CHAPTER 1

THREE DIMENSIONAL CELL CULTURE SYSTEMS AND THEIR APPLICATIONS: A REVIEW

Mammals are comprised of a large number of cell types with a variety of specialised functions including tissue scaffolding and structure; signalling and sensing; digestion and absorption; transfer of molecular oxygen; immunity; mobility and reproduction to name a few. One of the major developments in science and particularly cell biology has been the development of systems allowing the growth of these various cell types *in vitro*, literally meaning "in glass" or outside the body. *In vitro,* biologists have been able to study amongst others cell differentiation, function, development, growth, regeneration and death.

Tissue culture was first developed in the early 1900's as a method for studying the behaviour of tissue fragments and gradually biologists developed techniques to study the behaviour of single cells and changed the name to cell culture. In its simplest form, cell culture involves the dispersal of cells in an artificial environment composed of nutrient solutions, a suitable surface to support the growth of cells, and ideal conditions of temperature, humidity, and gaseous atmosphere. In such a system, a researcher can measure the cells' response to culture and genetic alterations, physiological signalling molecules, prospective drugs, interaction with other kinds of cells, carcinogenic agents and pathogens. As our ability to grow, manipulate and analyse cells in this way has developed, so has our knowledge of cell function and physiology.

Increasingly the concept of three-dimension(al) (3D) is being applied in relation to *in vitro* cell culturing. *In vivo* all tissues reside in an extracellular matrix (ECM), which comprises of a complex 3D fibrous meshwork. Additionally, depending on the cell type the 3D microenvironments differ significantly (1). For example, osteoblasts are found on the surface of bone organised in sheet-like structures as cuboidal cells; hepatocytes are packed closely together in hexagonal shaped lobules and lymphocytes are suspended freely in blood or lymph vessels (1). Today, there is increasing awareness of the drawbacks of 2D *in vitro* cell culturing and the related effect on the value of the research being performed as the dynamic range of structural organisation of various cell types *in vivo* cannot be accurately emulated using 2D cell culture technology. As a result, altered metabolism and reduced functionality are observed in 2D *in vitro* cell culture (2, 3) thus, 3D cell culture matrices also known as scaffolds were introduced to overcome these limitations (1).

An analysis of the number of publications in the field of 3D cell culture indicates an increasing interest in this field. Not surprisingly, more and more scientists are shifting their focus to cells cultured in 3D as intuitively one can appreciate that with cells, form affects function (4). Figure 1.1 provides a graphical representation of the increase in the number of publications arising from studies performed by using cells cultured in 3D.

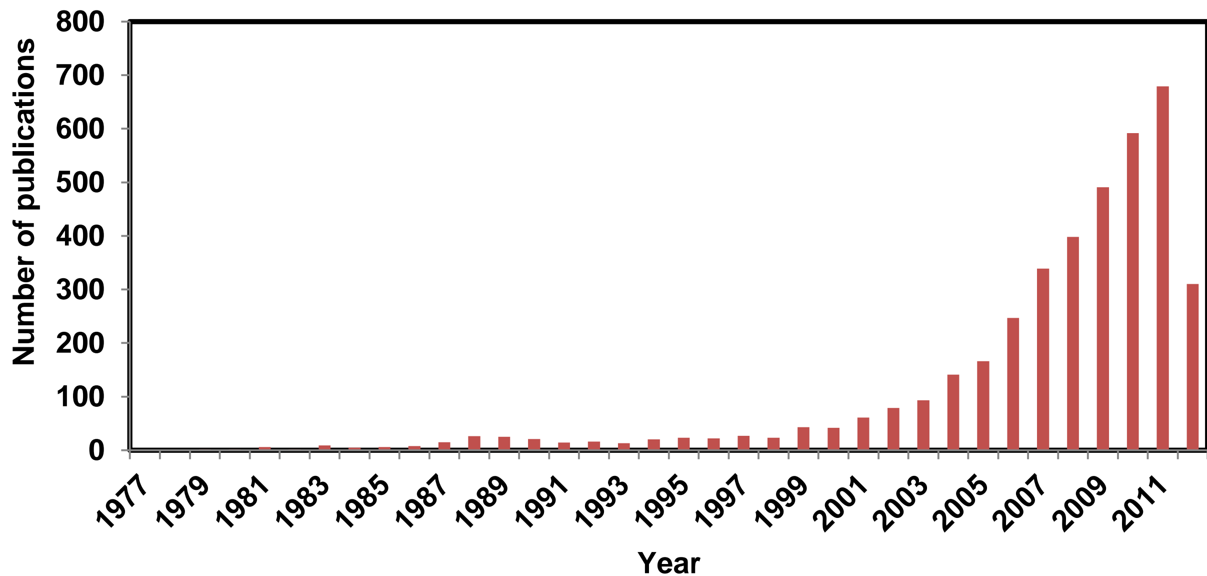

Figure 1.1. 3D cell culture publications per year (1977- June 2012). From 1977 a slow increase in the number of publications was observed in the field of 3D cell culture; a more rapid increase is observed after 2000. Source: Scopus statistics (accessed 25 June 2012).

This review looks at 3D cell culture and its growing importance in *in vitro* biology as well as the various parameters used to design and construct 3D scaffolds. Various applications of 3D cell culture in the fields of *in vitro* toxicology, anti-cancer drug screening and host-pathogen interactions are reviewed and the future of 3D cell culture is discussed.

1.1. 2D VS 3D: WHY DO WE NEED 3D?

Cells growing *in vitro* are traditionally grown on 2D surfaces of tissue culture plastic and this system has significantly improved the understanding of basic cell biology. However, 2D cell cultures bear little resemblance to the complexities of the 3D tissues from which they are derived (5). Two-dimensional monolayer cultures are convenient for routine work but impose unnatural geometric and mechanical constraints upon 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. This creates an unnatural stimulatory environment for the

cells resulting in a physical imbalance, which causes the aberrant spreading of the cells (6). As such, vastly different morphology is observed for cells cultured on 2D surfaces compared to 3D as illustrated in Figure 1.2.

