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DESIGN AND FUNCTIONAL EVALUATION OF A 3D *IN-VITRO* LIVER CULTURE SYSTEM FOR APPLICATIONS IN TOXICITY SCREENING OF NEW CHEMICAL ENTITIES

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SHIH-FENG LAN
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DESIGN AND FUNCTIONAL EVALUATION OF A 3D *IN-VITRO* LIVER CULTURE SYSTEM FOR APPLICATIONS IN TOXICITY SCREENING OF NEW CHEMICAL ENTITIES

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DEPARTMENT OF BIOENGINEERING

BY

Dr. Binil Starly, Chair

Dr. Edgar A. O'Rear III

Dr. David W. Schmidkte

Dr. Barbara Safiejko-Mroczka

Dr. Scott D. Russell

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ABSTRACT

Advancements in biomaterials and manufacturing processes have enabled the development of 3D cell encapsulated hydrogels as systems to mimic *in-vivo* like function for drug-screening. Accurate prediction of human response to potential therapeutic drugs and vaccines are through conventional methods of *in-vitro* cell culture assays and expensive *in-vivo* animal testing. Traditional *in-vitro* cell culture assays are time consuming, at times unreliable and expensive. Hence, there is a critical need to reduce the time and financial investment required to discover new drug cures for major illnesses through advanced tissue model systems. In this study, we have evaluated the use of 3D culture with HepG2 liver cells for applications in drug testing. The method is based on alginate hydrogels encapsulation. Two different ultra-sterile alginates, SLM100 (G:M::40:60) and SLG100 (G:M::60:40) have been used for our 3D matrix. In addition, we present a disc design and dynamic device for 2D-3D co-culture and 3D dynamic culture. The major research accomplishments reported in this thesis include:

I. Development of the encapsulation method for 3D culture. We have studied the cellular viability and metabolic capacity of the encapsulated cells in two different alginate structures SLM100 and SLG100. We have also developed protocols to characterize the encapsulated cells within the alginate structure using Scanning Electron Microscopy (SEM) and Laser Scanning Confocal Microscopy (LSCM). Liver-specific enzymes such as CYP1A1 and CYP3A4 after 14 days in culture indicates the viability and functionality of the encapsulated HepG2 cells. Phase II Glutathione activity of the encapsulated cells were also maintained in 3D culture conditions. The encapsulated cells within the

3D gels were also capable of metabolizing the pro-drug EFC (7-ethoxy-4-trifluoromethyl coumarin) to HFC (7-hydroxy-4-trifluoromethyl) in a linear fashion over a period of time.

II. Development of the porous poly-carbonate disc platform for 3D culture. We have developed an *in vitro* platform to enable high density 3D culture of liver cells combined with a monolayer growth of target breast cancer cell line (MCF-7) in a static environment as a representative example of screening drug compounds for hepatotoxicity and drug efficacy. Alginate hydrogels encapsulated with serial cell densities of HepG2 cells (10^5-10^8cells/ml) are supported by a porous poly-carbonate disc platform and co-cultured with MCF-7 cells within standard cell culture plates during a 3 day study period. The clearance rates of drug transformation by HepG2 cells are measured using a coumarin based pro-drug. The platform was used to test for HepG2 cytotoxicity 50% (CT_{50}) using commercially available drugs which further correlated well with published *in vivo* LD_{50} values.

III. Development of dynamic device for 3D culture. the design approach is (1) To design a liver bioreactor unit that is scalable, interchangeable and compatible with other scaffold materials; (2) To establish a long-term 3D culture dynamic environment; (3) compared the drugs toxicity result between dynamic and static.

The 3D encapsulation of cells within hydrogels represents an increasingly important and popular technique for culturing cells and towards the development of tissue engineering and drugs testing. This environment better mimics what cells live *in vivo*, compared to standard tissue culture, due to the tissue-like properties and 3D

environment. The following methods for the 3D encapsulation of HepG2 have been optimized in our lab to maximize cell viability and liver specific enzymes activity, minimize the of hydrogel processing steps using support disc design and integrated into dynamic device. The research will also enable scientists to expand their scope of research and study in the field of *in vitro* drug screening and toxicity study.

Chapter 1: Introduction and background

1.1 Introduction

Cell culture in two dimensions (2D) has been used for more than 20 years and still is the most common method for supporting cell growth and proliferation. A major criticism of 2D culture is that *in vivo* three dimensional (3D) physiologies cannot be accurately reproduced using a monolayer culture condition. Obviously, the cultivation of cells on a two-dimensional glass or plastic substrate is not an accurate representation of native tissue; many complex biological responses cannot be represented normally via 2D culture such as cellular migration characteristics or certain gene expression profiles. Led by *in vitro* toxicity researchers, biologists are increasingly turning to 3D hepatocyte cultures for accurately reproducing cell and tissue physiology, where they are discovering many liver specific-gene functions that closesly mirrors *in vivo* conditions. Metabolic studies, toxicity testing and pharmacokinetic studies are main activities in early drug discovery screening. *In vitro* systems that could predict the potential hepatotoxic effects and unsuitable pharmacokinetic properties of drug candidates would facilitate drug development. Reducing number of animal experiments would also provide a faster and cheaper way for analysis. In this thesis, we have developed a 3D liver cells alginate-based culture system for static 3D hepatotoxicity testing, 2D-3D co-culture for drug effect testing and dynamic bioreactor for long-term and high cell density 3D culture. The final goal is to design a reliable, simple, affordable and fast data collecting *in vitro* prediction device which helps to reproduce the native cellular environment for preliminary drug screening, toxicology studies and drug effects study.

1.2 Background information

Ideally, it is desirable to perform long-term and high density cultivation of liver cells in an *in vitro* environment for drug studies, but they are extremely difficult to maintain in culture, due to issues such as clogging, cell-cell contact inhibition or loss of liver specific functionality after a couple of days. In order to solve these challenges, a number of 3D culture methods have been developed for a variety of cell types, including liver cells by using different biomaterials (Lee *et al.* 2008a). A common goal for many of these studies is to bridge the gap between the uses of animal testing and clinical trials. It is necessary to create 3D cell-based testing sample that mimic to some degree the native tissue as close as possible. One possible way is to grow the desired cells on a biocompatible porous 3D matrix structure. Many parameters need to be considered for the complexity of 3D cultures. These criteria include cell source (liver slices or hepatocytes), material of scaffold (naturally derived or synthetic materials), culture method of cells (static or dynamic), cell culture medium and scaffold geometry.

