CHAPTER 4

CONCLUDING DISCUSSION

New insight into cell phenotype and function *in vitro* and the desire to have cells functioning as closely to their *in vivo* counterparts has intensified the demand for materials that support 3D cell growth to enhance cell function in ways that resemble tissues *in vivo*. Cells growing *in vitro* are traditionally grown on 2D surfaces of tissue culture plastic and bear little resemblance to the complexities of the 3D tissues from which they were derived (5). Two-dimensional monolayer cultures are convenient for routine work but impose unnatural geometric and mechanical constraints upon the cells. The inherent problem with cells growing on 2D surfaces is the lack of dorsal anchorage points, which affects the balance between cells spreading or retracting. In tissues, cells connect to each other as well as to the ECM and it has been shown in various culture applications that the growth and function of cells as multi-cellular, 3D structures is significantly different to the 2D monolayer cultures (3, 5, 10-12). A change of environment for the cells, therefore, translates into a change in function and capacity for growth and differentiation. It is thus evident that the context in which cells are grown plays a significant role in their proper functioning and it has been proposed that the predictive accuracy of the drug discovery process and the analysis of host-pathogen interactions can be enhanced by engineering the cell culture microenvironment to create growth conditions that more accurately mimic the *in vivo* behaviour of cells (195). Thus, much effort has been dedicated to the fabrication and testing of materials that can support 3D cell growth. This study aimed to contribute two aspects to this field, specifically a non-woven 3D scaffold capable of supporting high numbers of cells, specifically hepatocytes, metabolically superior to their 2D counterparts. Secondly the non-woven scaffold should permit on-demand non-invasive release of the cells growing on and in the scaffold.

Despite the obvious advantages of 3D cell culture, the application of this technology as a tool for routine tissue culture applications has been slow. This is largely due to a number of identified limitations including: poor reproducibility between batches of biomimetic scaffolds, limited ability to scale up or down, difficulty in post-culturing processing and/or cell extraction from the matrix, lack of proven automated solutions, little flexibility in accommodating the many different cell-lines and types, characterising cells cultured in 3D geometries is difficult, poor visualisation and cost (98). By combining 3D cell culture with thermoresponsive technology, this study set out to investigate the potential of 3 non-woven fabrics (PP, PET and nylon) grafted with PNIPAAm to promote 3D hepatocyte proliferation in the scaffolds and subsequent non-

invasive cell harvesting of viable cells to overcome the problem of cell extraction from the 3D matrix for downstream applications. Ultimately, this 3D culturing technology was aimed to contribute towards studies such as host-parasite interactions, using the malaria parasite hepatocyte invasion system as model.

This study was successful regarding establishment of thermoresponsive 3D cell culturing technologies. This was measured by several factors as outlined below, providing novel data on and extending the current *status quo* regarding 3D cell culturing. High-density cell culture was achieved in this work and the number of cells cultured on selected non-woven scaffolds was comparable to the commercially available hydrogel 3D cell culture system, Algimatrix™. Hydrogel scaffolds are of the most commonly used and well characterised 3D scaffolds on the market; our scaffolds can, therefore, be considered in line with what would be acceptable to the consumer market in terms of cell numbers supported on the scaffold. A PP scaffold grafted with PNIPAAm was identified as being capable of promoting both good cell proliferation and displayed thermoresponsiveness and thus, was capable of non-invasive thermal release of the hepatocyte within the scaffold. This demonstrated the first “on-demand” method of cell harvesting using the thermoresponsive properties of PNIPAAm from 3D scaffolds thus circumventing the need to use salts or enzymes for scaffold degradation; a previously difficult result to achieve for downstream cell processing. The time it takes to release the cells from the 3D matrix is an area upon which future research may wish to improve. Currently thermal release of cells from 2D thermoresponsive plates takes approximately 30-40 min; halving the time it takes to release the cells from the 3D scaffolds would place our technology in line with what is commercially available. This may be achieved by further optimising the grafting method to adjust the graft thickness on the surface of the non-woven scaffolds. By using the scaffold identified to be thermoresponsive in a semi-automated cell culture device we were able to culture a large number of hepatocytes, however, cell release from this device would require further optimisation. This device would potentially allow researchers to grow large numbers of cells in 3D and harvest them non-invasively. Future work may aim to miniaturise the bioreactor such that several experiments could be run in parallel.

