CHAPTER 7

Conclusions and Prospects

7.1 Conclusions of study

Although the conventional 2D monolayer cell culture method is currently the gold standard used to culture adherent cells, this method is highly tedious, prone to contamination, and is known to be an inaccurate representation of the manner in which cells exist in their physiological state. Nowadays there are numerous solutions being proposed to culture cells, however there is still a need for a bench-top type cell culturing system; capable of culturing cells in 3D with efficiency, minimal human intervention, and where cell release is non-invasive and achieved without damaging the harvested cells. The aim of this study was to develop a unique 3D highly porous scaffold containing poly(*N*-isopropylacrylamide) for use in non-invasive cell culture.

A summary of the main findings from phases 1-3 of this work is given below:

- **Phase 1: Development of P(NIPAAm-*co*-MBA) hydrogels**

We initially developed P(NIPAAm-*co*-MBA) hydrogels with the aim of investigating the effect of crosslink-density and the use of mixed solvents on the physical properties of PNIPAAm hydrogels. Cross-linking maintained the LCST of the hydrogels, and increasing the cross-link density, improved the viscoelastic properties of the hydrogels. However when water was used as the co-polymerisation medium the swelling-deswelling response rate of the gels was very poor. The swelling-deswelling response was significantly improved using mixed solvents as the co-polymerisation medium; however these gels displayed very poor stability and handling and were deemed unsuitable for use in a robust bioreactor. Additionally at 25 °C the PNIPAAm-co-MBA hydrogels displayed relatively small pores (<10 µm) which may present a challenge for cell release, whereas at 37 °C (temperature used for cell seeding and proliferation), porosity was completely lost due to the dense skin layer, implying that the hydrogels may be unsuitable with respect to providing 3D support for cells during culture.

- **Phase 2: Development of PNIPAAm grafted 3D NWF scaffolds**

We successfully synthesised highly porous 3D PNIPAAm grafted NWF scaffolds based on PP, PET and nylon using needle-punching and a facile oxyfluorination-assisted graft polymerisation (OAGP) method. The NWF scaffolds displayed highly porous 3D structures, with high porosity content. The mean flow pores for the PP, and PET NWF ranged between 100-200 µm which was the desired range for culturing cells, whereas the nylon NWF

scaffolds displayed substantially smaller pores. We showed that OAGP could be successfully used to covalently attach PNIPAAm onto the surface of the 3D NWF scaffolds, while preserving the open porous structure in the grafted scaffolds. The OAGP method resulted in new functional polar groups forming on the surface of the NWF scaffolds. No changes were seen to the crystalline phase of bulk PP after OAGP; however, twin-melting thermal peaks were detected, indicating crystal defects. The LCST of PNIPAAm was maintained in the grafted scaffolds. Graft yields was highest for the PP-*g*-PNIPAAm NWF which was pre-oxyfluorinated. A free radical mechanism was proposed for the OAGP method with initiation via $SO_4^{\bullet-}$; $OH^{\bullet}$ and $RO^{\bullet}$ radicals. This OAGP method used in this study, proved to be an attractive alternative to other polymerisation technologies (such as gamma radiation, plasma-radiation etc.) since it is simple to perform, does not require the use of expensive equipment or toxic solvents, and it is relatively less invasive compared to other radiation methods.

- **Phase 3: Proof-of-concept for temperature-induced cell culture**

Finally in phase 3 of this work, we showed temperature-triggered cell release from the PNIPAAm grafted NWF scaffolds whereby confluent cells were released spontaneously and non-destructively from the surface of the grafted NWF by cooling the cell culture medium from 37 ℃ to 20 ℃, and without requiring destruct ive enzymes. We demonstrated that hepatocyte cells attached onto the surface of the PP-*g*-PNIPAAm NWF scaffolds, and remained viable during 21 days of culture. PP-*g*-PNIPAAm NWF scaffolds which were pre-oxyfluorinated, demonstrated superior thermal cell release capability compared to the other scaffolds. During cell growth, cells arranged themselves as 3D multi-cellular constructs or spheroids and were released as cell aggregates. Temperature-induced cell release was also demonstrated in the T3D bioreactor with the potential for high-density cell culture.

7.2 Significance of study

With technological innovations in regenerative medicine, gene-therapy and stem-cell research, the demand for large quantities of mammalian cells closely resembling physiological state and new and improved cell culture systems for manufacturing such cells are projected to accelerate in the future. The CSIR has developed a simple-to-use, cost-effective bench-top type device (T3D) which contains a PNIPAAm grafted 3D NWF scaffold in a perfused bioreactor to improve the efficiency of the cell culture process. The T3D device

of the present study represents a substantial improvement to the way in which cells are cultured, compared to static 2D monolayer cell culture method, as well as other PNIPAAm scaffolds which have been developed. The combination of a perfused bioreactor capable of culturing cells on a 3D scaffold capable of high-density cell culture and non-invasive cell release has not been reported / demonstrated previously in literature. Currently most of the bioreactors capable of 3D cell culture rely on either enzymes or chemicals to release cells, which are destructive to the cell membrane. Additionally most of the literature pertaining to "3D PNIPAAm scaffolds" focus on scaffolds with relatively small pores (sub-micron) only for the purpose of facilitating oxygen supply, and nutrient to cells, as well as hydration from the basal cell layer. Also the focus of many of these studies is still on the release of cell monolayers for cell sheet engineering, and not on cell expansion, propagation and release of 3D cell cultures. No other study reported the growth, propagation and release of 3D multicellular structures in large volumes non-invasively using PNIPAAm 3D scaffolds.