1.2.1 Three-dimensional cell culture

1.2.1.1 Liver slices

3D culture models can be divided to several groups such as organotypic explant culture, cellular spheroids, microcarrier cultures and tissue-engineered models (Pampaloni *et al.* 2007). Not all three-dimensional culture models require a scaffold. For example, liver slice is one of the most important models for drug testing. Recent studies have shown that isolated hepatocytes are difficult to mimic native liver functions because primary liver cells are unable to function and survive without supporting cells such as the endothelial cells and stellate cells (Bhatia *et al.* 1997). Human liver are

composed of various sets of cells arranged in specific architectural patterns which is very hard to mimic *in vitro*. Culturing a liver slice could be a useful tool for metabolism and toxicity testing (Moronvalle-Halley *et al.* 2005). However, maintaining the cellular activity for a liver slice is difficult especially since hepatocytes have a very high oxygen consumption rate (Allen and Bhatia 2003). Various culture methods have been developed to achieve the survival of hepatocytes in tissue slices (Vickers and Fisher 2004). All of these methods aim to improve the oxygen and nutrients mass transfer from the surface to the inner perfusion of the tissue. The cultivation methods employed involve static culture or dynamic culture systems to improve the maintenance of liver slices (Olinga *et al.* 1997). The main advantage of liver slices is that preserving hepatocytes in their natural environment and architecture albeit for a very short incubation time period (<48hr).

1.2.1.2 Cellular spheroids

Cellular spheroids are simple 3D models that can be generated from many cell types and from cell aggregates. Spheroids do not require scaffold and can be easily imaged by fluorescence or confocal microscopy. One of the most famous commercial products for making cellular spheroids is AlgiMatrix® (Invitrogen). AlgiMatrix® sponge is extracted from brown seaweed and mixed animal collagen. It is suitable for cardiomyocyte organogenesis studies, or co-culture studies. The spheroid cell culture has several advantages compared to mono-layer cell culture. (1) AlgiMatrix® possesses a tissue-like structure and cells can contact each other for communication. (2) Adhesion molecules can be mixed in spheroids that are required for cell proliferation and the reconstruction of cellular polarity (Tamura *et al.* 2008). (3) Spheroids can be

used in modeling solid tumor growth or metastasis studies for therapeutic studies, e.g. for high throughput screening (Ivascu and Kubbies 2006).

1.2.1.3 Microcarrier cultures

To meet the principal needs of bio-artificial liver functions, microcarrier technique was used to cultivate high density liver cells to improve the cultivation efficiency and yield. The productivity of large-scale cell culture can be increased either by scaling up to larger volumes with cell densities of $2–3\times10^6$/mL, or by intensifying the process in smaller volumes but with higher cell densities (up to 2×10^8 cells/mL) (Reiter *et al.* 1990). When compared with traditional monolayer or suspension techniques, stirred microcarrier cultures yield up to 100-fold as many cells for a given volume of medium. Superior yields have been reported for a wide variety of systems including guinea pig keratinocytes (Griffiths *et al.* 1983) and HepG2 (Lupberger *et al.* 2006). Microcarrier culture method reduced the requirement for culture medium particularly when expensive serum supplements such as fetal calf serum are used. The growth of liver cells on microcarriers also can be observed and the specific functions of liver cells were determined periodically (Gao *et al.* 1999). Microcarrier technology results in a homogeneous culture system that is scalable for large volume of incubation. The advantages of microcarrier culture for vaccine production include increased productivity, lower costs and reduced contamination when compared with other cell culture methods.

1.2.1.4 Tissue-engineered *in vitro* models

Tissue engineering models are made by biological fabrication or semi-synthetic living tissue, the main usage is for damaged tissue replacement. This technique has been

widely used for many tissues including skin, kidney or liver (Howard *et al.* 2008). Tissue engineering models have the potential to provide new *in vitro* toxicology models or organ transplantation. The most important role for tissue engineering models is its potential ability to mimic key morphological, physiological, and biochemical properties of the natural tissue as closely as possible (Suuronen *et al.* 2004).

1.2.2 Biomaterial scaffolds for cells seeding

3D scaffold biomaterial has been become more and more important because the demand of biotechnology usage and complexity of scaffold design. Cells require careful exchange of nutrients and oxygen in addition to geometry control in a 3D matrix. However, cell viability is an issue when scaffold or cell aggregate thickness of 1-2 mm arise through a lack of mass transfer, especially through a limited exchange of nutrients and waste (Griffith and Swartz 2006). Diffusion problems can be overcome by making highly porous scaffolds or increasing the flow of oxygen and nutrients. Different cell types have varied micro and macro-environment requirements which dictate different properties of scaffolds. For example, liver cells must be surrounded within a soft environment. In contrast, osteoblasts adhere to a hard surface just like the bone tissue. Consequently, the design of the scaffold must reflect the native tissue in human environment to represent the cells normal functionality (Lee et al., 2008a). For implantation field, a functional implant requires a biodegradable and biocompatible scaffold, which, after implantation, is replaced by the regenerating tissue (Walles *et al.* 2003). In this situation, the scaffold must support cell proliferation and differentiation; furthermore, the scaffold can be degraded and replaced by human body without immune-rejection. On the other hand, these scaffold can also be applied in 3D *in vitro*

model for drug screening and cosmetics (Canton *et al.* 2007). Here, it is necessary to reproduce an accurate artificial tissue for cell functions and response evaluation.

The varieties of materials that can be used for scaffold fabrication, including polymers, metals and ceramics. Polymers are used commonly for bio-fabrication scaffolds, which are typically grouped into synthetic and natural materials. Synthetic polymers such as polyethylene glycol (PEG), polyactic acid (PLA) and polycaprolactone (PCL), are included as well as natural polymers such as collagen and alginate. However, natural scaffolds tend to exhibit better biocompatibility properties than synthetic materials (MacNeil 2007). The general role for these 3D scaffolds is to reproduce an extracellular matrix (ECM) for supporting cell growth. In mammalian tissues, cells not only connect to each other, but also communicate through extra cellular matrix (ECM) molecules. ECM contains proteins, such as collagen, laminin and elastin that provide communication between cells and matrix. The receptors, embedded within the matrix, called the integrins, play a role in cell singling. These receptors are very important the functional profile of cells. For example, in 1997, Bissell's group (Weaver *et al.* 1997) found that surface receptor, called β-integrin, can influence the behavior of cancerous breast cells in 3D culture. Consequently, 3D culture environment can alter cell behavior compared with 2D culture.

