CHAPTER 6

Temperature-induced Cell Culture

Published in Biotechnology and Bioengineering
Part of work has been published in Colloids and Surfaces A: Interfaces and Engineering aspects
6.1 Introduction

It is well-known that cells grown onto 3D surfaces show closer similarities to their in vivo counterparts in terms of cell morphologies, behaviour, and function than 2D surfaces (Justice et al., 2009; Pampaloni et al., 2007). Nowadays 3D scaffolds are routinely used to culture cells, however cell release from 3D scaffolds remains challenging. Often enzymatic digestion of the extracellular matrix (ECM), mechanical scraping of the cells, or chemical degradation of the matrix is required in order to release adherent cells. Such release mechanisms cause irreversible damage to cells leading to disruptions in essential receptor-ligand interactions on the cell surface (Canavan et al., 2005). Damage to the ECM is known to adversely influence cell signalling pathways which affects important cellular processes for downstream applications (Guillame-Gentil et al., 2010). Furthermore, concerns exist with regards to the animal origin of some of the 3D ECM scaffolds and the associated production variability (Justice et al., 2009). This, combined with the additional wash steps and extra handling requirements, results in high costs, well-to-well variations, and culture inconsistencies.

Poly(N-isopropylacrylamide) (PNIPAAm), a temperature responsive polymer, has revolutionised the cell culture fraternity by providing a non-invasive means of harvesting adherent cells, whereby confluent cells can be spontaneously released by simply cooling the cell culture medium and without requiring enzymes. The pioneering work by Okano et al showed that cells could be released as intact monolayer cell sheets from the surface of PNIPAAm coated tissue culture polystyrene trays with their deposited ECM and preserved cell-cell and cell-ECM interactions (Okano et al., 1995). PNIPAAm switches its properties reversibly between hydrophobic (cell adhesive) and hydrophilic (non-cell adhesive) states at temperatures higher and lower than its lower critical solution temperature (LCST) (~32 °C) respectively (Schild, 1992). In recent years, advances were made in the area of regenerative medicine where stacking of homotypic or heterotypic cell sheets onto patterned surfaces has shown the formation of functional 3D tissues and organ-like constructs (Yamato et al., 2007).

PNIPAAm monolayer cell culturing is a promising tool for engineering tissue but the current technology is essentially based on the use of flat 2D substrates. The method of culture lacks structural and organisational cues for cells since the 2D environment does not replicate the complexity of physiological tissue (Isenberg et al., 2008). Where layered or patterned co-culture cell sheets are used, the process requires multiple steps and does not address the need for a structural matrix to enable cell growth in three dimensions. In recent years, some
3D PNIPAAm scaffolds have been developed based on hydrogels, grafted porous 3D membranes and micro-textured PNIPAAm surfaces (Isenberg et al., 2008; Kwon and Matsuda, 2006; Ohya et al., 2005). Additionally a PP-g-PNIPAAm non-woven membrane with adsorbed antibodies has recently been reported in the literature for selective cell separation and enrichment of target cells (Okamura et al., 2008). However, to date little work has been done regarding applying the PNIPAAm technology to highly porous 3D scaffolds for the purpose of 3D cell propagation and non-invasive recovery of 3D cell constructs. Additionally many of these studies still focus on the formation of 2D cell monolayers for the purpose of tissue engineering and regenerative medicine (See Table 6.1).

We have developed a new 3D scaffold based on PNIPAAm for use in 3D non-invasive cell culture. The scaffolds were developed to firstly support cell attachment, enhanced proliferation, and the growth of 3D cellular aggregates or constructs as opposed to cell monolayers. Furthermore the scaffolds were modified such that 3D cellular structures could be released non-invasively from its surface with surface proteins and extracellular matrix proteins remaining largely intact. We are also developing a novel cell culturing device (T3D) (Section 6.1.1) which will contain a bioreactor containing the 3D scaffolds for improving the efficiency of the cell culture process. The device is novel since such a device does not exist for cell culture anywhere in the world. 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.

In this study three different scaffolds were developed i.e. PP-g-PNIPAAm, PET-g-PNIPAAm, and nylon-g-PNIPAAm non-woven fabric (NWF) scaffolds, using an oxyfluorination-assisted graft polymerisation method as detailed in Chapter 5 which included functionalisation of the NWF scaffolds using fluorination followed by graft polymerisation in an aqueous NIPAAm solution. This chapter focuses on proof of concept or validation for the use of the developed scaffolds in non-invasive 3D culture of hepatocytes. Cell culture studies in the T3D bioreactor are also discussed.