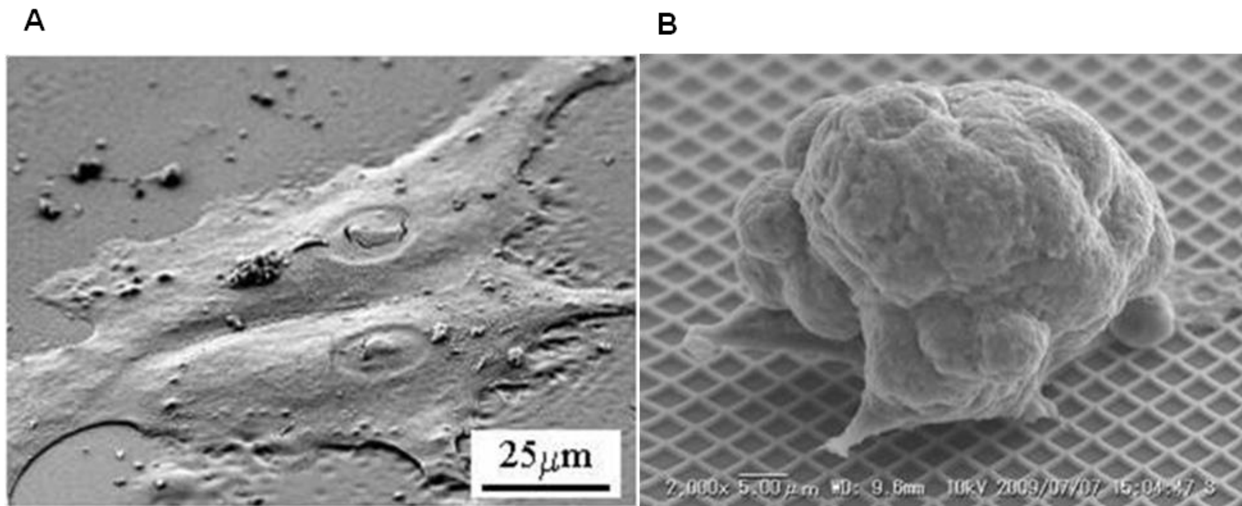

Figure 1.2. 2D and 3D cell culture morphology. **A**. Cells growing in a 2D format on the surface of tissue culture plastic show distinct flattening and spreading. **B.** Cells aggregated to form a 3D spheroid, replicating the cells' natural 3D conformation and morphology. Micrographs from (7).

Typically, cells growing in 3D are ellipsoids with dimensions of 10-30 μm whereas cells growing in 2D are flat and on average 3 μm thick (8). Cells in 3D have nearly 100% of their surface area exposed to other cells, the 3D matrix and culture medium whereas cells in 2D have approximately 50% of their surface area exposed to the cell culture media and approximately 50% exposed to the flat culture surface; only the marginal areas of the cell periphery is exposed to surrounding cells (8). Thus, 2D cultures do not mimic *in vivo* tissues as well as 3D cultures with regards to cell shape and cellular environment (9).

In addition to 3D cultures maintaining cell shape, it is also more successful in mimicking cell functionality. In native tissue, cells connect to each other as well as to the ECM to provide mechanical and biochemical signals that guide cell function. Various culture applications have shown that the growth and function of cells as multi-cellular, 3D structures are significantly different and more representative of the *in vivo* physiological state compared to 2D monolayer cultures (3, 5, 10-12). The difference lies primarily in the formation of chemical signal or molecular gradients which are important for biological differentiation, cell fate, organ development and signal transduction to name but a few. Other changes in metabolic and gene expression patterns as well as a significant reduction in the production of ECM proteins have been observed for 2D monolayer cultures (2). A change of environment for the cells, therefore, translates into a change in function and capacity for growth and differentiation. These enhanced

interactions in the 3D environment enable the cells to interpret the multitude of biochemical and physical cues from the immediate environment (6).

3D systems exhibit a much closer approximation to the *in vivo* cell microenvironment as a result of improved nutrient, oxygen and waste exchange leading to the maintenance of cell viability and function.

3D cell cultures have been used in a broad range of cell biology research, including tumour biology, cell adhesion, cell migration, metastasis, angiogenesis, epithelial morphogenesis, drug discovery applications and transport studies. Figure 1.3 illustrates several observed benefits of using 3D cell culture in place of conventional 2D cultures. Selected examples of these enhanced differences have been studied in, for example, 3D liver (6), bone (13) and neural (14) models.

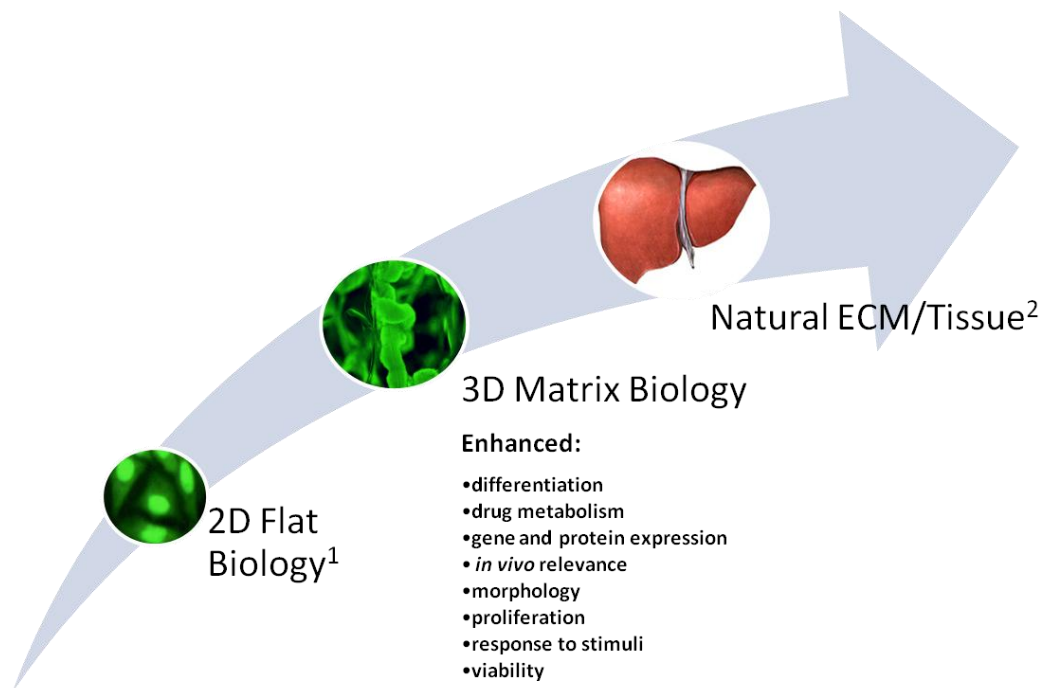

Figure 1.3. Bridging the gap between 2D cell culture and natural tissue. 3D cell cultures have demonstrated enhanced cell differentiation, drug metabolism, gene and protein expression, *in vivo* relevance, morphology, proliferation, response to stimuli and viability as compared to cells growing in 2D; [1]2D HepG2 hepatocytes (15); [2]the liver (16).

