL21C, despite its largest average pore size. The reason for this was the low pore interconnectivity as the consequence of increasing size of and coalescence of polymer-poor phase, as described previously.

Among all the prepared scaffolds, scaffold SL-25C induced the highest A549 cell activity. It is interesting to know whether modification of the surface of such scaffolds can induce even higher A549 cell activity than the unmodified scaffolds and, therefore, it was decided to modify scaffold SL-25C. Ethylenediamine introduces amino groups into the polymer backbone by breaking the ester bonds using aminolysis, a trans-esterification reaction [33]. For the aminolysis modification of scaffold SL-25C, 4% ethylenediamine solution in isopropanol was used for 5 min. 4% ethylenediamine solution was chosen because the preliminary study demonstrated that PDLLA films treated by ethylenediamine solution at a concentration higher than 4% can be too fragile for further branching modifications. Amine-terminated or carboxylic acid-terminated branched architectures were also prepared by repeatedly introducing “spacer” and “tri-functional molecules” to the 4%EDiamie. Branched architectures can enhance the concentration of the functional groups in the vicinity of each branched architecture and, therefore, the functional groups are not homogeneously distributed on the surface of the scaffolds. The effect of these modifications of PDLLA films on A549 cell behaviour, including attachment, activity and population size, had been tested previously [304]. The major difference between cells cultured on films and 3D scaffolds is that cells did reach 100% confluency quickly when they are cultured on films, while 3D scaffolds offer sufficient surface area for the cells to migrate and proliferate. Therefore, A549 cells cultured on surface-modified 3D scaffolds may present different activity levels compared to A549 cells cultured on PDLLA films. A549 cells were therefore seeded on surface-modified scaffolds and the activity of the A549 cells was investigated. The result demonstrated that both 4%EDiamine and bTREN3 increased the A549 activity during 16 days in culture compared to those cultured on the unmodified SL-25C scaffold, whereas bADA3 did not induce higher A549 cell activity compared to the unmodified SL-25C scaffold. As 4%EDiamine and bTREN3 possessed amines with different distributions on their surfaces, it can be inferred that surface amines at certain concentrations can enhance the A549 cell activity, regardless of their distribution. A further study should be performed to quantify the amine concentration on the surface of both 4%EDiamine
and bTREN3. The A549 attachment and their number on scaffolds were not quantified in this study because these would require the complete isolation of the DNA of A549 cells cultured within the scaffolds, and the preliminary test demonstrated that erroneous results can be obtained due to any residual DNA trapped within the scaffolds.

7.5. Conclusion

This experiments presented in this chapter provide basic information about the effect of the preparation conditions of porous 3D PDLLA scaffolds on their pore structure and morphology. The surfaces of these 3D scaffolds were modified and the impact of the surface modification on the subsequent culture of pulmonary epithelial cells was investigated. It was demonstrated that scaffolds with pores of similar dimensions to pulmonary alveoli can be fabricated using liquid-liquid phase separation of the PDLLA-dioxane-water tertiary systems. The pore size of these scaffolds can be tuned by controlling the coarsening temperatures. Scaffolds prepared using solid-liquid phase separation using freezing temperature of -25°C possessed large ladder-like pores with dimension ranging between 150-250 μm. Cell culture of pulmonary epithelial cells A549 demonstrated that pore sizes of the scaffolds and surface modification on the scaffolds can affect the overall level of the A549 cell activity over time.
Chapter 8. Conclusions and future work

8.1. Summary and conclusions

The overall aims of this project were, firstly, to increase the differentiation efficiency of type II pneumocyte progenitors from mESCs by enhancing the established differentiation protocol, and secondly, to develop 2D environments and 3D scaffolds made of poly(D,L-lactide) (PDLLA) suitable for the culture of pulmonary epithelial cells.

To achieve the two aims, the following three questions were proposed at the start of this thesis, and investigated in the five experimental chapters;

**Question 1: Can the differentiation of murine embryonic stem cells into type II pneumocyte progenitors be enhanced by:**

- coating the culture vessel with ECM protein/s?
- grafting bioactive peptides to the culture vessel?
- adding fibroblast growth factors to the cell culture medium?

Protein coatings on tissue culture plastic (TCP), specifically with the ECM protein laminin and ECM replacement Matrigel, were shown to enhance the expression of the mRNAs for markers of pulmonary epithelial progenitors, including surfactant protein C and D (SPC and SPD) and aquaporin 5, as compared to the control gelatin, which was used in the original “three-step differentiation protocol” for the differentiation of pulmonary progenitors from mESCs. Furthermore, coating with either laminin or Matrigel was found to increase the number of SPC-eGFP positive cells.