6.1.1 CSIR’s cell culturing device T3D

The Council for Scientific and Industrial Research (CSIR) in South Africa is developing a thermoresponsive 3D (T3D) cell culturing device for culturing of adherent (or anchorage-dependent) cells. The device consists of a 3D thermoresponsive NWF scaffold which is contained in a sterile perfused bioreactor allowing for non-destructive and high-density cell
culture in a continuous manner (Moolman et al., 2009). The T3D bioreactor was designed and constructed by Mr Kobus van Wyk from BlueLine Designs (Pretoria, South Africa).

The specific attributes of the T3D device is as follows:

- **A bioreactor**: Cells are cultured in a closed, sterile, perfused bioreactor more closely simulating the physiological conditions, with sufficient media circulation, oxygen and nutrient supply, and waste removal.

- **A 3D scaffold**: Cells are cultured on a highly porous 3D scaffold based on a NWF to enhance cell-to-cell and cell-to-media interactions, with a high surface area to more closely resemble the physiological microenvironment compared to 2D culture trays. The NWF contains an open porous highly permeable polypropylene non-woven scaffold that enables cells to be cultured in a 3D environment allowing for cell-to-cell and cell-media interactions as well as direct perfusion of media containing nutrients and oxygen.

- **Non-destructive cell harvesting**: The device allows for the proliferation and non-destructive harvest of cells by containing a PNIPAAm graft layer on the 3D NWF. Cell release is then possible by changing the temperature of the cell culture media/or gas without requiring harsh detachment methods (such as using enzymes). The cell membrane of cultured cells is preserved whereby critical cell surface proteins remain intact after cell release.

- **Bench-top system**: The device is a small, cost-effective bench-top system which is very easy to operate.

- **Automation**: It is envisaged that the T3D device will be automated whereby cell culturing will be possible with minimal human intervention since the manual cell culturing steps are automated (such as cell seeding, cell proliferation and cell release) with automatic regulation and real time monitoring of system parameters such as temperature, pH, flow rates, oxygen consumption, etc.

A schematic of the final T3D prototype device is given in Figure 6.1. The device works by circulating cell culture medium maintained at 37 °C between the medium reservoir and the bioreactor with axial flow using a peristaltic pump; whereby the bioreactor will contain the NWF disks; cells are then seeded into the bioreactor via the sampling port and allowed to settle via gravity onto the NWF disks in the bioreactor; sufficient time is given for the bioreactor to be populated with cells; and then cold media is perfused through the bioreactor to detach cells from the scaffold. The media would then be recovered and centrifuged separately for cell recovery, or a continuous centrifuge would be incorporated into the
system for automatic separation of cells. The cells can then be stored in the cell reservoir until required.

**Figure 6.1:** Schematic of the CSIR’s T3D cell culture device showing the process-flow diagram for the bioreactor containing the 3D thermoresponsive non-woven scaffold.

The completed bioreactor system, including the bioreactor housing, the cell scaffold, the piping, the reservoir etc., would be off-the-shelf components, which would be easily sterilisable or pre-sterilised. All the system hardware (pumps, heating /cooling equipment, valves, etc.), instrumentation, electronic control and user interface and consumable location unit, are once-off acquisition that will be re-used for consecutive cell culturing experiments. The consumable set contains the “wetted” perfusion components (incl. the perfusion tubing, media reservoir, etc. and the bioreactor itself) and will be for a once-off single-use.

6.2 Experimental

To show proof-of-concept for the PNIPAAm grafted NWF as a new 3D scaffold for non-invasive culture of adherent cells, cells were cultured onto the scaffolds by Claire Rossouw at CSIR Biosciences and several assays were performed to determine cell viability,
metabolic activity, and thermal release potential. Assays which were conducted include AlamarBlue® cell viability assay, DNA cell quantification using Hoechst 33258, fluorescent staining of viable cells using fluorescein diacetate, metabolic activity using albumin blue, cytochrome P450 mRNA expression, and enzymatic and thermal-induced cell release (Rossouw et al., 2012). Comparisons were also made to a commercially available 3D scaffolds i.e. Aligimatrix™. This chapter focuses on validation of the scaffolds for 3D cell culture and temperature-triggered cell release.

