CHAPTER 1

Introduction

1.1 Background

With the global expansion in the biotechnology industry there is now a growing demand for mammalian cells and their products. High-density cell cultures are typically required for a large number of applications including drug screening, cytotoxicity testing, cancer research, stem cell research, tissue engineering, genetic engineering, regenerative medicine and for the production of cell culture therapeutics. The global cell culture market was estimated at $1.02 billion in 2005 and is projected to reach $1.86 billion by 2010 (Kulkarni, 2006). This market is predominantly driven by the larger biopharmaceutical and biotechnology companies with the main contributor being manufacture of therapeutics including vaccines, hormones, blood factors, thrombolytics, interferons, monoclonal antibodies, and therapeutic enzymes; all of which require mammalian cell cultures for their production. The cell culture market is also supported by research laboratories at universities, and contract manufacturing and research organizations that utilise large numbers of cells for new research and development or routine testing and analysis.

Mammalian cells can be in the form of either primary cells or cell lines. Primary cells are obtained from normal healthy tissue (i.e. from a human or animal source) while cell lines are typically obtained from immortalised tissue or through random mutations. Primary cells are ideal since the cells are uncompromised and exist in their native physiological state. However their use is limited in research, due to difficulty in isolation, ethical issues, challenges with cell proliferation, high costs, and typically they have a limited lifespan where they stop dividing and undergo senescence (Gomes and Reis, 2004; Salgado et al., 2004). Conversely cell lines have the ability to proliferate indefinitely, and at a much faster rate than a typical primary cell. Since they are more readily available and easier to work with, cell lines are routinely used for *in vitro* cell culture.

Cell lines however are still expensive and typically a small vial of cells is purchased (e.g. from the American Tissue Culture Collection (ATCC®)) and frozen away until required. When cells are required for a specific assay, cell stocks are thawed, and the cells are subjected to a cell culture process as shown in **Figure 1.1a**. For adherent cells, typically this process involves the use of two-dimensional (2D) tissue culture polystyrene (TCPS) trays, onto which cells are seeded and attach under

appropriate growth conditions, and the cells and then allowed to proliferate and reach confluence (**Figure 1.1b**).

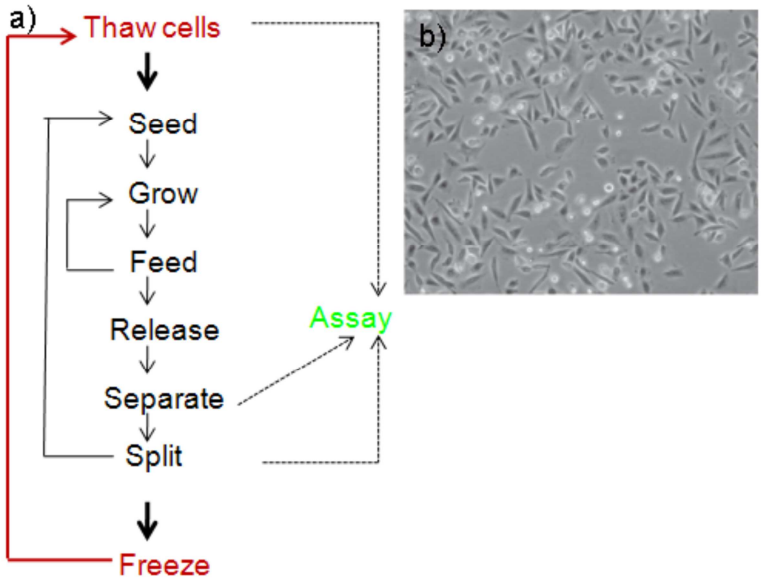

Figure 1.1: (a) Schematic showing processing steps involved in conventional cell culture process; and (b) micrograph showing ATCC® CCL-61™ cell lines growing on TCPS (ATCC®, 2012).

Confluent (or semi-confluent) cells are released from the TCPS typically using proteolytic enzymes (such as trypsin). If left to grow beyond the confluent state, cells can undergo cell death by necrosis due to space and nutrient deprivation. After cell release, cells are washed in sterile centrifuge tubes to inactivate and remove proteins such as trypsin, and other cell products. After this separation / washing step, the cell mass is now split and seeded into more 2D cell culture trays in a sub-culture process. Typically several such cycles of seeding, growth, release, washing and reseeding are required to grow sufficient numbers of cells. The cultured cells can then finally be used directly in a biological assay or frozen away for down-stream processing.