As the conformation of an adsorbed protein is determined by the surface properties of the material that the protein is adsorbed to, laminin and Matrigel were further coated onto the PDLLA films and the effects of the coatings on the differentiation of mESCs tested. It was found that laminin coating on PDLLA films induced the highest levels of SPC mRNA expression by the differentiated cells as compared to Matrigel or gelatin coating. The adsorption of the proteins on TCP and PDLLA films was confirmed by the change in the IEP, where the \( \zeta \) potential \( \zeta = f(\text{pH}) = 0 \), and the \( \zeta_{\text{plateau}} \).
of the protein-coated surfaces. Moreover, all protein-coated TCP and PDLLA films had lower water contact angles as compared to non-coated counterparts. The water contact angles of the protein-coated surfaces, however, did not correlate to the SPC expression of the differentiated pulmonary progenitors.

The SPC mRNA expression of the differentiated pulmonary progenitors can also be enhanced by adding fibroblast growth factor (FGF) into the differentiation medium. High concentrations (500 ng/ml and 1000 ng/ml) of FGF-1 were found to enhance the expression of SPC mRNA of the differentiated cells compared to the control culture of cells fed with no growth factors. Interestingly, results were found using cells fed with the differentiation medium containing FGF-10. FGF-10 at 60 ng/ml did not induce a change in the expression of SPC mRNA when the mESCs were differentiated on a gelatin coating, but, in contrast, FGF-10 enhanced the SPC mRNA expression of the differentiated pulmonary progenitors when the gelatin was replaced with Matrigel, suggesting synergic effects between the growth factors and ECM proteins.

It was further demonstrated that the positive effects of laminin coating of PDLLA films on the differentiation of pulmonary progenitors from mESCs can also be observed on cells differentiated on YIGSR-grafted PDLLA films. YIGSR is the peptide that mimics the cell adhesion function of laminin. YIGSR-grafted PDLLA films induced greater SPC mRNA expression of the differentiated cells than was observed with cells cultured on the gelatin-coated PDLLA control. When RGD peptides were co-grafted with YIGSR onto PDLLA, the expression of SPC decreased, suggesting a concentration-dependent effect of grafted YIGSR on the expression of SPC mRNA.

**Question 2: How do the pulmonary epithelial cells respond to the surface of PDLLA scaffolds grafted with RGD and YIGSR peptides or modified with amines or carboxylic acids and how do the surface-modified PDLLA scaffolds differ from unmodified PDLLA scaffolds?**

Pulmonary epithelial A549 cells do attach better to RGD-grafted than YIGSR-grafted PDLLA surfaces. Focal adhesion points formed within A549 cells cultured on RGD-grafted PDLLA films outnumbered the focal adhesion points formed within the A549 cells cultured on YIGSR-grafted PDLLA films. The number of focal adhesion
points in A549 cells was positively related to the amount of the grafted RGD. All peptide-grafted PDLLA films demonstrated lower water contact angles and rougher surfaces than the unmodified PDLLA surface. YIGSR-grafted surfaces, on the other hand, possessed only slightly lower water contact angles but rougher surfaces than RGD-grafted surfaces.

Functional groups were introduced to the surface of PDLLA films using aminolysis and branching modifications. Aminolysis introduced amines onto the surfaces of PDLLA films, while the branching modification created amine-terminated and carboxylic acid-terminated tree-like branched architectures on the amine-functionalized PDLLA. All of the functionalized PDLLA films possessed lower water contact angles than unmodified PDLLA films. The attachment of A549 cells was better on PDLLA films possessing more functional groups. PDLLA films functionalised with amine-terminated branched architectures can induce higher metabolic activity of the cultured cells than unmodified PDLLA film and PDLLA films functionalised with carboxylic acid-terminated branched architectures.

**Question 3: What are the parameters needed to prepare 3D synthetic scaffolds with a pore structure suitable for the culture of pulmonary epithelial cells?**

Experimental results demonstrated that, when scaffolds possessed pores with diameters exceeding 150 μm and with good interconnectivity between these pores, pulmonary epithelial cells can penetrate into the scaffolds and be active within them for at least 16 days, as evaluated using the WST-1 assay. This is because large pores allow nutrition diffusion in and waste diffusion out of the scaffolds. Consequently, pulmonary epithelial cells can penetrate into and survive within the interior of such scaffolds.