This insight into cell phenotype and function *in vitro* and the desire to have cells functioning closely to their *in vivo* counterparts has intensified the demand for the best materials able to support 3D cell growth, organisation and differentiation.

1.2. 3D SCAFFOLDS

1.2.1. Materials of choice

Material selection for 3D scaffold development is based on three fundamental criteria: the biomaterial of choice, the bulk properties of the selected material and the surface properties and/or surface modifications of the selected biomaterial as illustrated by Figure 1.4. The criteria will be different for each scaffold depending on its intended application.

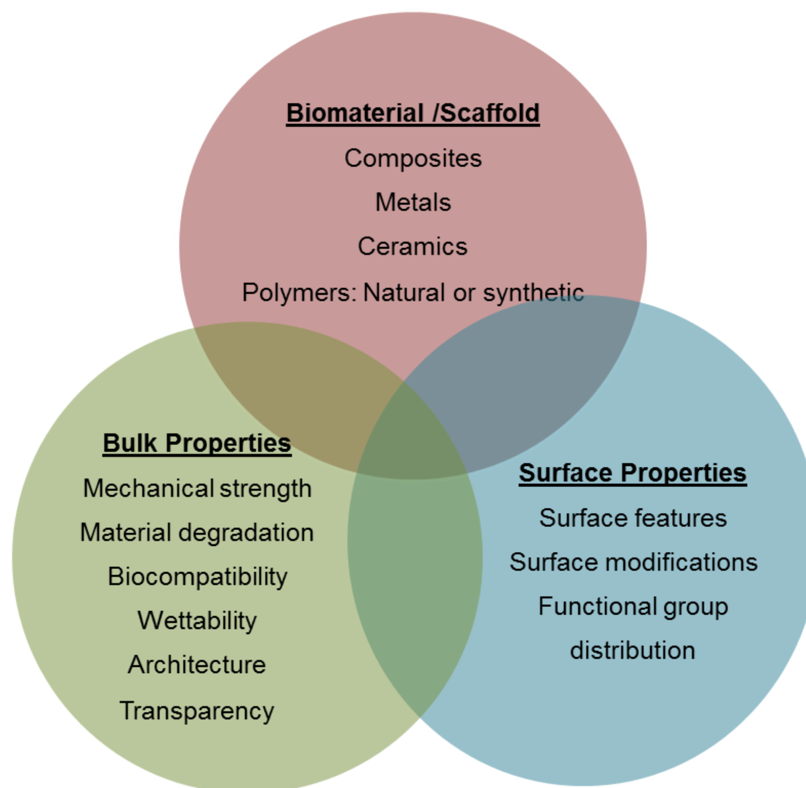

Figure 1.4. The three criteria used to design 3D scaffolds. These include the selection of the bulk material and its bulk and surface properties.

Bulk material selection is the first consideration of 3D matrix design as this will dictate various fundamental properties of the matrix, from biological effects to processability (1). For example, clinical work that requires a functioning implant may only require a temporary, biodegradable scaffold, which breaks down into metabolites without a toxic or immunogenic response. Alternatively a scaffold used as a 3D *in vitro* model needs to accurately reproduce the native tissue structure containing cells at a given stage of differentiation as well as a greater need to image these models for cell function and response (17).

Many new biomaterials have been developed to mimic the unique characteristics of natural ECM for *in vitro* cell culture applications. These biomaterials can be broadly divided into 4

categories: polymers, composites, metals and ceramics (1, 18). Among them, polymeric materials have received substantial attention because of the great flexibility in designing the composition and structure of scaffolds for specific needs (1). Polymeric 3D scaffolds can be further divided into natural and synthetic polymers as illustrated in Figure 1.5.

