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Optimization of an *in vitro* System to Study the Exo-erythrocytic Stage of the Human Malaria Parasite, *Plasmodium falciparum*

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INTRODUCTION

Much research remains to be done in order to understand the *Plasmodium*-liver interaction process. While previous *in vitro* studies have focused on two-dimensional (2D) cell culture, it is becoming more evident to researchers that the context in which cells are grown plays a significant role in their proper functioning. It is with this in mind that we hypothesise that a higher parasitemia may be achievable in cells with a three-dimensional (3D) morphology, thus much effort has been dedicated to the fabrication and testing of materials that can support 3D cell growth. In addition to the advances in 3D cell culturing, in the past few decades extensive research has been conducted in the use of thermo-responsive "smart" polymers for non-invasive cell harvesting. Pioneering work by Okano in the early 1980's revealed the use of a temperature-sensitive polymer, namely poly-N-isopropylacrylamide (PNIPAAm) for culturing cells. PNIPAAm is covalently grafted onto the polystyrene cell culture tray surface, facilitating cell attachment and spontaneous cell release through a temperature change without requiring trypsin (Okano *et al.*, 1995). The major advantage of using PNIPAAm for cell culturing is that cells are non-invasively harvested as intact cell sheets or clusters with critical cell surface proteins, growth factor receptors, and cell-to cell junction proteins remaining intact. The overall aim of this study is, therefore, to combine these two technologies to create novel thermo-responsive 3D scaffolds for HC04 hepatocyte proliferation and thermal release. HCO4 hepatocytes (Sattabongkot *et al.*, 2006) are able to support the complete development of the EE stage parasites *P. falciparum* and *P. vivax*. Ultimately, improved hepatocyte models may facilitate higher rates of parasitemia, leading to improved opportunities to study this infection process.

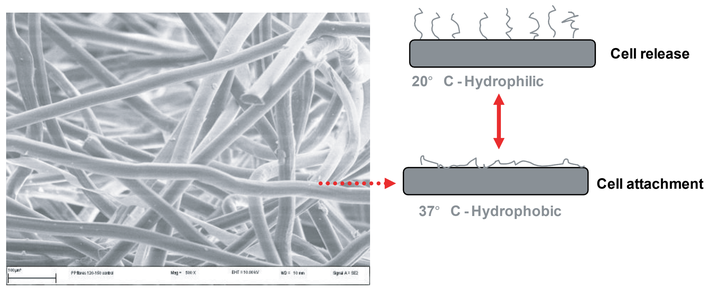

Figure 1: A schematic representation of the functionality of a thermo-responsive 3D scaffold.

METHODOLOGY

Polymers to be investigated

- Polypropylene (PP) non-woven, thermofused (145 °C), needle punched
- Polyethylene terephthalate (PET) non-woven, thermofused (180 °C), needle punched
- Nylon 6.6 non-woven, needle punched
- Also included in this study as the 2D control were polystyrene (PS) 96-well tissue culture plates (NUNC) and an additional scaffold which is commercially available was included in the study to serve as a positive control, namely, the AlgiMatrix™ 3D culturing system from Invitrogen.

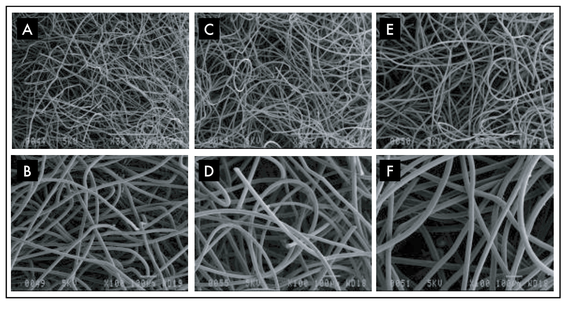

Figure 2: Scanning electron micrographs of the three non-woven polymers investigated in this study. A-B: Nylon, C-D: Polyethylene terephthalate, E-F: Polypropylene.

Table 1: Non-woven polymers and PNIPAAm grafting methods

Set Number	Polymer	Grafting method		
		Initiator	Temperature	Time
PP Control	PP	-	-	-
PET Control	PET	-	-	-
Nylon Control	Nylon	-	-	-
July Set 5	PP	Ceric ion/nitric acid	70°C	24 h
July Set 7	PP	Ceric ion/nitric acid	50°C	24 h
July Set 11	PP	APS	70°C	24 h
September Set 1	PET	APS	70°C	8 h
December Set 1	PP	**A**[1]: APS	70°C	7 h
		B[2]: APS		
December Set 1	PET	**A**[1]: APS	70°C	7 h
		B[2]: APS		
December Set 1	Nylon	**A**[1]: APS	70°C	7 h
		B[2]: APS		

1: Dynamic oxyfluorination method 5:95 F:N2, 3 min exposure
2: Static oxyfluorination method 20:80 F:N2 and other gas mixtures, 3 min exposure

Cells used

Human hepatocyte cell-lines HepG2 and HC04 (ATCC) were used in all experiments.