The second objective of this study was to test the hypothesis that hepatocytes growing in 3D would be more permissive to *P. falciparum* sporozoite invasion as a model system compared to hepatocytes growing on conventional tissue culture plastic in 2D. However, this was dependent on the successful production of *P. falciparum* gametocytes *in vitro* and the subsequent infection of *Anopheles* mosquitoes. We were successful in generating high yielding gametocyte cultures using NF54 *P. falciparum* parasites; gametocytogenesis reached 6% on occasion, a percentage

higher that currently reported in literature. We were, however, unsuccessful in infecting the mosquitoes with the parasite and were thus unable to generate an in-house source of sporozoites.

Due to the biological complexity of the malaria-*Anopheles* system, we may have been better positioned to study a less complex host-pathogen interaction, to show proof of concept of our 2D vs 3D system. The major vector of *P. falciparum* in Africa is *An. gambiae* which is widely distributed throughout the Afro-tropical belt (196) *An. gambiae sp.* is divided into two morphologically indistinguishable molecular forms, known as M and S. The susceptibility of the S and M molecular forms to *P. falciparum* relay contrasting findings; S and M forms exhibited similar susceptibility in Cameroon whereas in Senegal the S form were more susceptible than M form mosquitoes.(196-198), thus, it can be inferred that the susceptibility of *Anopheles* mosquitoes to *Plasmodium* infection is under genetic control. Multiple lines of evidence also suggest that mosquito bacterial communities influence vector competence (196). The protective role of *Anopheles* midgut bacteria against malaria infections was demonstrated by using antibiotic treatment to clear the gut microbiota, which resulted in enhanced *Plasmodium* infections (199, 200) and it has been observed that co-infections of bacteria with *Plasmodium* reduced the number of developing oocysts in the mosquito midgut, in the laboratory as well as field conditions (196). However, in a study by Boissiere (196) it was found that an abundance of *Enterobacteriaceae* is higher in *P. falciparum*-infected mosquitoes, suggesting that some microbe-parasite interactions may, in fact, contribute to the successful development of the malaria parasite. The inability to infect the mosquitoes in our insectary may hinge on one of the afore-mentioned factors which would need to be more extensively studied in the future.

When the *in vitro* invasiveness of commercially sourced *P. falciparum* sporozoites was tested in hepatocytes growing in 3D it was ultimately unsuccessful; this complex parasite may ultimately require a more advanced *in vitro* system to enhance *in vitro* sporozoite invasiveness. The results of this study can, however, be used as the basis to hypothesise a different strategy, perhaps one involving a different type of 3D scaffold or basement membrane, or possibly investigating macrophage-hepatocyte co-cultures. The possibility remains that this may be a host-pathogen interaction that will always be difficult to work with *in vitro.* However, due to the fact that the pursuit for novel drugs and vaccine strategies is expensive and at present animal testing currently offers the best means to study the malaria liver stage, this is an important area of research where all options for an *in vitro* model system need to be exhausted. *In vitro* assays are invariably cheaper, would allow high throughput screening of novel compounds and are ethically more acceptable.

In conclusion, this work demonstrated the potential of 3D cell culture and on-demand cell release in the grafted non-woven scaffolds and the possible application of an automated 3D cell culture and non-invasive release was demonstrated. In the human body virtually all organs are functionally integrated and looking to the future of 3D cell culture for the study of toxicology and host-pathogen interactions, investigators are already developing integrated 'human-on-a-chip' models that consist of interconnected compartments, each containing a cell type representing a different organ, linked through a microfluidic circulatory system (201); this concept is illustrated in Figure 4.1. This *in vivo* mimicry would vastly improve upon the speed with which new drugs reach the bedsides of patients and enhance our understanding of complex-host pathogen models for improved rational design of drugs and prophylaxis.