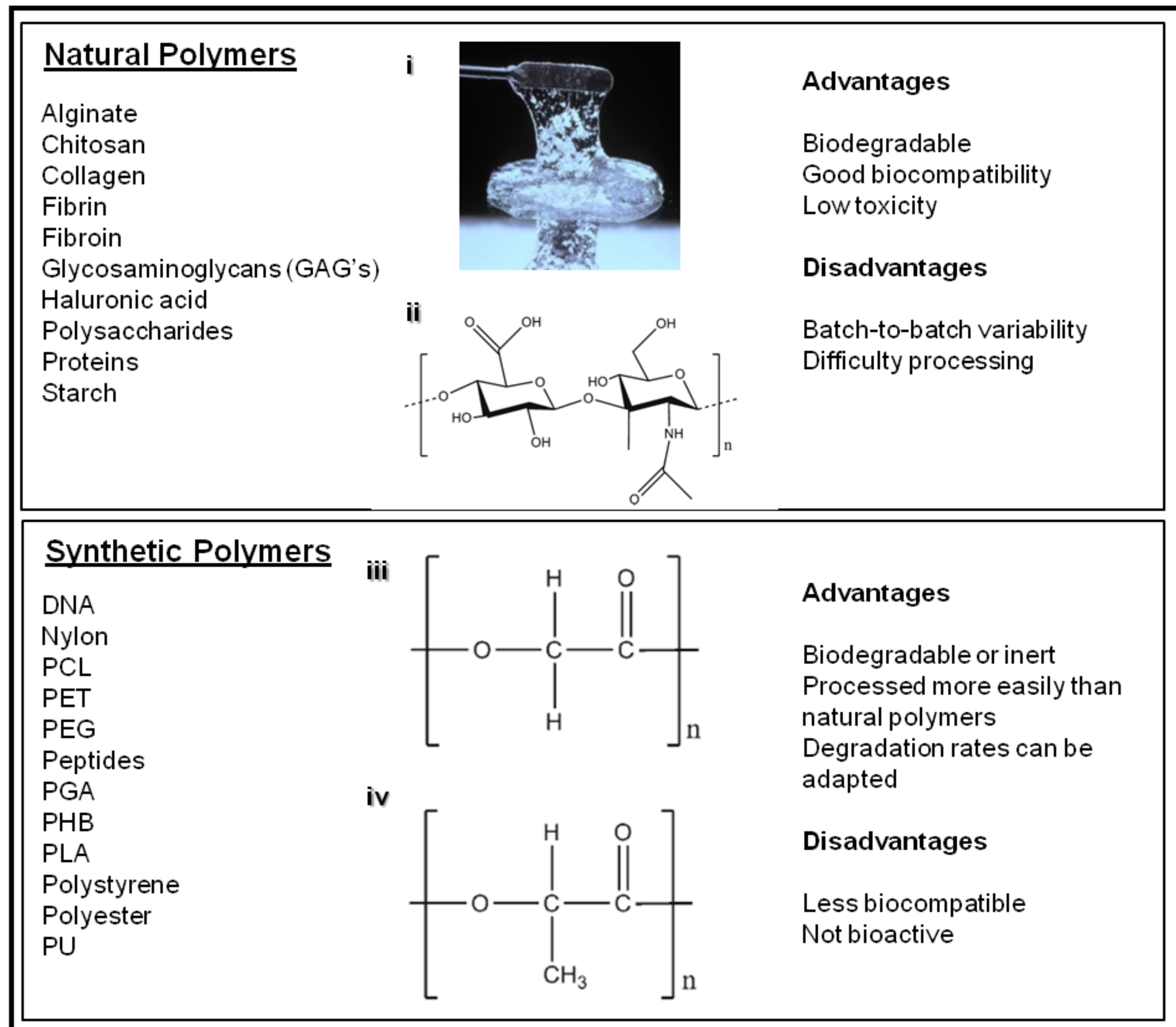

Figure 1.5. Polymer scaffolds for 3D cell culture. Polymeric scaffold are made from either natural or synthetic polymers. i-ii: An example of a natural polymer material, biodegradable haluronic acid and its chemical formula (19, 20). iii-iv: the chemical formulae of the most commonly used degradable synthetic polymers poly(glycolic acid) and poly(lactic acid) (20).

When selecting a material for scaffold fabrication various factors are important to consider. The biocompatibility of the material is an important characteristic; this is especially true if the scaffold is to be implanted into the body. The biocompatibility of the biomaterial determines the ability of the scaffold to perform its specified function without eliciting inappropriate cellular and/or host responses. Biocompatibility can be considered a graded characteristic for which the requirements change depending on the specific application of the scaffold; natural materials are typically more biocompatible than synthetic materials (1). Controlled biodegradability is an

essential requirement for implantable 3D scaffolds as the scaffolds should degrade at the rate that in-growing tissue replaces them (1). Synthetic materials generally degrade by hydrolysis (21) and natural materials generally undergo enzymatic degradation (22). Mechanical properties of the bulk materials are an important set of characteristics to consider in 3D matrix design, especially in tissue engineering for structural tissues i.e. bone. The scaffold must be able to endure sufficient loads so as not to fracture but, should not be too strong thus damaging adjacent tissue. Mechanical properties also directly shape surface mechanical properties such as surface stiffness or elasticity, which elicit clear cellular responses (1). Natural ECM is a fully hydrated gel, thus wettability is another bulk material consideration; a hydrophilic biomaterial is better at mimicking the *in vivo* aqueous environment. Transparency is another important parameter for 3D *in vitro* modelling applications where cellular behaviour within the scaffolds is to be visualised using optical, fluorescence or confocal microscopy (1).

Many types of biodegradable polymeric materials have been used for scaffold fabrication in tissue engineering applications. The gradual degradation of a biodegradable polymer is an important feature of a scaffold to aid the integration of the cells they carry with host tissues. Natural-based materials include polysaccharides (starch, alginate, chitin/chitosan, hyaluronic acid derivatives) or proteins (soy, collagen, fibrin gels, silk) and, as reinforcement, a variety of bio-fibres such as lignocellulosic natural fibres (23). Synthetic polymers include poly(lactic acid), poly(glycolic acid), poly(ε-caprolactone) (PCL) and poly(hydroxyl butyrate) (24). Many advantages and disadvantages characterise these two different classes of biodegradable polymers. The synthetic polymers have relatively good mechanical strength and their shape and degradation rate can be easily modified, but their surfaces are typically hydrophobic and lack cell-recognition signals. Naturally derived polymers have the potential advantage of biological recognition that may positively support cell adhesion and function, but they generally have poor mechanical properties (24). Synthetic polymers can be produced under controlled conditions and therefore exhibit, in general, predictable and reproducible mechanical and physical properties such as tensile strength and elastic modulus. A further advantage of synthetic polymers is the control of material impurities. Possible risks such as toxicity, immunogenicity and favouring of infections are lower for pure synthetic polymers with constituent monomeric units having a well-known and simple structure (23).

Natural and synthetic polymers exist as three main morphological formats to develop various 3D scaffolds; these are hydrogels, open- and closed cell-porous scaffolds and non-woven scaffolds.

Hydrogels have been utilised as scaffold materials for drug and growth factor delivery, engineering tissue replacements and a variety of other applications. Due to their ability to simulate the nature of most soft tissues, hydrogels are a highly attractive material for developing synthetic ECM analogs. A hydrogel is a network of polymer chains that are water-insoluble. The gels possess a high water content, facile transport of oxygen, nutrients and waste, as well as realistic transport of soluble factors (25). Furthermore, many hydrogels can be formed under mild, cyto-compatible conditions, they possess a degree of flexibility very similar to natural tissues due to their significant water content and are easily modified to possess cell adhesion ligands, desired visco-elasticity and degradability (26).

A variety of synthetic and naturally derived materials may be used to form hydrogels for tissue engineering scaffolds. Synthetic materials include poly(ethylene glycol) (PEG), macroscopically self-assembled peptides and DNA (1). DNA hydrogels are formed from branched DNA molecules with complementary sticky ends which hybridise to one another via DNA ligase to form hydrogels. Natural gels for cell culture are typically formed by proteins and ECM components (27) such as collagen (28) fibrin (29), hyaluronic acid (30), or Matrigel™ (31), as well as materials derived from other biological sources such as chitosan (32), alginate (33), or silk fibrils. Since they are derived from natural sources, these gels are inherently biocompatible and bioactive (34).