Liquid-liquid phase separation using 5% PDLLA in 13% water and 87% dioxane generates porous scaffolds with round pores. The “coarsening” phenomenon, a tendency of the decreasing interfacial area and the reducing interfacial energy of the separated phases over time, was used to control the pore structure and pore sizes within the scaffolds. Longer coarsening times generated scaffolds with large pores because of the thermodynamic tendency to reduce interfacial energy between the polymer-rich and polymer-poor phases and the subsequent coalescence of the
polymer-poor phases in the polymer solution. When a coarsening time of 30 min and coarsening temperature between 21°C to 15°C was used, scaffolds possessing pores with diameters exceeding 150 μm were obtained. These scaffolds possessed structures similar to the pulmonary alveolus and allowed the penetration and the culture of pulmonary epithelial cells as mentioned, thus, they may be good candidates for the implantation purposes.

Solid-liquid phase separation using 5% PDLLA in 100% dimethylcarbonate generates scaffolds with ladder-like porous structures. The pore dimensions are determined mainly by the growth of solvent crystals, which is controlled by the freezing temperature and rate of the polymer solution. Using a freezing temperature of -25°C prior to freeze-drying, the size of the ladder-like pores can be as large as 300 μm. A549 cells proliferate better on these scaffolds than on all other scaffolds studied. This is because of the much larger pores which facilitated nutrition and waste exchange. This scaffold, therefore, can be considered suitable for the in vitro expansion of the pulmonary epithelial cells.

In conclusion, the differentiation of pulmonary progenitors from murine embryonic stem cells can be influenced by the external signals from the culture system, including extracellular matrix, fibroblast growth factors and bioactive peptides. Furthermore, PDLLA films modified using bioactive peptide grafting, aminolysis and branching modification and 3D PDLLA scaffolds can be applied as cell culture environments for pulmonary epithelial cells.

8.2. Suggestions for future work

The results obtained in this project provide a basis for the engineering of pulmonary epithelial tissue. For example, the differentiation protocols of pulmonary progenitors from mESCs established in this study can be further applied to the differentiation of human pulmonary progenitors from hESCs. However, a number of issues encountered during these studies will need to be resolved and more research performed. These issues and possible solutions are summarised below.

The differentiated cell cultures derived from ESCs in this study only expressed the
mRNA for pulmonary epithelial markers, specifically surfactant proteins. The RT-PCR method used appeared to be reliable and had appropriate controls, so there was no reason to suggest the data were erroneous. However, the corresponding surfactant proteins could not be detected using immunocytochemistry, despite extensive tests using different antibodies. This has long been a problem of the three-step differentiation protocol [203]. This may have been due to the technical difficulties encountered during sectioning of the attached embryoid bodies (EBs) on the protein-coated TCP or PDLLA. It may also have been a result of surfactant protein synthesis being restricted to the mRNA level and consequent immaturity of the type II pneumocyte progenitors. If the latter is the case, more studies should be performed to find the missing key, which can guide the pulmonary progenitors to differentiate into mature pulmonary epithelial cells.

The positive effects of laminin and Matrigel on mESC differentiation to distal airway epithelium were demonstrated in this study. The molecular mechanisms by which laminin and Matrigel exerted their effects are still unknown. Investigations of the expression of the integrins and proteins in the downstream cascades of the cells in the attached EBs should be performed. Furthermore, it needs to be understood whether the future pulmonary progenitors are influenced by laminin and Matrigel in a direct or indirect manner.

Laminin was found to affect the expression of SPC of the differentiated cells at varying levels when it was adsorbed to the surface of different materials. This might have been due to the fact that, when laminin is adsorbed to different materials conformational change and may affect the exposure of its bioactive moieties [175]. To establish this, further investigations could be performed aiming to quantify the conformation of the adsorbed laminin and the exposure of its bioactive moieties.

FGFs were shown to be effective in increasing the SPC expression levels of differentiating mESCs. The FGF concentrations used in the experiments were based on those reported in the literature for the maintenance of the type II pneumocytes or for the study of the FGFs effect on the excised embryonic lung in vitro. A broader range of FGF concentrations, more FGF combinations and different feeding regimes could be tested to further improve the differentiation protocol. As with the positive
effects of the ECM coatings on the derivation of pulmonary progenitors, the mechanisms by which FGF influence on differentiation of mESCs are still unknown. All FGFs used in this project, i.e. FGF-1, 2, 7 and 10, can bind to FGF receptors, then the signals pass through a series of downstream protein cascade to MAPK/ERK pathway, that can regulate the activities of several transcriptional factors, such as c-Myc, Sap1a, Elk-1 and CREB [2-8]. Some of these transcriptional factors controlled by FGF can affect the organogenesis and the branching morphogenesis of the embryonic lung [87, 88, 247, 251, 307-309], especially via mesenchymal-epithelial interaction [309]. Thus, it would be interesting to find out how the FGFs affect the differentiation of pulmonary progenitors via the regulation of the above-mentioned transcriptional factors.