1.2 Problem statement

Although the 2D monolayer TCPS method is simple and easy to perform, this method is found to be unreliable and sometimes inaccurate, hence *in vitro* data often cannot be used to accurately predict the cellular responses of living organisms (Pampaloni

et al., 2007). Some of the main challenges with the conventional cell culture method are addressed below.

a) **Use of a 2D surface to grow cells**

The conventional TCPS trays (**Figure 1.2**) used for cell culture are flat, 2D, non-porous, and rigid, and does not represent the complex 3D cellular environment found in living tissue (Pampaloni et al., 2007).

Figure 1.2: Examples of two dimensional tissue culture polystyrene trays, flasks and plates used for cell culture of adherent cells.

Due to the unnatural constraints imposed on cells when grown in 2D, 2D cell cultures bear only limited resemblance to the complexity of the 3D dynamic environment in which cells exist naturally (Bokhari et al., 2007). Essential cellular interactions, and signalling pathways present in living tissue are absent in 2D cell cultures (Pampaloni et al., 2007). It is now well-known that cells grown in 3D display closer similarities to their *in vivo* counterparts in terms of cell migration, morphology, differentiation, phenotype, gene expression and function (Bokhari et al., 2007; Justice et al., 2009; Liu, 2008; Pampaloni et al., 2007) when compared to their 2D counterparts. A 3D scaffold is required to resemble the extracellular matrix (ECM) onto which cells naturally attach in physiological tissue. The ECM is a highly porous 3D mesh consisting of a complex mixture of proteins and sugars which is secreted by cells during normal growth. The ECM serves a number of functions, which includes providing structural support to cells; enabling diffusion of oxygen, nutrients, and removal of waste products; maintaining cell-cell interactions; and regulating signalling pathways and other important biochemical and mechanical cues (Justice et al., 2009).

b) **Harsh methods used to release confluent cells**

Confluent cells are either released using enzymes, chemicals or by mechanical scraping. The use of trypsin is by far the most popular means of releasing harvested cells. However many studies have reported disruption to the ECM and integrin receptors by trypsin use during cell release (Canavan et al., 2005). Trypsin is a proteolytic enzyme which cleaves cell adhesive proteins present in the ECM into smaller peptides and amino acids. Damage to the ECM is known to adversely influence the cell signalling pathways affecting a number of important cellular processes such as adhesion, proliferation, differentiation, migration, structure, gene expression and cell fate (Geiger et al., 2001; Guillame-Gentil et al., 2010). Furthermore trypsin is of animal origin and a potential source of contamination to cells. Over-exposure of cells to trypsin has been shown to lead to slow cell growth, unhealthy rounded morphology, and cell heterogeneity even in the same TCPS culture flask. Chelating agents (such ethylenediamine tetraacetic acid - EDTA) are also often used in conjunction with trypsin for cell release. EDTA is used to inactivate divalent cations such as Ca^{2+}, Mg^{2+}, which are known to inhibit typsin activity. However EDTA use, has alos been reported to disrupt the ion channels and important cell-to-cell junctions (Canavan et al., 2005). Ion-channels are pore-forming membrane proteins present in every cell, and changes in the ion concentration across the cell membrane affects secretion of fluids, hormones, ions. Other commonly used approaches include mechanical scraping, however this has been reported to break cell walls in particular the lipid-membrane leading to cell inflammation which induces changes to the morphological appearance of the harvested cells (Canavan et al., 2006). This implies that the current cell release methods is a major contributor to the poor repeatability, high contamination, and high variability of the conventional cell culture process (Canavan et al., 2006).

c) **Highly labour intensive and prone to contamination**

Often millions of cells are required for a specific biological assay, and the cell seeding-splitting steps must be repeated manually several times to achieve sufficient cell mass (Felder and Gildea, 2005). Typically this would involve seeding one flask, then four, then 16, then 64 etc. Due to the extra handling requirements and human operator involvement, conventional cell culture is thus prone to poor repeatability, batch to batch inconsistency, and contamination. If a specific culture is contaminated with microorganisms, typically it would be discarded and the whole process repeated.