Hepatocyte proliferation on the scaffolds

- **Alamar Blue assay**

All of the scaffolds listed in table 1, the AlgiMatrix™ alginate scaffolds and the 2D control (96-well tissue culture plate), were each seeded with 200 µL of a 1 x 10^6 cells mL^{-1} HC04 cell suspension. Parallel samples of n=6 were used for each scaffold or 2D control per sampling point. The Alamar Blue assay was performed using the scaffolds 0, 3, 7, 11, 15 and 21 days post inoculation (dpi), these time points were selected based on previous work by Mandal and Kundu (2009), Shor *et al.* (2007) and Wagner *et al.* (1997). The time point day 0 served as a leak test and was used to ascertain how many cells were retained in the different scaffolds after the 2 h incubation period post inoculation.

On the day of the assay five scaffolds per treatment were transferred to the wells of a 24–well tissue culture plate containing a 10% Alamar Blue solution. After incubation the fluorescence was read at excitation and emission wavelengths of 570 nm and 585 nm respectively. The blank values were subtracted from the fluorescent values and the data were converted to viable cell number per scaffold using a standard curve. The media was removed from the wells in which the scaffolds were placed as well as from the 2D controls and were frozen at –70°C for subsequent DNA quantification. This experiment was repeated independently using HepG2 cells.

- **Fluorescent microscopy**

The HC04 and HepG2 cells growing on the scaffolds were visualised using fluorescein diacetate (FDA). Cell attachment and morphology was monitored on 3, 7, 11, 15 and 21 dpi.

- **DNA quantification using Hoechst 33258**

On the day of assay 500 µL of distilled water was added to the wells containing the individual scaffolds and then incubated at 37°C for 1 h. This was then followed by 3 freeze-thaw cycles to lyse the cells on the scaffolds during which time the plates were stacked and swirled regularly to ensure equal and even mixing of the DNA in the water. Equal volumes of the cell lysate and the working dye solution (20 µg mL^{-1}) were added in duplicate to wells of black-walled 96–well plate and fluorescence measured at excitation and emission wavelengths of 350 nm and 450 nm respectively. A blank was included to serve as a negative control and was subtracted from the fluorescent values obtained. The number of viable cells per scaffold was calculated by extrapolation from a standard curve.

Thermal release A

Thermal release A was conducted to ascertain which scaffold and grafting method would release its cells when cooled to 20 °C. Nine scaffolds per grafting method were seeded; three (3) were used as control scaffolds which remained at 37°C, 3 were allotted 1 hr for cell release at 20°C and 3 were allotted 2 hr for cell release at 20°C. The scaffolds were gently rinsed in warm, sterile PBS to remove any loose or dead cells from the scaffolds. Thereafter the scaffolds were placed (3/well) into a 6 well plate which contained room-temperature culture media. Periodically over the allotted time course the 6-well plates were stacked and gently swirled or lightly tapped to help agitate any trapped cells off/out the scaffold. Equal agitation was applied to all scaffolds. After the 1 and 2 hr thermal release respectively the scaffolds were removed from the wells and the cells that had been released from the scaffolds and settled at the bottom of the 6 well plate were photographed at 40x magnification. Relative thermal release efficiency could then be compared between scaffolds by looking at the number of cells dropped relative to the other scaffolds.

Thermal release B

Based on the results of thermal release A, PP-g-PNIPAAm scaffolds which were prepared by the 2 step grafting methods involving functionalisation either by static (PPb) or dynamic (PPa) oxyfluorination (table 1) appeared to have released more cells/cell clusters at room temperature after 2 hr than the controls and other grafted scaffolds. To confirm the results the experiment was repeated using the PPa and PPb scaffolds using both HC04 and HepG2 hepatocytes. The method was the same as above but using only a 2 hr release period. It was also noted if, while thermal release was taking place, a constant gentle agitation would help release more cells as compared to scaffolds that remained static during the 2 hr period and were only swirled/tapped at the end of the incubation period; agitation was achieved by placing the cells in the 12 well plates onto a plate shaker at 50 rpm.