As described above, the surface chemical and receptor properties are fundamental for cellular adhesion, proliferation and signal transduction. For instance, the Vroman effect, is exhibited by protein adsorption to a surface, in particular for serum proteins. The highest mobility proteins arrive to surface first and are later replaced by less motile proteins that have a higher affinity (Vroman 1962). This effect is

known to correlate to cells adhesion on biomaterial surface, where cells interact with the protein layer (Allen *et al.* 2006). A biomaterial surface can be modified to increase or decrease cell adhesion. An example is plasma acrylic acid layer that can improve Schwann cell adhesion ability (Murray-Dunning *et al.* 2011). On the other hand, the deposition of allyl amine can prevent Schwann cell attachment. Cell adhesion can also be controlled by structural motifs into a biomaterial. The most widely used adhesion ligand is RGD peptide (Arg-Gly-Asp), discovered in 1991 (Massia and Hubbell 1991), was proved to improve fibroblast cells adhesion ability *in vitro*. RGD peptide covalently immobilized surface has proved to be effective for HepG2 biotransformation activity, particularly in the presence of diclofenac. Also the biotransformation functions were expressed at high levels (De *et al.* 2005). In contrast, chemical reaction surface modification techniques such as plasma-enhanced chemical vapor deposition have proved to enhance cells adhesion in 3D scaffolds. 3T3 fibroblast attachment was found to be greater for the plasma deposits than the untreated poly (D,L-lactic acid) (PDLLA) tissue-engineering scaffolds (Berry *et al.* 2005). UV and ozone have often been used for biomaterial surface modification to improve the surface wet ability for cell adhesion performance (Liu *et al.* 2010); the major advantage of this approach is in the rapidity and reproducibility for modifying 3D scaffolds. Treatment of adhesion proteins with biomaterials for 3D culture is one of the most important techniques in tissue engineering.

1.2.3 Scaffold scales

The scaffold design can be characterized for macro-, micro-, and nanoscale. If constructs are for implantation, the size, usually macro-scale, can be followed by

computer-aided design and fabrication by stereolithography. The micron scale design has been widely used for liver tissue engineering in the evaluation of drug metabolism, toxicity and other evaluations (Griffith & Swartz, 2006). However, hepatocytes *in vivo* are complex metabolic cells and their functionality is dependant on their microenvironment such as cell-cell and cell-matrix interactions, especially sinusoid structure. To improve *in vitro* micro-scale culture conditions, microfluidic devices have been developed for studying pharmaceutical and toxicological problems over the last years. L.G. Griffith's group used perfused multi-well plates with an integrated filter to accumulate rat liver cells and nonparenchymal cells (Griffith and Naughton 2002). This bioreactor supported a viable culture for up to 7 days, allowing for high throughput and continuous perfusion of the culture. Another approach to microfluidic hepatocyte cultures was recently shown by Chao et al. (Chao *et al.* 2009). After first seeding cells onto a substrate and then assembling the microfluidic components, the group cultured the cells for up to 24 h. Subsequently, they measured the hepatic clearance rate of six marketed model compounds and compared the performance with *in vivo* data. The main advantages for microfluidic device including (1) Volume reduction can reduce the cost of expensive drugs. (2) Sinusoid structure cab is modified as a place of drug reaction and also increase the surface area for a faster reaction time. (3) Multi-channels have the potential to test many samples at the sample time. (4) In *vivo* microenvironment can be mimicked as real as possible.

1.2.4 Bioreactors for 3D constructs

An important consideration of 3D cultures is the maintenance of mass transfer (Martin *et al.* 2004). When 3D cultures are too thick, there are diffusion limitations into

the cells at the center of 3D cultures. Major challenges in 3D cultures include the oxygen and nutrients penetration and removal of waste products. Traditional 3D cultures are based on static culture condition, however, the design and use of bioreactors are moving towards utilizing 3D culture systems (Martin *et al.* 2004). Bioreactors are able to control many parameters required for cell culture. These include medium flow rate, oxygen and nutrient supply. In additional, some complex bioreactors can mimic the oxygen gradient across the tissue that is similar to *in vivo* liver sinusoids (Allen & Bhatia, 2003).

Several bioreactors can be grouped into hollow fiber, flat plate and monolayer systems, and direct perfusion systems with scaffolds or encapsulated cells (Table 1). Hollow fiber systems are applied for cells which have a high metabolic rate (Haycock 2011). Hepatocytes can be suspended in a collagen solution which is injected into hollow fibers systems. Nutrient medium is circulated through the fibers into cells (Gordon *et al.* 2005). Perfusion systems allow the culture medium pass through the construct to increase the mass transfer. Cells are usually seeded within a flat plate, 3D scaffolds or encapsulated with biomaterials. Some considerations when designing these flowing systems are scaffold porosity and mechanical properties of constructs. For example, mechanical stimulation of bone induces new bone formation *in vivo* and increases the metabolic activity and gene expression of osteoblasts (Frias *et al.* 2010). Several bioreactors have received FDA approval for clinical studies. For example, the BioLogic-DT artificial liver system, appears to be safe in treatment of patients with hepatic insufficiency and coma in clinical trails (Ash *et al.* 1992).

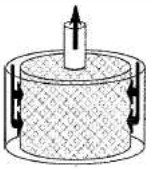
 Hollow Fiber	 Flat Plate and Monolayer	 Perfused Beds/Scaffolds	 Encapsulation and Suspension
Pros: attachment surface, potential for immunoisolation, well characterized, cells protected from shear	Pros: uniform cell distribution and microenvironment	Pros: ease of scale-up, promotes 3-dimensional architecture, minimal transport barrier	Pros: ease of scale-up, uniform microenvironment
Cons: nonuniform cell distribution, transport barrier with membranes or gels	Cons: complex scale-up, potential large dead volume, cells exposed to shear, low surface area-to-volume ratio	Cons: nonuniform perfusion, clogging, cells exposed to shear forces	Cons: poor cell stability in suspension, transport barrier due to encapsulation, degradation of microcapsules over time, cells exposed to shear forces
• Extracapillary cryopreserved cells on microcarriers (Rozga et al.[91]) • C3A cells cultured in extracapillary space (Sussaman et al.[118]) • Multicompartmental interwoven fibers with extracapillary seeding and oxygenation (Gerlach et al.[81]) • Cells entrapped in contracted gel in interlumenal space (Nyberg et al.[119]) • Cells entrapped in collagen gel in extracapillary space (Naka et al.[63]) • Tricompartmental coaxial hollow fibers (Macdonald et al.[120]) • Extracapillary seeding with in-line oxygenation (Patzer et al.[106]) • Dialysis against circulating hepatocytes (Greg Szebo; Exten, Inc, San Diego, CA) • Spirally-wound fabric scaffold and integrated hollow fiber oxygenation (Flendrig et al.[83])	• Dialysis against cell suspension (Matsumura et al.[33]) • Flat membrane reactor with cell in sandwich culture (De Bartolo et al.[72]) • Stacked plates of monolayer culture (Sheil et al.[121]) • Stacked plate reactor with monolayer culture (Uchino et al.[36]) • Monolayer coculture with membrane oxygenation (Tilles et al.[71]) • Collagen gel sandwich culture bioreactor (Taguchi et al.[122])	• Radial flow through packed bed, cells on glass microcarriers (Kawada et al.[78]) • Microchanneled polyurethane packed bed with spheriods (Gion et al.[79]) • Polyvinyl resin cubes seeded with cells in a packed bed (Yanagi et al.[77]) • Murine cell line on porous carriers in packed-bed (Fassnacht et al.[123]) • Radial flow through polyester fabric cell scaffold (Naruse et al.[82])	Encapsulation: • Spouted bed perfusion with encapsulated spheroids (Takabatake et al.[124]) • Fluidized bed of alginate encapsulated cells (Dore et al.[76]) • Encapsulated spheroids in perfusion chamber (Dixit et al.[75]) • Multicomponent capsules containing rabbit hepatocytes (Matthew et al.[125]) • Entrapped aggregates in glass bead packed bed (Li et al.[126]) • Hydrogel entrapped cells on rotating disks with perfusion (Yanagi et al.[74]) Suspension: • Perfusion chamber with membrane isolated cell and charcoal suspension (Margulis et al.[90]) • Cell suspension with a centralized spinning filter (Sakai et al.[89])

Table 1. Liver cells-based bioreactor designs (Allen *et al.* 2001).