Solid but highly porous scaffolds are an alternative for growing cells in 3D. The scaffolds are convenient to use, they can be manufactured in a controlled and reproducible manner, they can be moulded and shaped as needed and their structure remains stable over time. The porosity of these structures can be controlled during the production process and can, therefore, be tailored towards the types of cells to be cultured (35). Porous scaffolds fabricated from polystyrene can be used to support *in vitro* cell growth as previously described by Hayman *et al.* (35, 36) and modified by Bokhari *et al.* (5). These solid scaffolds were manufactured by polymerisation in high internal phase emulsions (HIPEs); the PolyHIPE material is characterised by its low density and open cellular construction. Bokhari *et al.* (5, 6) document the improved cell structure and function of Hep2G cells growing on this 3D culture device and an enhanced function during toxicological challenge.

Non-woven fibrous matrices are widely used scaffolds in tissue engineering (37). Many different polymers have been used to manufacture non-woven fabrics for use as scaffolds in 3D cell culture. These include polyester (38), poly(etherurethane) (39), poly(ethylene terephthalate) (PET) (37), nylon (40) and PCL (41). Non-woven fibre production is performed by the polymer of

choice being melted and extruded though capillaries which form microfibers or via electrospinning, which uses an electrical charge to draw very fine fibres (typically on the micro or nano scale) from a liquid. Non-woven materials are then formed in numerous ways including thermal bonding, needle-punching, hydro-entanglement, ultrasonic pattern bonding, chemical bonding or melt blowing (42). The advantage of using non-woven scaffolds is their large surface-area to volume ratio and the opportunity to manipulate scaffold structure and mechanical properties (43).

1.2.2. Bulk modifications

Among the scaffolds currently in use, the majority are either “simple” or “ill-defined” and many of the simple matrices are often made from only one or two components such that the physiological properties can be controlled (44). A drawback of these “simpler” polymers, however, is that cellular recognition is limited, thus restricting natural cell-matrix adhesions or abolishing them all together. These variations may impact on important cellular signalling mechanisms. On the other hand there are complex, ill-defined matrices such as Matrigel™ (44), which is a solubilised basement membrane preparation extracted from Engelbrecht-Holm-Swarm (EHS) mouse sarcoma, a tumour rich in ECM proteins. The inherent complexity of these scaffolds makes it difficult to understand cell signalling and batch-to-batch variability can negatively impact on the reproducibility of experiments (44).

“Biomimetic materials” are able to elicit specific cellular responses and direct new tissue formation mediated by biomolecular recognition (45) and modulate many aspects of synthetic matrices in an attempt to overcome the limitations of simpler polymers (46). This is achieved by altering design parameters of the selected bulk material via chemical or physical methods with bioactive molecules, such as a native long chain of ECM proteins as well as short peptides derived from intact ECM proteins that can affect specific interactions with cell receptors (45).

Bulk properties are frequently modified to replicate the multi-functional tasks of natural ECM on an artificial 3D matrix. One approach involves the incorporation of cell signalling peptides into the biomaterial such that the recognition molecules are present, not only on the surface but in the bulk of the material. One of the applications of bulk modification is the introduction of enzymatically degradable sequences, for example, photo-polymerised hydrogels containing Ala-Pro-Gly-Leu (APGL) or Val-Arg-Asn (VRN) peptides are sensitive to collagenase or plasmin degradation, respectively (45).

1.2.2. Surface modifications

Surface modification of biomaterials is a simple way to generate biomimetic materials. The surface chemical properties of a biomaterial are fundamental for dictating the adhesion and spreading of living cells (47) and one approach to endow biomaterials with bioactivity involves the incorporation of cell binding peptides into biomaterials via chemical or physiological modification to develop brand new materials (45). The incorporation of peptides into biomaterials may, for example, enhance the adhesive properties of the scaffold (48) or may render the scaffold biodegradable by specific proteases (49) such as the amino acid sequence 'A_9' for elastase mediated degradation (45).

Early work involved coating the surface of biomaterials with long chains of ECM proteins such as fibronectin, vitronectin and laminin (45). More recently, however, short peptide fragments (signalling domains of long ECM proteins) are used for surface modification. These peptides include the sequences: 'RGD' from fibronectin/vitronection for cell adhesion (48); 'IKVAV' from lamnin for neurite extension (50, 51); 'VPGIG' from elastin to enhance the elastic modulus of artificial ECM (52) or 'FHRRIKA' from the heparin binding domain to improve osteoblastic minerilization (53). These short peptide sequences have greater stability than the long chain proteins during the modification process and due to their shorter length do not form complex folded structures on the surface making them sterically available for binding (45). Additionally, the shorter peptide sequences are less expensive to produce and can be synthesised on a large scale. Surface modification peptides can be introduced through physical (54), chemical (48, 55), photochemical (56, 57), and ionic crosslinking techniques (58).

Considerations when engineering a biomimetic scaffold include the concentration and spatial distribution of the peptide or protein as well as the "spacer" for peptide modification. High peptide density may aid in cell attachment but may be antagonistic to cell migration and/or proliferation. For example, studies have shown that *in vivo,* bone scaffolds coated with the 'RGD' adhesion protein motif promotes maximal tissue growth only at intermediate values of ligand surface density (59). The spatial organisation of the RGD peptides on the surface of the biomaterials is also important as the peptides more effectively induce cell adhesion and migration when organised as clusters and not a random distribution (60, 61). The "spacer" for peptide modification is a polymer chain between the scaffold and the peptide sequence, which allows the peptide sequence to be freely extended outward of the network, increasing its availability to the target (45). Bioinert chains such as PEG with certain molecular weights (48,

57) or repetitive sequences of non-specific peptides e.g. 'GGGG' (62) have been used as spacers (45).

1.3. APPLICATIONS OF *IN VITRO* 3D CELL CULTURE

The growing body of research performed using 3D cell cultures has led to the increased use of 3D models to complement 2D cell culture and animal model studies (63). Figure 1.6 represents several applications of *in vitro* 3D models specifically in the fields of 3D cell modelling and migration, toxicology, anti-cancer drug screening and discovery, and host-pathogen interactions.