This often results in low turn-around times and additional costs for consumables, media, etc.

d) **Static growth conditions**

Conventional cell culture is typically performed in a static environment which does not mimic the dynamic environment in living tissue. In native tissue, cells are subjected to a dynamic perfused environment which ensures a continuous supply of oxygen and fresh nutrients by blood capillaries and removal of waste products, while at the same time the fluid flow stimulates cellular behaviour. The oxygen concentration in the culture media is known to affect various cellular mechanisms, including cell cycle, cell proliferation, apoptosis, and glucose metabolism (Volkmer et al., 2008). To maintain homogenous growth rates, and cell viability, static culture requires regular manual feeding, i.e. supplementation of the media and monitoring the oxygen content which further adds an element of variability, also contributing to inconsistencies amongst operators. A dynamic fluid flow environment is also required for physical and mechanical stimulation of cells (such as in the case of bone or heart tissue) (Bancroft et al., 2002), which does not occur in a static state.

1.3 Recent advances in cell culture

It is well-known that the process in which cells are grown *in vitro* directly influence cell behaviour, growth, differentiation, gene expression and other important biological activities (Bokhari et al., 2007; Justice et al., 2009; Mueller-Klieser, 1997). Due to the limitations mentioned above, the conventional TCPS monolayer method to culture mammalian cells cannot be used to accurately predict the cellular responses of living organisms (Pampaloni et al., 2007). Hence expensive *in vivo* trials are required at an early stage of research. In the recent decades, much effort has been made towards developing more reliable cell culture scaffolds and systems.

3D scaffolds are nowadays available to culture cells. The vast majority of the commercially available 3D scaffolds are biomimetic-based and include Matrigel™, AlgiMatrix™, GEM™, Extracel™ and Cytodex™ (Justice et al., 2009). However concerns exist with regards to the production variability of some of the scaffolds, and in some cases animal components are used (Justice et al., 2009). Also the available

3D scaffolds are typically expensive, and cannot be used for routine cell-culture work. Recently bioreactors have also been developed for high-density cell proliferation, to increase oxygenation, culture media circulation, cell volumes and outputs, and include for e.g. multiple-stacked plates (e.g. AcCellerator™), spinner flasks, fluidised-bed, hollow-membrane fibre bioreactors (e.g. Cellmax) and the like. Some of these systems are also automated to alleviate contamination and the human intervention required. Although some 3D bioreactors are appearing on the market (BioLevitator™), the majority of the automated systems are still based on the use of 2D surfaces. For e.g. AcCellerator™ allows the traditional cell culture steps based on 2D trays to be automated by robots. However despite these advantages, one of the main challenges still remaining in the field is that cell release from scaffolds still largely involves the use of enzymes to degrade the cell surface proteins, or harmful chemicals to dissolve the scaffold in order to release the cells. This combined with the additional wash steps and extra handling requirements results in well-to-well variations and culture inconsistencies.

A major breakthrough in the field of cell-culture is the use of a temperature-responsive polymer i.e. poly-*N*-isopropylacrylamide, (i.e. PNIPAAm) to non-destructively release adherent cells by merely cooling the cell culture medium. PNIPAAm is a temperature-sensitive polymer that is characterised by a lower critical solution temperature (LCST) of approximately 32-33 °C (Schild, 1992). PNIPAAm switches its properties reversibly between hydrophobic (cell adhesive) and hydrophilic (non-cell adhesive) states at temperatures higher and lower than its LCST respectively. The pioneering work by Okano's group reported for the first time that cells could be released spontaneously as intact sheets from the surface of PNIPAAm coated TCPS with preserved cell-cell and cell-extracellular matrix (ECM) interactions by simply cooling the cell culture medium (Okano et al., 1995) as shown in **Figure 1.3**.

While PNIPAAm cell sheets serve as a promising tool for engineering tissue, a limitation of the current technology is that it is primarily based on the use of 2D flat substrates which lacks structural and organisational cues for cells (Isenberg et al., 2008). Existing 2D PNIPAAm substrates do not enable *in situ* cell growth in three dimensions.