1.2.5 The source of hepatocytes for *in vitro* studies

1.2.5.1 Primary hepatocytes

Hepatocytes can be obtained from human or other animals such as rat. Today human hepatocytes are the most widely used for *in vitro* studies. Freshly isolated hepatocytes can exhibit most *in vivo* functions but they have lost the attached surface and they do not survive for a long period of time. In order to improve the surviving time of primary hepatocytes, many papers have been published to several methods to improve hepatocytes function in *vivo*.

For example, the use of the sophisticated medium for human hepatocytes (Ferrini *et al.* 1997), the use of extracellular matrices that improve cell adhesion such as matrigel (Bissell *et al.* 1987) and co-cultivation with human hepatic stellate cells or nonparenchymal cells (Guillouzo *et al.* 1990; Okamoto *et al.* 1998). Various other models have been established including bioreactors providing primary hepatocytes maintained under continuous perfused oxygen (Vinci *et al.* 2011). Encapsulation of primary hepatocytes with alginate or collagen gels has been also developed to allow hepatocytes to survive for several days instead of several hours (Guyomard *et al.* 1996). Although most *in vitro* studies for drug metabolism are performed using microsomes, primary cells have several advantages: (1) Intact cell membrane. Xenobiotics in liver cells are usually involved in active transport. Level of xenobiotics in hepatocytes is higher than in plasma. Some transporter proteins have been demonstrated for drug metabolism such as pravastain (Okamoto *et al.* 1998). (2) Complete drug metabolic pathways. Enzymes involved in drug metabolism are included in PhaseI/II enzymes. Some cytosolic-conjugating enzymes and co-factors also play a major role in Phase II biotransformation process (Li 1984). Microsomes, for instance, lack these enzymes during homogenization process. On the other hand, primary hepatocyes still suffer several disadvantages that are present *in vitro* studies. First, the cells source is not easy to get and maintain in every lab. Second, cytochrome 450 levels are not stable during time. Because of this phenomenon, experiments for primary hepatocytes usually are performed within 2 days (Flendrig *et al.* 1998). However, human primary cell-based assays represent the gold standard in cell-based analysis.

1.2.5.2 Human liver tissue

One of the main advantages for human liver tissue is reproducing tissue architecture. However, hepatocytes in liver tissue rapidly lose their function within a 1-2 day of culture, and the cells are not equally preserved and reproduced in each of repeated experiments. The stable source of liver slices is still the problem. Although liver slices are used for prediction of drug metabolism, clearance rate or drug-drug interaction, these limitations explain why liver slices have not been widely used for *in vitro* drug development model (Graaf *et al.* 2007).

1.2.5.3 Hepatocytes cell lines and derived hepatocytes

Hepatocyte cell lines can be derived from normal cell immortalization or from cancer cells. Hepatocyte cell lines have been widely used because of several advantages: (1) Major liver-specific functions exhibit stability. For instance, HepG2 has been widely used for drug metabolism or drug-drug interactions studies (Lan *et al.* 2010). A new cell line HepaRG has been established recently for drug screening studies. HepaRG can express the major CYP450s and phase II enzymes over two weeks (Cerec *et al.* 2007). (2) Data are reproducible and consistent. Unlike primary cells, hapatocytes cell lines are immortal and represent a promising alternative to non-proliferative normal hepatocytes. The most commonly used human hepatocyte cell lines (eg., HepG2, Hep3B, HBG) are derived from tumors. (3)Hepatocytes are suitable for high-throughput screening. For example, HepG2 cells express a variety of proteins in large quantities that can be used to a good model for high-throughput screening (Rodriguez-Melendez *et al.* 2005). Hepatocytes cell lines genes are also well characterized for microarray testing (Solorzano-Vargas *et al.* 2002).

In recent years, human embryonic stem cells (hESCs) have recently provided an alternative, unlimited source for human hepatocytes. However, differentiation of hESCs to hepatocytes remains a challenge (Hay *et al.* 2007). Some reports have indicated that the bone marrow of adult rodents contains progenitor cells with the potential to give rise to cells expressing the hepatocyte markers cell−cell adhesion molecule or albumin (Petersen *et al.* 1999; Theise *et al.* 2000). Carlos Semino et al (Semino *et al.* 2003) at MIT showed that they could take liver progenitor cells and differentiate them into mature hepatocytes in PuraMatrix synthetic nanofiber scaffolds. Induced hepatocytes from a patient's pluripotent stem cells (iPS) has also provided a alternative way without immune suppression (Espejel *et al.* 2010). Although hepatocytes derived from embryonic cells have not been widely used for liver cells studies, nevertheless, use of hepatocytes-like cells derived from stem cells may be expected for *in vitro* drug screening in the future.

Overall, it is desirable to perform long-term and high density cultivation of liver cells in an *in vitro* environment for drug and liver cells metabolism studies, but they have been difficult to maintain in culture at high cell density and contact inhibition or liver specific functionality are lost after couple days. 3D culture has been frequently used in tissue engineering, pharmacology and immunology. 3D matrices are superior to cell based assays and animal testing because: 1. 2D mono-layer cell based assays do not mimic the complex environment undergone by a potential therapeutic drug, especially for cancer drugs treatment; 2. Animal testing is often expensive, time consuming and at times irrelevant; 3. Some 3D devices are small, relatively cheap and can enable the parallel study of multiple candidate drugs for high throughput screening. This thesis

proposes an alginate encapsulation method for 3D hepatocytes cell culture under static and dynamic environment. Custom designed engineered liver matrices can be designed for applications such as high throughput drug screening studies or metabolism-dependent toxicity study in the future.

1.3 Research objectives

The research purpose of this study is to design an alginate-based 3D culture system which can be applied for static culture, 2D-3D co-culture and dynamic culture. Once this system is established, custom designed engineered liver matrices can be designed for applications such as high throughput drug screening studies or metabolism-dependent toxicity study. The major research objectives reported in this thesis include:

A. The development of a three dimensional culture system which embeds hydrogels encapsulated with high density of HepG2 liver cells. This includes the design of an alginate based manufacturing system; verify the encapsulated cells viability, phase-I/II metabolism activity and pro-drug EFC-HFC conversion rate and phenotype maintenance.