6.2.1 Materials

HC04 (MRA-156, MR4) and HepG2 (ATCC HB-8065TM) hepatocyte cell lines were used in this study and obtained from ATCC®, Manassas, VA, U.S.A. Dulbecco’s Modified Eagle Medium (DMEM) was obtained from Lonza (Walkersville, Inc. Maryland, USA). All other chemicals (glutamine, foetal calf serum (FCS), penicillin, streptomycin) were obtained from Sigma-Aldrich Chemie, GmbH. The NWF scaffolds tested included: PP NWF scaffold (T2-N6(1): Mean –flow pores (MFP) ~ 127 µm; and T4-N13 : MFP ~200µm); PET NWF (T2-N7(1) : MWF ~142 µm, and nylon NWF (T3-N8(1)) : MFP ~79 µm.

6.2.2 Cell–scaffold interaction

To assess if the PNIPAAm grafted scaffolds could be used as a potential new scaffold for cell attachment, and enhanced proliferation, hepatocyte cells were cultured in static on PP-g-PNIPAAm which were pre-oxyfluorinated. The grafted NWF was cut into 5 x 5 x 3 mm pieces and were ethanol sterilised. 2 x 10^5 HepG2 and HC04 hepatocytes were seeded separately into the scaffolds which were maintained in 2 mL DMEM with 2 mM.L^-1 glutamine and supplemented with 10% (v/v) FCS, 100 g.mL^-1 penicillin and 10 µg.mL^-1 streptomycin in a 12 well tissue culture plate for 21 days under standard conditions. Media changes were performed every 48 hours. The HC04 and HepG2 cells growing on the scaffolds were visualised with fluorescent microscopy using fluorescein diacetate (FDA, Sigma-Aldrich Chemie) to monitor cell attachment and morphology on 3, 7, 11, 15 and 21 days post inoculation. After a specified time period, cells on the scaffolds were incubated with a FDA solution (enough to cover the scaffold) for 5 min, after which the cells were gently rinsed in PBS to remove any residual dye (Rossouw et al., 2012). The non-specific esterase activity in the cytoplasm of viable cells converts non-fluorescent FDA to fluorescein, a green fluorescent dye. Both the top and bottom of the scaffolds were viewed for cell attachment at the days mentioned above at 40x magnification using a standard fluorescence microscope.
6.2.3 Temperature-induced cell release from PNIPAAm grafted NWF scaffolds in static culture

After it was ascertained that the grafted NWF scaffolds was suitable for cell culture, PP-g-PNIPAAm; PET-g-PNIPAAm; and nylon-g-PNIPAAm NWF scaffolds were all assessed for their potential to enable temperature-triggered cell release. Cells were cultured on the scaffolds as described above, however on day 10, the scaffolds were gently rinsed in warm, sterile phosphate buffered saline (PBS) solution to remove loose or dead cells and were placed (3 / well) into a six-well plate containing 2 mL of cooled (20 °C) culture media. Nine scaffolds were used for testing: three scaffolds remained in the incubator at 37 °C, three at 20 °C for 1 hr, and three at 20 °C for 2 hrs to establish the length of time necessary for temperature-mediated cell detachment. The cell culture plates containing the scaffolds were periodically agitated by gentle swirling during the incubations, where after the scaffolds were removed and the released cells on the bottom of the wells were photographed at 40x magnification using a standard microscope (Olympus BX41).