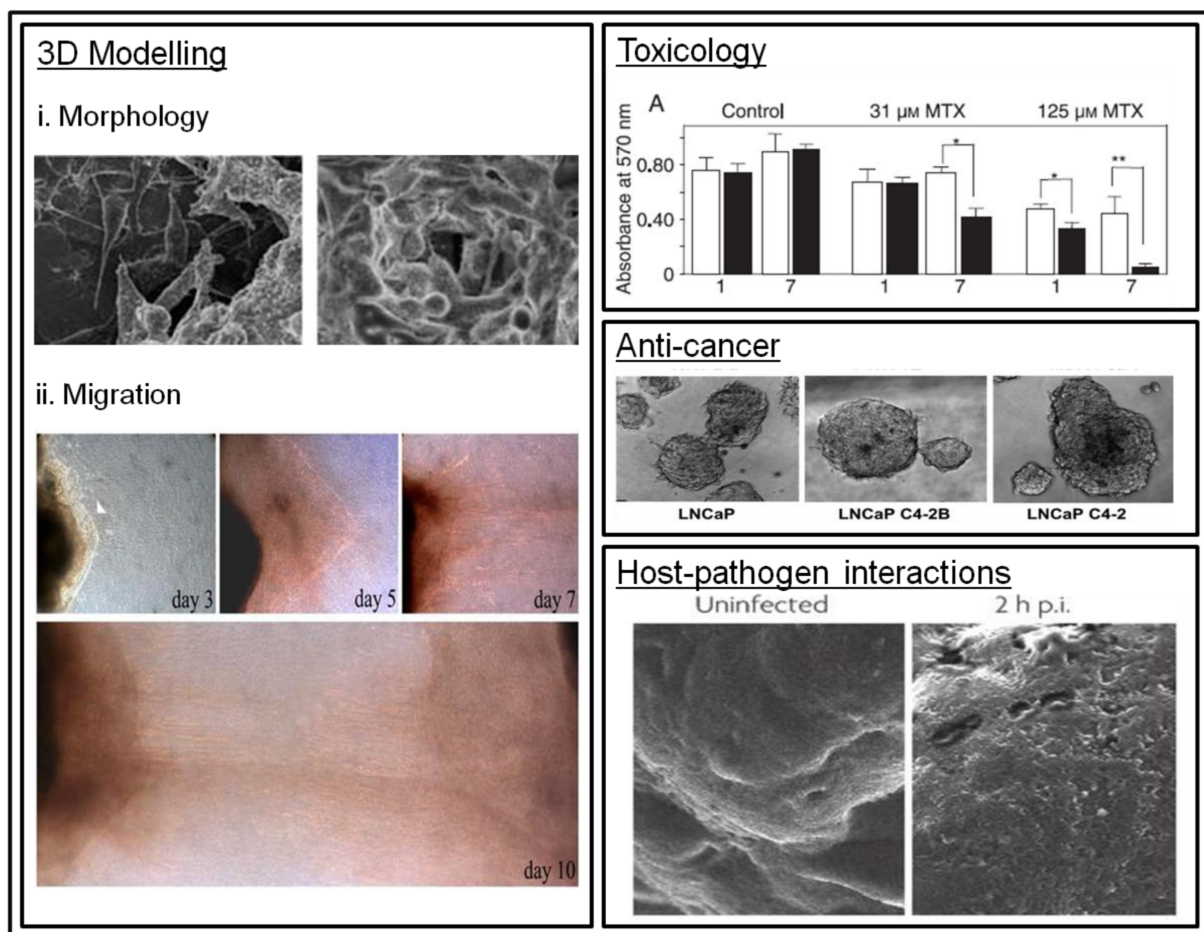

Figure 1.6. Selected *in vitro* applications of 3D cell culture. **3D Modelling: i. Morphology:** Scanning electron micrographs showing examples of HepG2 cells cultured on 2D (left panel) and 3D (right panel) polystyrene scaffolds for 7 days; SEM showed that HepG2 cells cultured on 2D surfaces were significantly more heterogeneous in structure compared to cells grown on 3D surfaces (6). **ii. Migration**: Phase contrast microscopy of the dominant axis of 3D invasion for human breast carcinoma cells explants over 10 days in a 3D collagen matrix (64). **Toxicology:** Functional activity of HepG2 cells grown on 2D (solid bars) and 3D (open bars) substrates when challenged by the cytotoxin, methotrexate (MTX) (6); **Anti-cancer:** Matrigel^{TM} strongly supports both growth and differentiation of normal and prostate cancer spheroids which can be used to screen new chemical compounds (65) and **host-pathogen interactions:** scanning electron micrographs of Int-407 3D aggregates (control and 2 hours post infection with *Salmonella enterica* subsp. *Enterica*) (66).

The contribution made by 3D cell culture using various polymer scaffolds for *in vitro* anti-cancer drug screening, toxicology and host-pathogen interactions is further reviewed below.

1.3.1. *In vitro* anti-cancer drug screens: Tumour tissue models

State of the art *in vitro* models aim to replicate the structural, functional and mass transport properties of tumours by culturing cells in 3D (63, 67). However, the development of *in vitro* tumour tissue models has been driven by the need for better principal methods for predicting the efficacy of anti-cancer drugs (63). The development of anti-cancer drugs and therapeutics relies on screening chemical libraries of compounds using these *in vitro* tumour models to identify potential drug candidates. Current 2D cell culture models tend to suggest that anti-cancer drugs have a much higher efficacy than is subsequently observed *in vivo* and studies have indicated that the 3D architecture of tumours and the ECM all contribute to higher *in vivo* resistance to anti-cancer drugs (63).

Two 3D models exist to study/screen anti-cancer drugs. Firstly, the multicellular tumour spheroid (MCTS) model and secondly, scaffold-based cancer models. In the MCTS model, spheroids contain both surface-exposed and deeply buried cells, proliferating and non-proliferating cells and well oxygenated and hypoxic cells, much like in tumours themselves (68). In addition to oncogenic mutations, aberrant tissue organisation and signal transduction are common features in neoplasia (68) and the 3D environment in which these spheroids are grown allows a better understanding of tissue architecture and signalling in cancer. Spheroids can be studied in suspension or in hanging drops of medium, in bioreactors or in 3D matrices and are usually composed either of a homogenous cell population (valuable for high-throughput drug screening (69)) or of a multi-cellular mixture of tumour, stromal and immune cells (68). The principal limitations associated with MCTS models is the absence of vasculature, which results in lower nutrient supply to the tumour than would be observed *in vivo* and the relatively long culture period.