B. The development of an *in vitro* porous poly-carbonate disc platform to enable high density 3D culture of liver cells for toxicity testing, and also combined with a monolayer growth of target breast cancer cell line (MCF-7) in a static environment as a representative example of screening drug compounds for hepatotoxicity and drug efficacy.

C. The development of a perfusion bioreactor approach for 3D dynamic culture studies. A meso-scale perfusion bioreactor was designed which can be stacked

multiply disc with hydrogels. This includes the design of bioreactor, long-term dynamic 3D culture, and study of the EFC-HFC metabolism activity and compared the drugs toxicity under static and dynamic condition.

The 3D encapsulation of cells within hydrogels represents an increasingly important and popular technique for culturing cells and towards the development of tissue engineering and drug testing. This environment better mimics how cells live *in vivo,* compared to standard tissue culture, due to the tissue-like properties and 3D environment. The following methods for the 3D encapsulation of HepG2 have been optimized in our lab to maximize cell viability and liver specific enzymes activity, minimize the hydrogel processing steps using support disc design and integrated into dynamic device. This research will also enable scientists to expand their scope of research and study in the field of *in vitro* drug screening and toxicity study. An overview of research accomplishments is presented in Figure 1.

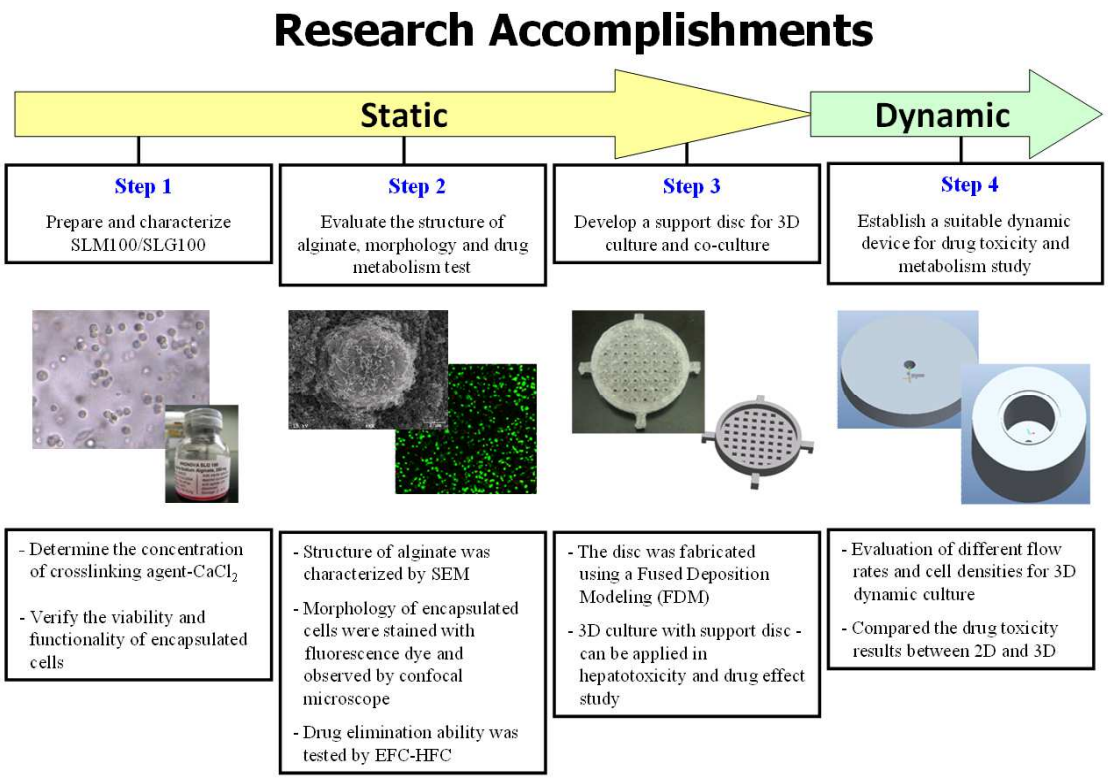


Figure 1. Summary of research accomplishments.

1.4 Research motivation

The objective of research is to develop an alginate-based 3D liver cell platform for hepatotoxicity, metabolism of hepatocytes and drug effect studies through an *in vitro* encapsulation technique. In this thesis, we will present an alginate-based 3D culture with combination of a support disc and perfusion bioreactor for possible applications in tissue engineering, characterization of hepatotoxicity and drug effects and analysis. In addition, the research conducted here has the following features:

A. 3D culture can mimic the complex environment and maintain to some degree the *in vivo* morphology *in vitro*.

B. Animal testing is often expensive, time consuming and any results derived

from animal testing may or may not be useful to humans. Our *in vitro* 3D culture design has the potential to provide a reliable and stable drug testing platform for reducing time and money during drug development.

C. High cell density and stable number of cells can be attained by 3D culture. 2D culture cells can only allow the growth of low density culture on mono-layer surface and proliferation activity is out of control.

D. For dynamic 2D culture through microfluidic devices, the cells tend to lift off from the chamber and clog the channels after extended usage. The clogging problem can be solved by the use of 3D matrices during dynamic culture.

E. Liver cell integrity can be maintained by encapsulation method and support disc design can be applied in co-culture study. For some high-throughput samples, some devices used microsomes or CYP enzymes for drug testing, but there may be loss of structural integrity and removal of cell membrane results in the loss of transporter proteins.

F. Current microfluidic devices or 3D culture modification techniques require specialized skills and expensive equipments for fabrication and operation, which makes it difficult to be used by non-experts. Alginate 3D hydrogel formation is relatively cheap and can enable the parallel study of multiple candidate drugs for high throughput drug screening.

1.5 Thesis outline

The objectives of this work were to develop 3D alginate-based cell culture system for application in toxicity testing and drugs metabolism studies. Background information on the research has been provided in Chapter 1 for better understanding of

this research that include: What is the 3D culture? What are the advantages of 3D cultures? What is the source of 3D culture? And how is the application for drug metabolism and toxicity studies. Chapter 2 describes the cultivation of HepG2 liver cells encapsulated in alginate hydrogels and the results obtained. Chapter 3 described the development of alginate based 3D hydrogels as an *in vitro* co-culture model platform for the toxicity screening of new chemical entities. Chapter 4 described the development of a perfusion bioreactor for high cell density cultivation. Chapter 5 summarizes the conclusion of this study and recommendations of future work.