YIGSR-grafting to PDLLA can replace the role of intact laminin and induce the expression of SPC, indicating differentiation of pneumocytes. Unfortunately, these experiments took place towards the end of the study and, thus, the results are only preliminary and there was not time to evaluate the expression of other pulmonary epithelial markers. Factors such as the concentration of the grafted peptide, the linker length between the grafted YIGSR and PDLLA surface, could be optimized to increase the SPC expression of the differentiated mESCs on the surface-modified PDLLA films. Recently, nanofibrous scaffolds made of self-assembly YIGSR peptides were reported [310, 311]. The nanofibrous scaffolds provide environments similar to the extracellular spaces of most tissues. These nanofibrous scaffolds may be used to culture mature pulmonary epithelial cells or to differentiate mESCs into type II pneumocyte progenitors. Had more time been available, this is something that would have been tested.

Using aminolysis and branching modifications, it was possible to demonstrate that functional groups on a PDLLA surface can alter the responses of a line of airway epithelium-like (A549) cells. However, it is still not known whether the effect of the functional groups on A549 responses is due to the groups themselves or the adsorbed serum proteins. To answer this question, it would be necessary to investigate the amount of the adsorbed protein and structure of the protein layer adsorbed from the A549 culture medium onto the functionalized PDLLA.
PDLLA scaffolds designed to mimic the structure of the distal lung were prepared and seeded with A549 cells as a first step to attempt a 3D culture of pulmonary epithelial cells. The mechanical properties of the scaffold should match its target tissue so that the implanted scaffolds should keep its structural stability in an *in vivo* physiological environment. The investigation of the mechanical properties, such as elastic modulus and tensile strength, of the manufactured scaffolds specifically under physiological conditions, i.e. in the tissue fluid and at 37°C, and the degradation profile of these scaffolds are needed as well as *in vivo* studies to evaluate the vessel in-growth.
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Appendix A  Step by step experimental protocol

A1. Protocols for immunostaining

1. Cells that formed a monolayer were fixed using 4% PFA (4% w/v paraformaldehyde in PBS) for 30 min after medium aspiration and several rinses of PBS. Aspire the PFA then rinse samples several times using PBS.

2. To fix unseeded EBs as described in Chapter 3, EBs were harvested, washed with PBS and fixed with 4% (w/v) PFA overnight, allowing PFA to penetrate the whole EB. Wash the fixed EBs with PBS for several times and cryoprotect the fixed EBs using 30% (w/v) sucrose in PBS at 4°C overnight. Cryoprotected samples were then immersed in O.C.T. embedding medium (Miles, Elkhart, IN) in a mould made of aluminum foil for 30 min at room temperature. The mould that contained samples was rapidly immersed into isopentane pre-cooled with liquid nitrogen. The frozen samples were either stored in -80°C for further use or cryosectioned to 15 μm thickness at -20°C immediately.

3. Use 3% (v/v) normal serum derived from the same species from which the secondary antibodies were derived to block the background antigens for 30 min.

4. Shake off the blocking solution, add primary antibodies in diluent comprising 0.05% (w/v) bovine serum albumin (BSA; Sigma-Aldrich, Dorset, UK) and 0.01% (w/v) NaN₃ (Sigma-Aldrich, Dorset, UK) in PBS at predetermined dilution ratios (Table 2-5) to samples overnight at 4°C followed by several rinses of PBS on the second day.

5. Appropriate secondary antibodies, raised against the donor species of the primary antibodies and conjugated with fluorophore, were diluted at predetermined ratios in azide-free diluent consisting of only 0.05% (w/v) BSA in PBS and were added to the samples for 1 h in the dark at room temperature.

6. Rinsed samples several times with PBS and mounted samples in Vectashield containing 1.5 μg/mL 4',6 diamidino- 2 – phenylindole (DAPI) (Vector Laboratories, Burlingame, CA).
Appendix B  Supplementary results

B1. Determination of optimal cycle number for conventional PCR

It is necessary to find the optimal cycle number for each PCR primer in order to obtain semi-quantitative information of the mRNA expression. The RNA was extracted from the cells differentiated from mESCs using the original three-step differentiation protocol, reverse-transcribed the extracted RNA into cDNA, and determined the optimal cycle number of the primer pairs as showed from Figure B-1 to Figure B-7. The density of each band on the left of Figure B-1 to Figure B-7 was measured by the Quantity One software. The density of the each band was plotted using a random logarithmic scale against the cycle number. The optimal cycle number was determined by choosing the cycle number that is corresponding to the middle point of the linear region of each plot.