6.2.4 Cell release from PP-g-PNIPAAm NWF in T3D bioreactor

Based on the device functional requirements, a preliminary prototype T3D device was initially developed as shown in Figures 6.2. PP-g-PNIPAAm NWF scaffolds (mean flow pore size: 200 µm) were cut into disks (15 mm in diameter) which were ethanol sterilised. Three disks were stacked horizontally in the preliminary prototype T3D bioreactor. 4.5 x 10^6 HepG2 hepatocytes were seeded into the bioreactor via the sampling port. The cells were then allowed to settle and attach to the grafted NWF disks for 3 hrs. DMEM Medium with 2 mM L^-1 glutamine and supplemented with 10% (v/v) FCS, 100 g.mL^-1 penicillin and 10 µg.mL^-1 streptomycin was perfused into the bioreactor at 2 mL min^-1 for a period of 10 days. A bioreactor running in parallel containing the pure PP NWF was used as the control. To ascertain thermal release potential of the grafted NWF, the bioreactor was perfused with cold media at 20 °C for 2 hrs. The media containing the released cells was then drained from the bioreactor, and the cells on the grafted NWF (before and after thermal release) were stained with FDA and visualised at 40x magnification using a standard fluorescence microscope (Olympus BX41) equipped with a 490 nm bandpass filter with a 510 nm cut-off filter for fluorescence emission.
Figure 6.2: Preliminary prototype of the CSIR’s T3D showing (a) i) sampling port through which cells are injected into the bioreactor; and ii) small volume bioreactor (without scaffolds); (b) close-up view of bioreactor containing stacked NWF disks (1.5mm diameter) with cells and media; and (c) full view of the preliminary prototype system showing the peristaltic pump used for pumping iii) media from (iv) media reservoir to the bioreactor; v) gas inlet (into the media); vi) gas vent; vii) gas line from tank; and viii) media return line (from the bioreactor).
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To ascertain if high-density cell culture was possible on the 3D NWF in the bioreactor, a preliminary trial was performed and cells were cultured for 14 days in the bioreactor as indicated above but at day 14 the media was drained and the NWF disks containing the proliferated cells were gently removed from the bioreactor, and the disks were trypsinated and the cells were harvested and counted using tryphan blue dye exclusion on a haemocytometer.

6.3 Results and discussion

6.3.1 Cell morphology and proliferation

To determine if the scaffolds were able to support cell attachment and growth, HC04 and HepG2 hepatocyte cell lines were used. HCO4 cells are a hepatocyte line immortalised from normal human liver tissue to support the in vitro development of human malaria parasite (Sattabongkot et al., 2006). HepG2 cells are a cell line from a human hepatic carcinoma and it is routinely used as a tool for drug toxicity screening.

To visualise the cells on the NWF scaffolds, and as an indicator of cell viability, fluorescence microscopy was used whereby only viable cells stain green with FDA. Representative cell morphology and proliferation of the HC04 and HepG2 hepatocytes on the PP-g-PNIPAAm scaffold over 21 days is shown in Figure 6.3.

Figure 6.3: Fluorescence micrographs of HC04 (top panel) and HepG2 (bottom panel) hepatocytes growing on the PP-g-PNIPAAm (pre-oxyfluorinated) after 3, 7, 11, 14 and 21 day post-inoculation (A-E) (Rossouw, et al., 2012).

Cells remained viable during the duration of the 21 day culture period due to the large surface area of the NWF, and scaffolds become densely populated with the cells.
Interestingly it was observed that the HC04 hepatocytes (Figure 6.3, top row) initially grew along the fibres in the NWF scaffold, and at later time points formed larger cellular clusters or constructs whereas the HepG2 hepatocytes (Figure 6.3, bottom row) formed spheroids soon after seeding. It is known that hepatocytes rapidly de-differentiate in monolayer cultures while hepatospecific functions are maintained for longer periods in 3D cultures (Pampaloni et al., 2007). Additionally it has been reported previously that 3D hepatocyte colonies with cell-to-cell contact, display an up-regulation of genes approaching that of native tissue (Pampaloni et al., 2007). We have recently reported significantly improved and superior gene expression and albumin production from the PP-g-PNIPAAm NWF scaffolds compared to cells cultured under the same conditions on 2D tissue culture polystyrene (Rossouw et al., 2012).

6.3.2 Temperature-induced cell culturing from PNIPAAm grafted NWF

An initial trial was conducted to see which of the grafted NWF scaffolds enabled optimal cell release from the scaffolds at 20 °C and to investigate the time required for cell release (Figure 6.4). PNIPAAm grafted PP, PET, and nylon NWF scaffolds were investigated when graft polymerisation was performed on direct fluorinated (gPP1, gPET1, and gnylon1), oxyfluorinated (gPP2, gPET2, and gnylon2) and the pure scaffolds (gPP3 and gPET3).