Chapter 2: Long-term cultivation of HepG2 liver cells encapsulated in alginate hydrogels: a study of cell viability, morphology and drug metabolism

2.1 Introduction

Methods to improve toxicology screening techniques for potential new chemical entities (NCE) are necessary to translate discovery of new drugs from the laboratory to actual patient consumption. The recent failure of Vioxx (Merck's pain killer drug) highlights the inadequacies in viable technologies able to successfully predict the safety and efficacy of a drug. It is estimated that about a billion dollars and 10- 15 years are invested for every successful drug in the market. Unfortunately, even with large amounts of investment, success is not guaranteed. Even today, macroscale animal testing endures as the prevailing model in the evaluation of toxicological and pharmacological profiles of chemicals and therapeutic agents. After successful animal studies, further testing progresses towards human clinical trials where about four out of five candidate drugs fail. Due to the sequential testing procedure, there could be hundreds of compounds that have failed in animal studies but may actually have therapeutic effects in humans. For example, penicillin is toxic to guinea pigs but non-toxic in humans (Green 1974). In addition to high cost, laborious process and ethical issues raised by animal right groups, newer technologies must be developed to limit the use of animal models during the drug discovery process (Durick and Negulescu 2001). In order to reduce the adverse effects of potential drugs, there need to be better, more efficient *in vitro* testing procedures that would be able to predict the ADMET

(adsorption, distribution, metabolism, elimination, toxicology) properties of a drug early on in the product development process.

To facilitate the expedited discovery of new viable drugs, current research efforts are geared towards developing viable *in vitro* human tissue models, for example liver, which will serve as a tissue model surrogate to predict candidate drug efficacy and safety in humans (Khetani and Bhatia 2008). A number of *in vitro* systems are currently under development to understand the biotransformation of potential drugs in the liver and in combination with other tissue types. A number of microsomes, cell and tissue-based *in vitro* systems have been developed to mimic human metabolism, including isolated liver slices (Onderwater *et al.* 2004), primary hepatocytes (McGinnity *et al.* 2004) and transformed cultured human hepatoma cell lines e.g., HepG2 (Hewitt and Hewitt 2004). Cell-based assays usually involve culturing cells as a monolayer on a two-dimensional (2D) surface. Schuler and coworkers have developed a cell based analog chip system to predict the human response to potential therapeutic drugs (Viravaidya and Shuler 2004). The device contained interconnected cell-specific chambers to simulate dose dynamics and drug metabolite-cell interactions. Flat substrates (2D) micro-scale culture has been developed for hepatotoxicity screening applications (Khetani and Bhatia 2008). Researchers cultured liver cells within tiny wells (100 µm-1 mm diameter) to mimic liver-like tissue for drug screening testing. A microfluidic device for primary liver cell culture was also established (Lee *et al.* 2007), wherein an endothelial-like barrier was created to control mass transport. The unit was seeded with primary hepatocytes within microfabricated channels. This micro-scale culture device mimics cell-cell contact and nutrient transport across the endothelial cell

barrier in liver sinusoids (Zhang *et al.* 2008b). Dynamic culture systems have been developed wherein liver cells were perfused within a microbioreactor to ensure physiological shear stress levels experienced by *in vivo* liver tissue (Hwa *et al.* 2007; Powers *et al.* 2002).

Developments in microbioreactor and computer aided fabrication technology have enabled researchers to expand the development of *in vitro* tissue model development towards a 3D environment (Sun and Lal 2002). Most cells respond to mechanical and chemical cues within a 3D microenvironment very differently from those on flat substrates (Rowley *et al.* 1999; Shachar and Cohen 2003). In addition, a 3D culture environment can enable higher cell density (Vukasinovic *et al.* 2009), allow cell-cell contact and cell-matrix interactions (El-Ali *et al.* 2006), control of matrix stiffness (Sun *et al.* 2004) and a tunable barrier to shear stresses (Powers *et al.* 2002) induced by fluid flow within the system. Such microenvironments are limited when flat substrate cell-based assays are used. A hydrogel based 3D environment integrated within a microbioreactor system has been developed wherein cells are encapsulated within alginate and deposited within a microfluidic chamber to form the *in vitro* drug screening system (Chang *et al.* 2008b). Drug detoxification also can be studied under a seal-less blood centrifuge (Sofer et al. 1979).

The hydrogels provide the necessary matrix for the encapsulated liver cells to be stationed within the matrix. However the exact mechanism of their behavior within the matrix is not understood. An understanding of the cell-matrix interaction at the micro-scale and the systemic behavior of the encapsulated cells within a 3D environment is necessary to further advance the 3D *in vitro* tissue model system technology.

To address this need, this chapter has conducted a series of quantitative and qualitative studies to study the viability of HepG2 liver cells within two different alginate based hydrogel molecular arrangements. As control, wherever possible, the results were directly compared with results obtained from flat substrate culture (2D). The results included the CYP enzyme metabolism, enzyme induction/inhibition phase of the encapsulated cells and measured the drug metabolic capacities of the encapsulated cells. Scanning electron microscope and confocal microscopy protocols have been developed as part of the research study to enable us to visualize and characterize the encapsulated cells. In addition, the cell encapsulated matrix was exposed to the pro-drug EFC (7-ethoxy-4-trifluoromethyl coumarin) and the metabolic response rate was measured.

2.2 Materials and methods

2.2.1 Culture of HepG2 cells

HepG2 were obtained from ATCC (American Type Culture Collection ATCC, Manassas, VA), passage 77. Hepatocytes were harvested between passage numbers 77 and 80 for all our experiments. The cells were maintained in standard Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen Co., Carlsbad, Calif., USA) and 1% penicillin G and streptomycin (Invitrogen). The cells were grown in 75 cm^2 tissue culture flasks at 37 °C in a 5% CO_2 humidified environment. At confluence, cells were washed with phosphate buffered saline (PBS), treated with 0.25% Trypsin/EDTA (Invitrogen) for 5 min to release cells from the flask, pelleted by centrifugation at 1500 rpm for 5 min and finally resuspended with fresh medium to the desired cell density.

2.2.2 Alginate preparation and encapsulation of HepG2 cells

The hydrogel was prepared using Pronova SLM100 and SLG100 (NovaMatrix Co., Sandvika, Norway), mixed at a final concentration of 1% (w/v) with DMEM medium. The SLM100 variety had a M:G ratio of 60:40, while SLG100 had an inverse ratio of 40:60. HepG2 cells were cultured as described above, trypsinized, counted using a hemocytometer and resuspended in DMEM. Cells suspension medium was mixed with alginate solution (1% w/v) at a concentration of 1:1(v/v). After mixing gently, the cells-alginate precursor solution (300µl) was placed in wells of a 24-well plate containing $CaCl_2$ solution (60µl, 45 mM, Sigma). The final cell density was 2 x 10^6 cells/ml. The ratio of alginate mixture and $CaCl_2$ solution is 5:1(v/v). After incubation at 37 °C for 30 min, the alginate hydrogel was washed with *PBS to* remove any uncrosslinked solution. Further, the gel was submerged in DMEM and incubated at 37 °C in a 5% CO_2 humidified environment. The cell medium was refreshed every 2-3 days. For experiments that involved 3D hydrogels, cell free alginate gels served as the control. For all flat substrate (2D) experiments, culture medium at the bottom of the 24-well plates served as the control.