RESULTS

Figure 3: Fluorescent microscope images showing viable HC04 hepatocytes on the PP-g-PNIPAAm non-woven scaffold "PPb". Cells were stained with Fluorescein diacetate and observed over the following time periods : a) day 3. b) day 7, c) day 11, d) Day 15 and e) day 21. (40 x magnification).

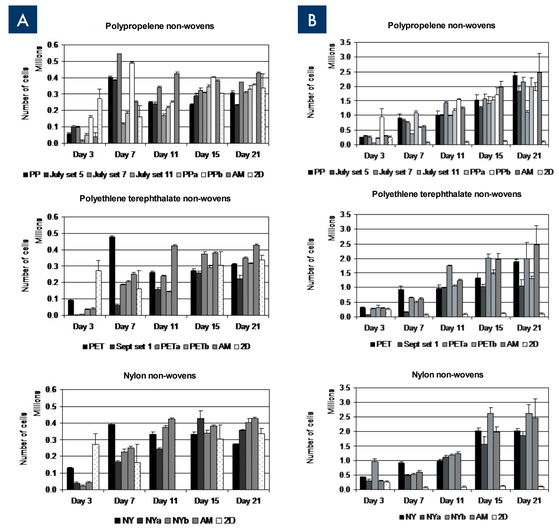

Figure 4: a) Alamar Blue and b) Hoechst 33258 results for HC04 hepatocytes growing on the non-woven scaffolds over 21 days.

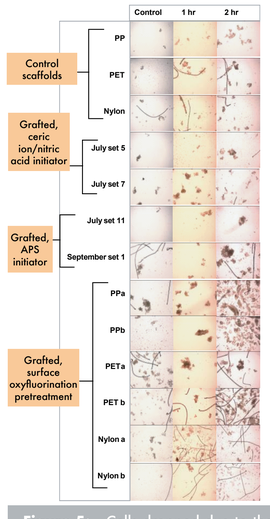

Figure 5: Cells dropped due to thermal release from the scaffold at 37 °C (control), 1hr at 20 °C and 2 hr at 20 °C respectively.

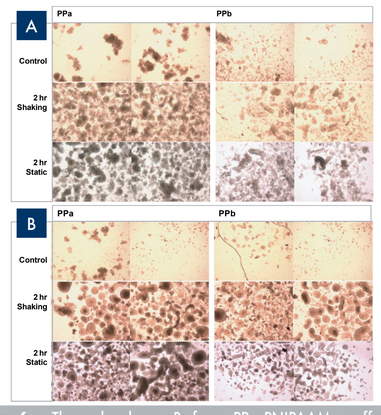

Figure 6: Thermal release B from PP-g-PNIPAAM scaffolds grafted using the 2 step grafting methods involving functionalisation either by static (PPb) or dynamic (PPa) oxyfluorination where a) HC04 hepatocytes and b) HepG2 hepatocytes are released from the scaffold.

DISCUSSION

Hepatocyte proliferation was monitored on the 3D scaffolds using the Alamar Blue assay and DNA quantification (Hoechst 33258). After 3 weeks of culturing it was observed that the HC04 and HepG2 hepatocytes proliferated better on the PP scaffolds as compared to the PET and nylon non-woven's. Specifically on the scaffolds "July set 7" (grafting with APS, and without functionalisation) and "PPb" (grafting by 2-step method involving oxyfluorination and APS initiation). The Alamar Blue results indicate an increase in cell number on the scaffolds for the first week, thereafter the number of cells appears to decrease or stabilise; this result can be attributed to the inability of the dye solution to penetrate through the well populated scaffold which is a result other researchers have encountered (Mandal and Kundu, 2009, Shor *et al.*, 2007). It was for this reason that a "destructive" assay, namely the DNA quantification assay was conducted. These data confirmed that although the Alamar Blue assay indicated the cell growth had slowed it had in fact continued and this is seen by the constant increase in DNA, which equates to more cells on the scaffold. The results were supported by the morphological data (Fig. 3).

Thermal cell release was successfully demonstrated on PP-g-PNIPAAm scaffolds which were prepared by the 2 step grafting methods (involving functionalisation either by static (PPb) or dynamic (PPa) oxyfluorination). A significantly higher number of cells were released from the PP-g-PNIPAAm non-woven scaffolds than from the pure PP non-wovens indicating that cell release was induced by the phase transition of the thermoresponsive PNIPAAm polymer.

Future studies include generating sporozoites and infecting the hepatocytes growing on the 3D scaffold. The parasitemia will be monitored and compared to that of infected HC04 monolayer's using qRT-PCR. Culturing on the thermo-responsive 3D scaffold and harvesting cells via the temperature change is currently being scaled up and a prototype bioreactor has been developed.

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Acknowledgments

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