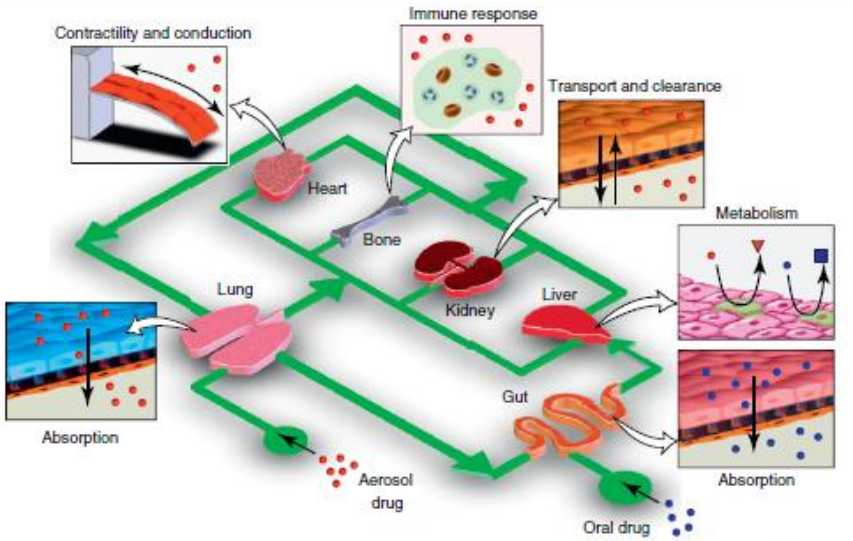

Figure 4.1. An illustration of the **"**human-on-a-chip" concept. Biomimetic microsystems representing different organs can be integrated into a single micro-device and linked by a microfluidics circulatory system in a physiologically relevant manner to model a complex, dynamic process of drug absorption, distribution, metabolism and excretion, and to more reliably evaluate drug efficacy and toxicity (202).

The investigation of useful biomaterials for *in vitro* mimicry is becoming more diverse bringing knowledge from a variety of disciplines (203) and bridging the divide between materials scientists and biologists alike. The changing future of cell culture will facilitate the translation of basic science to the clinical setting and the development and use of a series of increasingly complex 3D model systems. In order to achieve this, transdisciplinary research is fast becoming a more common practice and ultimately will be a priority for fast tracking novel materials from their conception to intended application. Our work was a collaboration between chemists, engineers, material scientists and biologists, all of whom played a critical role in the development of the novel thermoresponsive 3D scaffold. By engaging in transdisciplinary research we were able to address issues that could not otherwise be solved by one scientific discipline alone. The success of our collaborations can be summed up by four key factors as

outlined by Kessel and Rosenveld (204), these are: the willingness of the participating scientists to commit sufficient time to collaborative endeavours; openness to learning each other's disciplinary languages and jargon; the capacity to build mutual confidence and trust between researchers and overcoming the challenge of working as equals, with no knowledge or discipline or practice assuming priority. Additional factors contributing the success of this type of research between different scientific expertise and institutes is learning how to respect the value(s) of others and to be less concerned about submerging ones professional identity in the team process. To provide a broad foundation for such a process, the institutional infrastructure of scientific research, that is universities, journals, and funders, also all need to be aligned in support of transdisciplinary team science. Moreover, issues such as barriers between departments and faculties, authorship, peer review and funding applications can either support team science or constitute limiting factors (204). This study is a testament to the possibility of collaborative work. Much effort and time was dedicated to meetings and planning and the team co-operation lead to the successful development of the novel 3D scaffold.

This body of work represents the successful efforts to graft PNIPAAm onto 3D non-woven scaffolds for thermal release of cultured cell growing in 3D. We successfully demonstrated high density 3D cell proliferation using hepatocytes on the 3D scaffold comparable to that observed using the commercially available Algimatrix^{TM} system; thermal release of the hepatocytes from selected grafted non-woven's was also successfully demonstrated. Hepatocytes growing on the 3D scaffolds were observed to be metabolically superior to their 2D counterparts further validating the use of this scaffold for cell culture experiments. Applications of this scaffold may, therefore, be for drug and toxicity screening or high density proliferation and release of cells for the study of surface antigens and/or proteins that would have otherwise have been destroyed by enzymatic removal of the cells from the scaffold. A pilot scale-up experiment of the thermoresponsive scaffolds in a semi-automated system indicated that with further optimisation these scaffolds may be useful for very high density cell proliferation and recovery. When using the 3D scaffolds for a proof-of-concept experiment using the hepatocyte-sporozoite interaction we were not successful as hoped. However, these data obtained from this experiment will guide future experiments in the pursuit of an *in vitro* system that permits optimal sporozoite invasion – this would be an important development in the fight against this devastating pathogen.