2.2.3 De-crosslinking the alginate hydrogel

To de-crosslink the 3D alginate hydrogel, samples were immersed in DMEM (containing 1mM EDTA) and incubated for 5 minutes at 37°C. The gel de-crosslinks back to the solution state and then was spun down to retrieve the cells. The cell pellets were then resuspended in cell culture medium for further testing such as viability study. The 2D samples were also trypsinized by trypsin (0.02%) for 5 minutes at 37°C, and counted with trypan blue by hemocytometer (Figure 1).

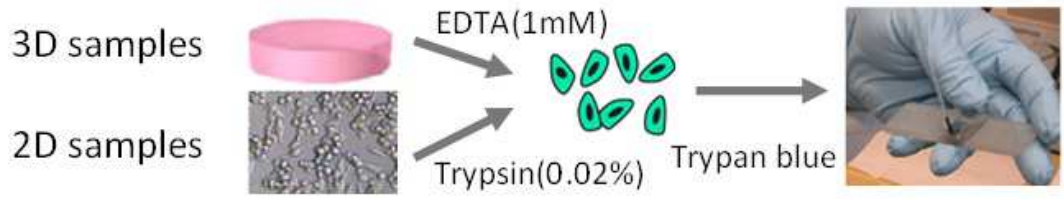


Figure 1. De-crosslinking and trypsination from 3D and 2D samples for viability test.

2.2.4 Direct cell viability counting

After detaching or de-crosslinking the cells from monolayer and encapsulated samples by trypsinization and EDTA treatment, they were assessed using a hemocytometer with trypan blue staining (Invitrogen). Measurements were performed on days 0, 5, 9 and 14.

2.2.5 Live/dead fluorescence viability testing

The viability of encapsulated HepG2 cells was quantified using the live/dead viability assay (Invitrogen) consisting of calcein-AM and ethidium homodimer. Hydrogels (SLM100 and SLG100) were formed as described above. At days 1, 4, 11 and 14, cell medium was aspirated from the wells and hydrogels were washed with PBS twice to remove FBS from the hydrogel. Samples were protected from light and incubated with 2 μg ethidium homodimer and 0.5 μg calcein-AM in 1 ml PBS solution at room temperature for 45 min. After multiple washing with PBS, the viability of encapsulated cells was quantified from z-series projections taken every 10 μm and counted as the percentage of dead cells compared to the total number of cells in encapsulated samples (Figure 2). Sections were taken using an Olympus BX61WI confocal microscope and software (Olympus, Center Valley, PA).

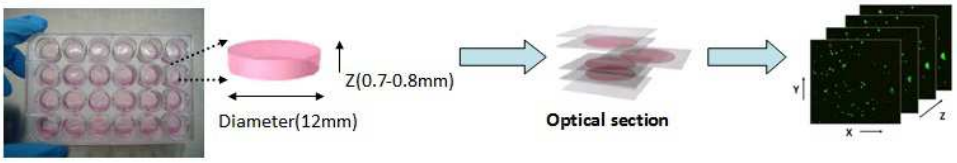


Figure 2. 3D cultures optical section by confocal microscope.

2.2.6 HepG2 filamentous actin (F-actin) staining

HepG2 cells were stained for F-actin and nuclei for observation using a confocal microscope. Samples were fixed in 4% paraformaldehyde in Hank's buffered salt solution (HBSS, Sigma) for 20 min at 37 °C, after fixation, samples were washed three times with PBS (Ca^{2+} and Mg^{2+} free) and rinsed in 0.1 M glycerine in PBS for 5 min at room temperature. For F-actin staining, the samples were incubated in dark with 5 mg/l FITC-phalloidin (Sigma) in PBS for 30 min at room temperature. After washing the samples three times for 5 min in PBS, cell nuclei were co-stained with 5 mg/L of 4',6-diamidino-2 phenylindole (DAPI, Sigma) in PBS for 30 min at room temperature. Samples were scanned with an Olympus Optical CV12 CCD camera and BX61WI confocal microscope to generate optically sectioned images. A series of optical sections taken successively at different focal levels were reconstructed with the image reconstruction program.

2.2.7 Sample preparation for scanning electron microscopy

HepG2 cells encapsulated in alginate were fixed with 2.5% glutaraldehyde in coagulation buffer (0.1 M) for 2 h at 4 °C. After washing in PBS, samples were immersed in 1% OsO_4 (aq.) solution for 60 min. Samples were dehydrated in ethanol (30%, 50%, 70%, 90%, 100%, 100%, 100%) for 20 min, three times for each respective ethanol change and dried using critical point CO_2 (Tousimis Autosamdri-814). Dried

samples were sputter-coated with gold/palladium for 15 min using a sputter coater (Anatech Ltd Hummer VI) and viewed in a JEOL JSM-880 scanning electron microscope.

2.2.8 Cytochrome P-450 and induction/inhibition activity testing

The Cytochrome P450 (CYP) superfamily of drug metabolizing enzymes is responsible for the metabolism of a variety of drugs and endogenous compounds. For testing CYP450 activity, cytochrome P-450 enzymes - CYP3A and CYP1A1 activities were measured by P-450-Glo™ CYP3A and CYP1A1 assay kit (Promega Co., Madison, WI., USA). The media were incubated with cells 24 h, removed from the wells and prepared for luciferase analysis at days 0, 5, 9 and 14. For induction/inhibition, CYP1A1 inducer omeprazole (Sigma–Aldrich., St. Louis, Missouri, USA) and CYP3A4 inducer dexamethasone (Sigma) were dissolved in DMSO to prepare stock solutions of 300 and 20mM, respectively. For our experiments, these solutions were further diluted in DMEM to a final concentration of 300 and 20μM respectively. The luminescent value was measured by FLUOstar OPTIMA microplate reader (BMG Labtech Co., Alexandria, VA., USA), 1 s duration time. Control cultures were treated with vehicle (DMSO at a final concentration of 0.1% in DMEM) alone for calculation of fold induction and inhibition. CYP1A1 activity was detected by the P-450-Glo™ kit.