From Figure 6.4, it can be seen that cell release was most significant at 20 °C (as compared to 37 °C) after 2 hours of incubation. Cell release was also most significant from gPP1, and gPP2 (when grafting was performed on the direct fluorinated and oxyfluorinated PP NWF scaffolds respectively), compared to the other scaffolds. Very few cells released at 20°C from the grafted PP surface when pure PP was used for grafting (Figure 6.4, gPP3). This can be attributed to the relatively poorer graft layer when grafting was performed on the pure PP scaffolds as compared to when the oxyfluorinated or direct fluorinated PP scaffolds were used and this result corroborates with the findings in Chapter 5. With respect to the PET-g-PNIPAAm and nylon-g-NIPAAm NWF scaffolds; some of the scaffolds appeared to have released cells however it was observed that these scaffolds also dropped a large number of fibres into the media indicating that thermal release alone was not responsible for the released cells. The release of fibres in the media may be due to poor stability of the NWF scaffolds such that upon handling and post-treatment, fibres were lost. It was observed that some cells did drop loose from almost all of the scaffolds at 37 °C which was attributed to agitation of the scaffolds during the cell release protocol.
Figure 6.4: Thermal release of HCO4 hepatocytes after 10 days post-inoculation at 37 °C (control), and at 20 °C after either one or two hours of agitation. Scaffolds investigated were gPP1; gPP2; gPP3 (PP-g-PNIPAAm) where grafting was on direct fluorinated,
The results indicate that of the developed scaffolds PP-g-PNIPAAm (pre-fluorinated) NWF was the most feasible for use in non-invasive cell culture. This may be attributed to the superior graft layer on the PP NWF compared to PET and nylon. Another factor that may influence thermal cell release is the pore size distribution of the NWF scaffolds. Further optimisation is required for the PET-g-PNIPAAm and nylon-g-PNIPAAm NWF scaffolds and to improve the PNIPAAm graft yield before these scaffolds can be considered for temperature-induced cell culture.

To confirm the above results, temperature-induced cell release was repeated for the PP-g-PNIPAAm scaffolds (mean flow pores of 200 µm) using HCO4 and HepG2 cells when graft polymerisation was conducted on either direct fluorinated or oxyfluorinated PP NWF scaffolds (Figure 6.5 gPP1 and gPP2 respectively). As can be seen in Figure 6.5, a significant amount of cells were again released from both PP-g-PNIPAAm NWF scaffolds at 20 °C compared to 37 °C indicating that cell release is temperature-triggered due to the thermo-responsive nature of the PNIPAAm graft layer contained in the NWF scaffolds. Although both PP grafted scaffolds released a similar amount of cells, cell proliferation and growth was assessed to be better on the grafted pre-oxyfluorinated PP compared to the grafted direct fluorinated PP NWF scaffolds (Rossouw et al., [2012]). For both grafted scaffolds, cells were released as 3D cellular structures; and for the HepG2 cells, cell spheroids were observed. The 3D cell clusters and spheroids observed in this work, are a desirable morphology for certain cell culture applications (such as drug screening) as spheroids are highly metabolically active and a more relevant physiologically cell model.

Some contradictions exist in the literature with respect to the influence of graft thickness on cell attachment and release. Akiyama et al demonstrated that thickness of the PNIPAAm grafted onto tissue culture trays plays a key role in regulating cell adhesion of endothelial cells, whereby cells did not adhere to PNIPAAm grafted layers > 20 nm due to restricted molecular motion of the thicker grafted polymer chains resulting in insufficient dehydration of the outermost gel surface (Akiyama et al., 2007). In this study, we show that a PNIPAAm graft layer of ~200 nm (see Chapter 5) was still effective for both cell attachment and release. However it was observed that 2 hrs before a sufficient amount of cells could be
released. Further investigations are required to shorten the cell release time; which may involve reducing the thickness of the PNIPAAm graft layer such that attached cells can be released more efficiently.

**Figure 6.5:** Temperature-induced cell release of HCO4 cells (a-d) and HepG2 cells (e-h) from PP-g-PNIPAAm NWF scaffolds; gPP1 (grafting on direct fluorinated PP NWF); gPP2 (grafting on oxyfluorinated PP NWF), where (a, b, e, f) and (c, d, g, h) shows cell release after 2 hours at 37 °C (control) and 20 °C respectively (Rossouw et al., 2012).
6.3.3 Temperature-induced cell release from PP-g-PNIPAAm NWF in the T3D device