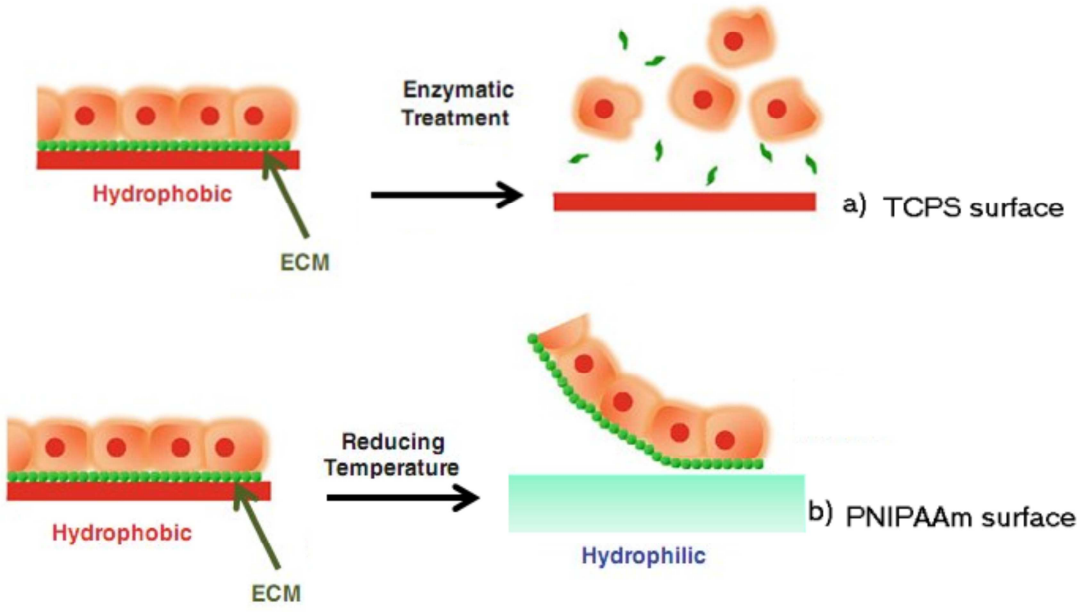

Figure 1.3: Schematic image showing cells in a monolayer whereby cell release is achieved either by a) enzymatic treatment or b) by lowering the temperature on a PNIPAAm surface. For enzymatic treatment, the deposited ECM (green) and membrane proteins are degraded and cells are released as single cells while for temperature-induced cell release, confluent cells spontaneously lift off the surface when the temperature is lowered to 20 ℃, with inta ct ECM and cell-cell junction proteins (Adapted from Kumashiro et al., 2010).

In recent years, some attempts have been made at the development of 3D PNIPAAm scaffolds based on sub-micron porous structures such as membranes (Kwon O.H., 2003; Murakami et al., 2006), hydrogels (Kwon and Matsuda, 2006; Ohya et al., 2005), micro-textured surfaces (Isenberg et al., 2008), and non-woven membranes (Okamura et al., 2008; Toshiyuki and Midori, 2006). However many of the studies focus on the culture and release of cell monolayers, and still to date little work has been done regarding applying the PNIPAAm technology to highly porous 3D scaffolds whereby cells are grown in a 3D environment and are released spontaneously as 3D cellular constructs.

Based on the literature, and discussions with various end-users (such as cell biologists, medical doctors, biochemists and molecular biologists etc.), the need for a new and efficient cell culturing system with minimal human intervention, efficiency, speed, and the ability to culture cells with minimal damage while maintaining their 3D

structure was identified. The key attributes of such a system should include the following:

- **3D porous scaffold:** A highly porous 3D scaffold should be used with large interconnected open pores to support cell-cell interactions. The porous structure should allow for oxygen and nutrient exchange, as well as cell-to-cell and cell-to-ECM interactions.
- **Non-destructive cell release:** Confluent cells should be released by a non-invasive method, without requiring harsh enzymes, chemicals, or scraping which are known to damage the cell surface. Cultured cells should retain their membrane constituents and remain in their natural state upon harvesting.
- **A bioreactor:** A bioreactor should be used to culture cells such that the cell culture medium can be perfused throughout the scaffold to enable, sufficient oxygen supply, nutrient exchange, as well as provide mechanical or physical stimulation to the growing cells.
- **High density cell culturing**: The system should allow for the cultivation of large numbers of cells (typically 1-2 orders of magnitude greater than a standard 75 cm^2 static culture flask), which would be possible with a 3D scaffold with a large surface area per volume ratio whereby a large number of cells can attach per cm^2 of scaffold.
- **Sufficient oxygenation:** To overcome diffusional constraints, oxygenation should be achieved by the use of either oxygen spargers, hollow-fibres, or oxygen carriers to support the oxygen requirements for high cell density cultures.
- **Automated operation**: The system should preferably allow for automation of the operating procedures as well as include instrumentation to automatically monitor, control and regulate the system parameters such as pH, temperature, dissolved oxygen content, agitation speed, nutrient/waste content etc. Automation would mitigate the labour-intensive process and human intervention requirement for manual cell culturing.