Currently cell culturing is a substantial bottleneck hampering advancements in a number of fields including tissue engineering, drug-screening, stem cell research and gene therapy amongst others. Successful implementation of the cell culture device will have far reaching implications not just for research but for the larger biotechnology industry.

The potential impact of the T3D device proposed in this project:

- Cells are cultured on a 3D surface in a contained sterile dynamic bioreactor environment to more closely resemble the cell physiological state (as compared to static 2D cell culture) thereby improving the quality of the outputs of *in vitro* cell culture research.
- Proliferation & harvesting of 3D cell clusters or spheroids are achieved which are more metabolically active and representative of the native state of cells.
- Confluent cells are released spontaneously and non-destructively from the surface of the scaffolds by cooling the cell culture medium from 37 ℃ to 20 ℃, and without requiring destructive enzymes, thereby preserving the ECM and surface proteins, which are important for downstream applications.
- Cell recovery time during passaging cycles is also reduced since cells have a preserved cell membrane composition, and are able to recover much faster.
- Cell culture is simplified since no enzymes are required thereby lowering costs since less washing and purification steps are needed.
- When the bench-top system is fully automated, minimal human intervention will be required hence the labour-intensive process required for manual cell culturing is

mitigated, thereby leading to reduced contamination, improved repeatability, and lower costs.

- The scaffold is supplied pre-fabricated, sterile and ready to use without requiring any preparation steps prior to use, hence simplifying the cell culture process.
- The scaffolds are highly permeable, allowing cells to easily pass through, and can be used directly for cell lysis (e.g. in a QiaShredder) without requiring any additional digestion steps, thereby saving time for the researcher, and improving the efficiency of the cell culture process. We have demonstrated this in our labs.
- Potential to improve quality of human life by contributing towards advancements in tissue engineering, stem cell research, *in vitro* drug screening and drug development research amongst others.

The PNIPAAm grafted 3D NWF scaffold and the T3D device could either be commercialised as one entity or separately; whereby typically any other similar scaffold could be used in the device, and the scaffolds itself could be used as 3D cell-culture inserts for culture trays as well as other suitable cell culture system. Both the scaffold and device has a relatively short route to market, with no preclinical or clinical trials required. Potential end-users include universities, research institutes, pharmaceutical companies, hospitals, etc., where large cell numbers are required and would include applications such as biomaterial and drug testing; drug screening of new actives, tissue engineering, and genetic engineering. The cell culture device of the present study aims to provide a reliable tool to culture cells with an *in vitro* cell proliferation system that minimises human intervention and potential error in a cost-effective, easy-to-use package that would enable widespread uptake.

7.3 Recommendations

The PP-*g*-PNIPAAm NWF scaffold developed in this study is suitable as a new 3D scaffold for non-invasive cell culture. However further investigations into the following aspects is suggested for this technology:

- Further improvements are required with respect to the homogeneity of the graft layer on the PP NWF surface. This will likely contribute to further improving the efficiency of cell release from the PP-*g*-PNIPAAm NWF.
- Improvements are required with respect to the structural stability of the NWF. Fibers dropped in the media from the NWF scaffolds during culture more so for the PET and

nylon fibres. Optimisation of the needle-punching technology is required which may involve improving the thermo-bonding process.

- We have shown that 2 hrs at 20 °C is required to release a significant amount of cells from the PP-*g*-PNIPAAm scaffolds. This cell release period however is relatively long. Currently the commercially available thermo-responsive 2D plates (UPCell™, Cell Seed Inc, Tokyo, Japan) require 30 min for release of a monolayer of cells. UPCell™ however is based on PNIPAAm grafted 2D surface. The grafted scaffold and the cell release protocol of the present study should be optimised such that maximum cell release is possible in a minimal amount of time. This may involve reducing the thickness of the PNIPAAm graft layer on the NWF scaffolds.
- In preliminary studies we have shown that high-density cell culture is possible in the T3D device, however cell numbers still are not significantly higher than 2D culture. Further optimisation is required to improve cell density in the system.
- At this stage we only showed proof of concept for hepatocyte cell lines. The present technology needs to be validated for a variety of different cell types (including primary cells and cell lines) before it can be adopted as a universal tool for cell culture.
- To improve the efficiency and reproducibility of cell culture, the system still needs to be automated such that human intervention is minimal. This will involve incorporating a programmable logic control system to automate the manual cell culture steps.