We also investigated temperature-triggered cell release when cells were cultured on PP-g-PNIPAAm NWF disks in the preliminary prototype T3D bioreactor (Figure 6.1). Hepatocytes were seeded on three PP-g-PNIPAAm NWF disks and cultured for 10 days in the bioreactor to determine non-destructive cell release at 20 °C. Figure 6.6a shows hepatocyte cells on the PP-g-PNIPAAm NWF disk at 37 °C at day 10 in culture while Figure 6.6b show cells remaining on the grafted PP NWF disk after temperature-induced cell release at 20 °C. Cells remained viable on the NWF scaffold during in culture in the bioreactor. A significant amount of cells were released from the PP-g-PNIPAAm NWF at 20 °C, confirming the thermoresponsive behaviour of the grafted NWF. Only minimal cell release was seen from the pure PP NWF disk at 20 °C due to loose cells falling off the scaffold (data not shown). As was observed previously, the hepatocyte cells grown on the PP-g-PNIPAAm NWF also arranged themselves as 3D multicellular aggregates (Figure 6.6a).

Fluorescence micrographs showing HepG2 cells on the PP-g-PNIPAAm NWF disks in the T3D device (preliminary prototype) (a) at 37 °C after 10 days of culture and (b) after thermal cell release at 20 °C for 2 hrs showing cells remaining on the NWF.

Fluorescent microscopy however did reveal that not all cells were released from the scaffold by a temperature change. Similar results were also observed for the thermal–release studies which were conducted in static culture. There are a number of factors which could contribute to cells still remaining on the NWF scaffolds after temperature-triggered cell release. This may possibly be attributed to inhomogeneity in the PNIPAAm layer on the NWF surface, whereby some of the fibre surface is not sufficiently grafted hence not all the attached cells
release using a temperature change. Another plausible explanation may be that due to the 3D cellular structural configuration of the cells in the NWF, not all cells may be easily able to navigate out of the matrix upon cell release.

An estimation of cell recovery from the PP-\textit{g}-PNIPAAm scaffolds after 10 days of culture was conducted for thermal cell release versus trypsin treatment (Rossouw et al., 2012). This study indicated that in most cases approximately similar cell numbers were obtained when either trypsin or thermal cell release protocol was used, and in each case fewer cells were released compared to the estimated total number of cells growing on the scaffolds. This finding can be attributed to the 3D nature of the cell clusters. The results are promising in that it shows that the temperature release mechanism of the grafted scaffolds under the present culture conditions is comparable to the number of cells released when trypsin is used, however temperature triggered cell release, displays the added advantage in that it is less destructive to the cultured cells, and cells are released with intact surface proteins and membrane constituents. However further optimisation of the scaffold and the cell culture process is needed to improve cell recovery.

A preliminary trial was also conducted to ascertain if high-density cell culture was possible in the preliminary T3D bioreactor. A total of $4.5 \times 10^6$ cells were initially seeded into the bioreactor containing three stacked pure PP NWF disks (15 mm diameter), and $39.7 \times 10^6$ and $49.9 \times 10^6$ hepatocytes were harvested from the bioreactor in two independent experiments at day 14, representing a 10 fold increase in cell numbers. Although these results are promising, further optimisation of the system is required to enable cell densities which are significantly higher than which can be obtained in conventional culture flasks.

Based on the results for the preliminary T3D device, the design of the T3D device was optimised and the final bench-top T3D system has now been constructed (Figure 6.7). Further studies are now on going to verify high-density cell culture in the T3D final prototype system.
6.4 Conclusions

In this study we have demonstrated proof-of-concept for temperature-induced cell release from PNIPAAm grafted NWF scaffolds. Hepatocyte cells attached on the scaffolds at 37 °C and remained viable for 21 days post inoculation. Cell release was most significant at 20 °C after 2 hrs of incubation from the PP-g-PNIPAAm NWF scaffolds compared to the grafted PET and nylon NWF scaffolds. Cells release was possible without using trypsin or other
destructive means. Additionally the cells were harvested as functional 3D multi-cellular spheroidal aggregates. The CSIR has also developed a novel T3D device containing a bioreactor for housing the PNIPAAm grafted scaffolds for use in 3D non-invasive cell culture. We have also demonstrated that cell release was possible from the PP-g-PNIPAAm NWF scaffolds in the T3D bioreactor. Due to the large surface area of the 3D scaffold NWF scaffold, we have also shown in a preliminary trial, that large increase in cell numbers is possible from the T3D bioreactor, however further optimisation of the scaffolds and device are required for improved performance.

### 6.5 References


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