Scaffold-based cancer models are the second method researchers use to screen anti-cancer drugs. Various 3D scaffolds have been used to enhance our understanding of cancer biology including chitosan-alginate scaffolds which mimic the glioma microenvironment (70), and poly(lactic-co-glycolic acid) porous scaffolds that allowed the relationship between the microenvironment and tumour malignancies to be studied *in vitro* (71). Hydrogels (including Matrigel™) have been used to study breast cancer (72), Matrigel™ and haluronic acid to study prostate cancer (65, 73) and alginate scaffolds were also explored as 3D models to study

cancer cell angiogenic capability (74). Cancer cells in 3D cell culture displayed high malignancies including over expression of pro-angiogenic growth factors, enhancement of tumorigenicity and insensitivity to drug treatment. For 3D cancer cell culture systems, many past studies have utilised collagen hydrogels thereby exploiting the importance of collagen as an ECM component in providing a proper platform for cell adhesion and migration (28, 75-77).

In addition to their utility in drug efficacy screens, 3D tumour models may prove useful in the development of new anticancer drugs. Both MCTS and scaffold-based models are known to better reflect *in vivo* gene and protein expression patterns and signalling pathways, making it possible to identify new cellular targets for anticancer drugs (63). It may even be possible to use 3D models for individualized therapy, that is, to predict the responsiveness of a particular patient's tumour to chemotherapy or radiotherapy (68).

1.3.2. *In vitro* toxicology models

It is estimated that for a new drug to reach the consumer market, it can cost up to $1.2 billion over a period of anywhere between 12 to 15 years. Approximately 1 in 5 drugs will fail in the human clinical trials at stage III due to hepatotoxicity (78) at an already considerable expense and cardiotoxicity is the leading cause of already approved drugs being removed from the market (63).

Friedman (79) states that "the holy grail of the [pharmaceutical] industry is to be able to predict [drug] toxicity from a cell culture." 2D cell cultures are able to predict acute hepatotoxicity (80) but cannot predict the toxicity of a drug that is metabolised *in vivo* to a toxic species - a phenomenon that cannot be adequately detected before stage III human trials (80). Due to the fact that rodents or other animals can metabolise or respond to drugs in a manner different to what humans would (81), 3D cell culture for "*in vivo*" like drug screening may help reduce the high failure rate in the development of new drugs.

The liver has many important functions in the body including albumin synthesis, fat and carbohydrate metabolism, bile secretion, elimination of harmful biochemical products such as bilirubin from the breakdown of erythrocytes and ammonia from the breakdown of proteins. Detoxification and metabolism of drugs, alcohol and environmental toxins is another important function of our liver and 3D cell culture offers opportunities for *in vivo* and *in vitro* liver toxicity models by culturing human hepatocytes (82).

Scaffold-free cellular spheroids and scaffold-based liver tissue models have been developed, and in perfused culture these systems can maintain excellent primary cell viability for two to three weeks (63). Further improvements in cell viability have been achieved by using co-culture and sandwich culture techniques, where hepatocytes are cultured with non-parenchymal cells such as epithelial cells and fibroblasts (63). 3D cell culture also enables liver specific functions, including urea and albumin production and cytochrome P450 enzyme activity, an important class of metabolic enzymes, to be maintained for up to 10 days at close to *in vivo* levels (63). Using a 3D scaffold-based model, increased cytochrome P450 enzyme CYP1A2 ethoxyresorufin to resorufin conversion activity was reported using the C3A human hepatocyte cell-line (33). An increased resistance to drug-induced apoptosis of the human hepatoma cell-line HepG2 (83) and increased albumin and urea production in hepatocytes (84) have also been observed.

In vitro cardiac tissue models have principally been developed for the study of cardiac disease and the prediction of cardiotoxicity (63). Primary cardiomyocytes would possibly offer more accurate toxicological results but, due to the logistical problems of procuring and maintaining primary tissues, 3D cardiac tissue models based on human embryonic stem cell-derived cardiomyocytes are being developed (63).

1.3.3. *In vitro* host-pathogen interactions

Despite great progress in our understanding of how pathogens successfully evade the immune system and cause disease, infectious disease still remains a huge health and economic burden particularly in the developing world, with 35% of all mortalities due to an infectious disease (85). This may potentially be reduced through the development of *in vitro* host-pathogen models that better mimic *in vivo* environments, thus, human 3D tissue models may provide superior systems for analysing the pathogenesis of disease (68).

Very little research has been published on host-pathogen interactions using 3D cell culture models, however, organotypic models cultured using the rotating wall vessel bioreactor, a cylindrical, rotating apparatus, filled with culture medium developed at the National Aeronautics and Space Administration, USA, have been used to study host pathogen interaction and are reviewed extensively by Barilla *et al.* (66). Organotypic models are formed by the natural sedimentation of cells due to gravity balanced by the bioreactor's rotation, resulting in a gentle falling of cells within the media in the chamber. The cells are attached to porous microcarrier beads or scaffolding allowing for cellular responses to chemical and molecular gradients in

three dimensions. Using this technology, host-pathogen interactions of the colon (*Salmonella enterica subsp. serovar* Typhimurium, *Cryptosporidium parvum*) (86, 87), small intestine (*S. typhimurium*, Norovirus) (88, 89), lung (*Pseudomonas aeruginosa*) (90), bladder (uropathogenic *Escherichia coli*) (91), lymphoid tissue (HIV, *Borrelia burgdorferi*) (92, 93) and lymphoblastoid (Epstein-Barr virus) (94) have been successfully studied and improved upon.

The liver pathogen hepatitis C virus (HCV) has also been studied using this technology. HCV is a liver trophic, positive-stranded RNA flavivirus causing acute and chronic hepatitis and hepatocellular carcinoma (95). HCV has infected more than 170 million people worldwide and is currently the leading cause of liver failure in the United States (96). Since its discovery in 1989, a major obstacle impeding HCV research has been the lack of robust cell culture and small animal infection models (97). Infecting cultured liver cells with the virus is extremely difficult as human hepatocytes quickly lose many of their liver-specific functions, including susceptibility to viral infection and ability to metabolise drugs, when they are cultured *in vitro*. Sainz *et al.* (97) found that when cultured in 3D, the Huh7 hepatocyte cell-line acquires a more differentiated hepatocyte-like phenotype that is highly permissive for HCV infection, thus providing an opportunity to study HCV entry and the effects of HCV infection on host cell function.

With this evidence, future engineered 3D tissues may provide a cheap *in vitro* system to replace the more expensive, and in some ways less accurate animal models, with greater control of variables for studying pathogens.