Figure B-1 Saturation curve of albumin primer

Figure B-2 Saturation curve of aquaporin 5 primer
Figure B-3 Saturation curve of β-actin primer

Figure B-4 Saturation curve of CC10 primer

Figure B-5 Saturation curve of GAPDH primer
B2. Optimization of reaction concentration of murine SPC primers for real-time PCR

Optimization of primer concentration was performed according to the supplier’s protocol. Primer pairs that have the best amplification efficient generate the lowest Ct when sample amount of the sample is added to each reaction.

As Table B-1 shows, three pairs of SPC primers, forward primer and reverse primer at 50 nM, 300 nM or 900 nM, were compared using MLE-12 cDNA. MLE-12 is a murine pulmonary epithelial cell line. SPC primer 3 with forward primer at 900 nM and reverse primer at 900 nM had the lowest Ct compared with the other primers at all concentrations and the primers in other combinations, thus, it was chosen for this study. The details of the primer sequences are listed in Table B-2.
Table B-1 Optimization of the reaction concentration of four different pairs of murine SPC primer

<table>
<thead>
<tr>
<th></th>
<th>SPC primer 1</th>
<th>SPC primer 2</th>
<th>SPC primer 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Forward</td>
<td>Forward</td>
<td>Forward</td>
</tr>
<tr>
<td></td>
<td>concentration</td>
<td>concentration</td>
<td>concentration</td>
</tr>
<tr>
<td>50 nM</td>
<td>50 nM</td>
<td>300 nM</td>
<td>900 nM</td>
</tr>
<tr>
<td>300 nM</td>
<td>30.38</td>
<td>28.47</td>
<td>39.34</td>
</tr>
<tr>
<td>900 nM</td>
<td>39.66</td>
<td>37.91</td>
<td>32.7</td>
</tr>
<tr>
<td>Reverse</td>
<td>UD</td>
<td>UD</td>
<td>UD</td>
</tr>
<tr>
<td>Concentration</td>
<td>38.86</td>
<td>33.12</td>
<td>29.6</td>
</tr>
<tr>
<td>50 nM</td>
<td>UD</td>
<td>UD</td>
<td>UD</td>
</tr>
<tr>
<td>300 nM</td>
<td>34.03</td>
<td>35.72</td>
<td>29.21</td>
</tr>
<tr>
<td>900 nM</td>
<td>33.83</td>
<td>28.45</td>
<td>28.14</td>
</tr>
</tbody>
</table>

UD stands for undetermined Ct, which is larger than 40 cycles.

Table B-2 Four pairs of primers designed using primer 3 software

<table>
<thead>
<tr>
<th>Primers</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPC primer 1</td>
<td>ACCCTGTGTGAGAGCTACCA Forward TTTGCCGAGGGTTTCTTTCT Reverse</td>
</tr>
<tr>
<td>SPC primer 2</td>
<td>CTTTCCTAGGCGCTTGTTG Forward GTTTCTACGCCGACTGTGGA Reverse</td>
</tr>
<tr>
<td>SPC primer 3</td>
<td>CAGCTTCAGGAACTACTGC Forward GCGAAAGCCTCAAGACTAGG Reverse</td>
</tr>
</tbody>
</table>

B3. Standard curve for fluorescamine assay

The standard curve, obtained using Sigmaplot software (Systat Software Inc., San Jose, CA), can be described by $y = y_0 + a \left\{ 1 - e^{-\frac{x-x_0+b\cdot \ln 2}{c}} \right\}^c$, where $a = 794.167$, $b=2.435^{-7}$, $c = 1.288$, $x_0 = 1.8371^{-7}$ and $y_0 = 6.739$. It was used as a reference to evaluate the numbers of surface amine groups in other figures. Black circles indicate the serial diluted ethylenediamine and the curve indicates the fitted nonlinear regression curve using Weibull 5 parameter formula.
B4. Standard curve for DNA quantity determination using Hoechst 33258

Figure B-8 Standard curve of ethylenediamine solution diluted in acetone at different concentrations.

Figure B-9 Standard curve of DNA quantity determination using Hoechst 33258