The Council for Scientific and Industrial Research (CSIR) in South Africa is developing a thermoresponsive 3D (T3D) cell culture device for culturing of adherent cells. The device consists of a 3D PNIPAAm scaffold of the present study, onto which cells grow and proliferate in a bioreactor and whereby cell release is non-destructive. The system will also preferably be automated to minimise contamination and human

steps. The device will enable the growth and release of 3D cellular aggregates in high-density, while enabling non-invasive temperature-induced cell harvesting without the need for destructive enzymes. A detailed description of the T3D device is given in **Chapter 6**.

1.4 Research objectives

The scope of this study is based on the design, development, and validation of a 3D thermoresponsive scaffold based on PNIPAAm for use in non-invasive temperature-induced culture of adherent cells. This project was divided into three phases: Phase 1 (preliminary study) involved development and characterisation of cross-linked PNIPAAm hydrogels using free-radical polymerisation to investigate the effect of cross-link density and mixed solvents on the physical properties of PNIPAAm hydrogels; phase 2 involved synthesis, and characterisation of highly porous 3D non-woven fabric scaffolds grafted with PNIPAAm using oxyfluorination-assisted graft polymerisation, while phase 3 focussed on showing proof of concept for use of the 3D thermoresponsive scaffolds for non-invasive cell culture.

The specific research objectives of this study were as follows:

- Phase 1 (preliminary study)
    - Synthesis of PNIPAAm hydrogels cross-linked with N,N'-methylenebisacrylamide (MBA) using free-radical polymerisation
    - Study the effect of crosslink density and solvent : water mixtures on the physical properties of the PNIPAAm hydrogels
- Phase 2 (primary focus of dissertation)
    - Development of 3D porous non-woven fabric scaffolds (based on PP, nylon and PET) grafted with PNIPAAm using oxyfluorination-assisted graft polymerisation (OAGP)
    - Physical and chemical characterisation of the PNIPAAm grafted non-woven fabric scaffolds and verification of the thermoresponsive behaviour
- Phase 3 (proof-of-concept study)
    - Cell culture of hepatocytes onto the PNIPAAm grafted NWF scaffolds at 37 ℃ and temperature-induced cell release at 20 ℃

- Cell culture of hepatocytes in the T3D cell culture device at 37 ℃ and temperature-induced cell release at 20 ℃

1.5 Research questions

The questions which this work attempts to answer are the following:

- Can the physical and mechanical properties of PNIPAAm hydrogels be improved for use in cell culture?
- How can we covalently attach PNIPAAm onto a 3D scaffold?
- Will the open porous 3D structure be maintained in the grafted scaffold?
- How does the grafting affect the properties of the non-woven material?
- Will the grafted PNIPAAm maintain its thermoresponsive properties?
- Will cells attach onto the grafted PNIPAAm surface and remain viable at 37 ℃?
- Will cells release from the grafted PNIPAAm surface at 20 °C without requiring enzymes?

1.6 Delineations and limitations

The project scope is limited to development of a new 3D scaffold for the purpose of *in vitro* cell culturing and focuses on PNIPAAm and its temperature responsive behavior and will not consider other non-destructive cell release agents. Only the following polymer scaffolds are included in this study: i.e. polypropylene (PP), polyethyleneterephthalate (PET), and nylon 6.6. Proof-of-principle studies for non-invasive cell culturing are limited to hepatocyte cell lines only.

1.7 Brief chapter overview

The chapters which are included in this dissertation will cover the following aspects:

Chapter 1: Introduction
Chapter 2: Literature review
Chapter 3: Instrumentation and characterisation techniques
Chapter 4: Development of cross-linked PNIPAAm hydrogels

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Chapter 5: Development of PNIPAAm grafted 3D NWF scaffolds

Chapter 6: Temperature-induced cell culture

Chapter 7: Conclusions and Perspective

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