1.4. 3D CELL CULTURE: FUTURE DIRECTIONS

The motivation to mimic the 3D structure and composition of natural cellular environments has driven the rapid development of materials and fabrication techniques (1). The various 3D cell culture systems available and the major potential of these systems to improve on our knowledge of cancer biology, toxicology and how pathogens interact with their host cells have been well documented. With the demonstrated advantages of 3D cell culture, the fact remains that it is not as widely accepted as 2D cell culture due to the large deviations of structure and composition in various 3D matrices (1). Such inconsistencies prevent reproducible data and systematic analysis. Standardisation of 3D matrices should, thus, be a priority for materials scientists and biologists alike. A 3D culturing system needs to be easy to use and any technical and experimental challenges will have to be solved before 3D culture can become routine (8).

In a survey conducted by Comley in 2010 (98), participants responded with the following concerns about 3D cell culture: 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 these geometries is difficult, poor visualisation and cost. Post-culturing processing and/or cell extraction can usually be achieved by waiting for a biodegradable scaffold to degrade or by dissolving the scaffold, usually a hydrogel, with salts. An alternative method for on-demand cell release would thus be a highly desirable feature of 3D scaffolds for *in vitro* cell analysis.

Looking to the future, optimised 3D models for toxicology screening are a priority to replace or minimise human and animal models helping to alleviate the overall cost of the drug discovery process. 3D skin models for testing pharmaceutical cosmetic compounds are also receiving wide-spread attention and currently two validated commercial skin model tissues, Episkin and EpiDerm are approved in the USA and Europe for *in vitro* corosity testing (99). Thus the investment of time towards developing and validating such *in vitro* models will likely have a significant impact on the overall success and efficiency of pharmaceutical development in the future (100).

Other niche areas set to utilise 3D cell culture lie in the areas of co-culturing, embryonic stem cell cultures (8) and 3D models for studying infectious disease (66). Co-culture of two or more cell types on scaffold systems can produce highly sophisticated models that mimic *in vivo* tissue more accurately than 2D cultures (8). Embryonic stem cells in scaffolds can promote their growth, differentiation and the formation of complex 3D structures (101); with the use of appropriate growth factors embryonic stem cells have the potential to provide an unlimited supply of pluripotent stem cells for tissue regeneration (8). As touched upon in section 1.3, 3D culture models can be a valuable tool for studying the mechanisms of infectious disease and promise to facilitate the translation of basic science from the lab to the clinic (66). Although no single *in vitro* model system will provide a complete understanding of the fundamental mechanisms governing pathogenesis, a combination of advanced cell culture models and animal models will advance our mechanistic understanding of “normal versus diseased”.

1.5. OBJECTIVES

The objective of this study was to develop a novel 3D scaffold allowing 3D cell proliferation and non-invasive cell harvesting, and to investigate the use of this technology as a tool to study host-pathogen interactions.

This study aims to address two of the issues raised above. Firstly, to address the problem of post-culturing processing and/or cell extraction from a 3D matrix by developing a scaffold capable of on-demand cell release. This system will be optimised using anchorage dependent mammalian hepatocytes; the hepatocytes will be necessary for the second aim of this thesis where the hypothesis that culturing hepatocytes in 3D would achieve superior levels of malaria sporozoite invasion *in vitro* is tested.

To address the first aim three different non-woven polymers, PP, PET and nylon were grafted with a thermoresponsive polymer poly(*N*-isopropylacrylamide) (PNIPAAm). The scaffolds were tested for their ability to permit hepatocyte proliferation and enhanced metabolic function in 3D and non-invasive cell harvesting using the various grafted scaffolds was tested (Chapter 2). Cell culture on the thermoresponsive scaffolds was scaled up using an automated cell culture device. The purpose of developing the device is for the proliferation of large numbers of cells and then, by using the thermoresponsive properties of the selected grafted non-woven scaffold, to non-invasively harvest the hepatocytes for downstream applications.

Subsequently, we aimed to test a possible application of the hepatocytes growing in 3D on the scaffolds by focussing particularly on malaria parasites as infectious agents targeting hepatocytes as a model system (Chapter 3). Mosquitoes were infected with malaria parasites (*Plasmodium falciparum*) to produce sporozoites which were harvested from their salivary glands. The ability of liver-infective sporozoites to invade hepatocytes more readily if they are growing in 3D compared to 2D was then assessed.

Chapter 1: Three dimensional cell culture systems and their applications: A review

The knowledge gained from the study has led to the following contribution in scientific journals and conference proceedings:

Papers:

Claire L. Rossouw, Avashnee Chetty, Francis Sean Moolman, Lyn-Marie Birkholtz, Heinrich Hoppe, Dalu T. Mancama. Thermoresponsive non-woven scaffolds for "smart" 3D cell culture. Biotechnology and Bioengineering 2012; 109(8):2147–2158. (Impact Factor 3.7)

Avashnee S. Chetty, Viktoria Vargha, Arjun Maity, F. Sean Moolman, Claire Rossouw, Rajesh Anandjiwala, Lydia Boguslavsky, Dalu Mancama, and Walter W. Focke. Development of thermoresponsive PP-*g*-PNIPAAm non-woven 3D scaffold for smart cell culture using oxyfluorination-assisted graft polymerisation. Colloids and Surfaces A: Physicochemical and Engineering Aspects, 2013; 419: 37-45. (Impact Factor 2.359)

Conference proceedings:

Poster presentations:

Claire L Rossouw, Avashnee Chetty, F Sean Moolman, Lyn-Marie Birkholtz, Heinrich Hoppe, Dalu T Mancama. Optimization of an *In vitro* System to Study the Exo-erythrocytic Stage of the Human Malaria Parasite *Plasmodium falciparum*, Keystone Symposia F1 Malaria: New Approaches to Understanding Host-Parasite Interactions held at Copper Mountain Resort, Copper Mountain, Colorado, USA on Apr 11 - Apr 16, 2010.

Oral presentations:

Claire L Rossouw, Avashnee Chetty, F Sean Moolman, Lyn-Marie Birkholtz, Heinrich Hoppe, Dalu T Mancama. Thermoresponsive Non-Woven Scaffolds for "Smart" 3D Cell Culture, presented at the Emerging Researcher Symposium, CSIR, Pretoria, 13 October 2011.

Claire L Rossouw, Avashnee Chetty, F Sean Moolman, Lyn-Marie Birkholtz, Heinrich Hoppe, Dalu T Mancama. Thermoresponsive Non-Woven Scaffolds for "Smart" 3D Cell Culture, presented at the South African Society of Biochemistry and Molecular Biology Congress, Champagne Sports Resort, Kwa-Zulu Natal, 29 January – 1 February 2012.