Fold induction was calculated as follows:

$$\text{Fold induction for 3D}(\%) = \frac{A-B}{B} \ (\%)$$

A: Luminance for inducers **treated** samples/1000 cells

B: Luminance for inducers **untreated** samples/1000 cells

Inhibitors of CYP1A1 and CYP3A4 were SB203580 (Promega) and actinomycin D (Sigma) which were made in DMEM (0.1% DMSO) at a concentration of 10 μM and 10 μg/ml respectively. Cultures were treated with SB203580 inhibitor for 1 day and with actinomycin D for 30 min. Control cultures were treated with vehicle (DMSO at a final concentration of 0.1% in DMEM) alone. Fold inhibition was calculated as follows:

$$\text{Fold inhibition for 3D(\%)} = \frac{A-B}{B} \ (\%)$$

A: Luminance for inhibitors **treated** samples/1000 cells

B: Luminance for inhibitors **untreated** samples/1000 cells

2.2.9 Glutathione assay

During phase II reactions, some activated xenobiotic metabolites are conjugated with charged species such as glutathione and produce more polar metabolites than can be eliminated from human body. A change in GSH levels is important in assessment of toxicological responses and is an indicator of phase II metabolism ability. In this study, glutathione (GSH) level was measured by GSH-Glo™ assay kit (Promega Co., Madison, WI., USA). Three-dimensional samples were de-crosslinked with EDTA and the encapsulated cells retrieved. For 2D samples, the medium was removed from the plate containing samples. After removing the medium, both 2D and 3D samples were resuspended with GSH-Glo™ reagent (Luciferin-NT and Glutathione-S-Transferase included). After 30 min incubation, the samples were mixed with reconstituted luciferin

detection buffer and luminance was measured by FLUOstar OPTIMA microplate reader (BMG Labtech Co., Alexandria, VA., USA).

2.2.10 Pro-drug metabolism by HepG2 liver cells encapsulated within alginate

In vitro tests for drug metabolism are used widely. 7-Ethoxy-4-trifluoromethyl coumarin (EFC) has been described in the literature as an easy and sensitive method (DeLuca et al. 1988). EFC is an analog of ethoxycoumarin, a widely employed cytochrome P450 test substrate. The reaction studied is given in Figure 3. The fluorescence emission spectrum of the product, 7-hydroxy-4-trifiuoromethyl coumarin (HFC) is different from EFC and can be monitored by fluorescence reader. CYP1A2, CYP2B6 and CYP2E1 have been studied which may involved in metabolism of EFC to HFC(Ekins et al. 1997).

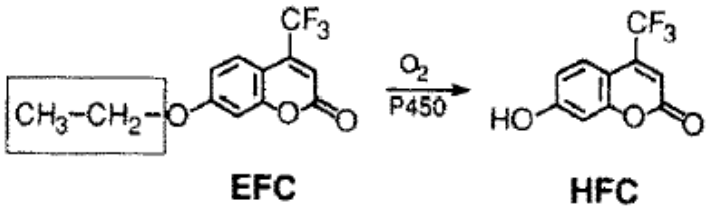


Figure 3. The cytochrome P450 mediated 0-deethylation of EFC to HFC.

Drug substrate EFC (Invitrogen) was mixed with DMSO to create a 10 mM stock solution of EFC. Pro-drug HFC (Sigma-Aldrich) was also mixed with DMSO to create a 10 mM stock solution of HFC. HFC standard curve range was prepared from 1 – 64 μM. Alginate hydrogel encapsulated with liver cells were incubated with 120 μM EFC at day 1, day 7 and day 14. At each time point, the concentration of HFC was

monitored during 40 h with a fluorescence reader using an excitation wavelength of 360 nm and an emission wavelength of 520 nm.

2.2.11 Statistical analysis

Analysis of variance (ANOVA) was used to analyze data and significance was considered at $p < 0.05$.

2.3 Results

2.3.1 Alginate matrix structure

SLM100 and SLG100 alginate were sectioned to several pieces, dried in a critical point drier (CPD) and coated with gold and examined in a scanning electron microscope (SEM). The images showed a varying pore structure for the SLM100 and SLG100 alginate molecules (Figure 4). The SLM100 with the lower G-content resulted in a more open network structure with larger pores when compared to the SLG100 pore network. Due to the denser network, the SLG100 alginate gels were stiffer than the SLM100 gels and hence were easier to handle.

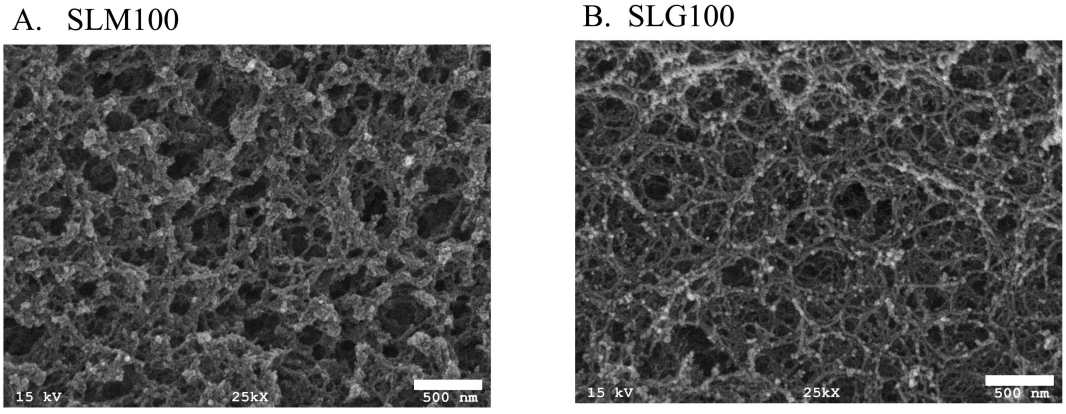


Figure 4. Scanning electron micrograph of a cross-section of SLM100 and SLG100 hydrogels. (A) SLM100 structure image. Scale bar 500nm; (B) The internal pore network within SLG100. Scale bar 500nm.

2.3.2 Cell proliferation and morphology

To determine the proliferation and viability of cells encapsulated within the 3D matrix, the alginate hydrogel sample sets were de-crosslinked by EDTA and the cells released from the gel. The total cell number during each day of culture is shown in Figure 5A. On day 0, the total number of cells within each sample set is the same due to the constant initial seeding density among all sets. As expected, after day 5, the number of cells cultured on the collagen coated well plate (2D culture) increased dramatically. After 2 weeks of culture, the cell number in the well plates was 4.5 times higher than day 0, while the cell number in the 3D gels increased gently from 2 to 3×10^6 cells/ml.

The microenvironment influences the morphology of the cells when grown on flat substrates as opposed to encapsulation within the hydrogel. In order to investigate the morphology difference between 2D and 3D culture, samples were stained with DAPI and fluorescently labeled phalloidin. Figure 5B shows F-actin as green and nuclei as red in HepG2 which are labeled with phalloidin and DAPI. Cells grown on the flat substrate spread out and adhered to the bottom of the well plates. Figure 5C shows HepG2 liver cells labeled with phalloidin. Cells encapsulated in the hydrogel display a spherical shape configuration with pockets of HepG2 aggregation and cell isolation within the gel. The spherical shape of the encapsulated cells in the 3D gel clearly suggests that the cells are entrapped within the gel and not adhered to the alginate molecular chains. However the cells remained viable during the period of culture.

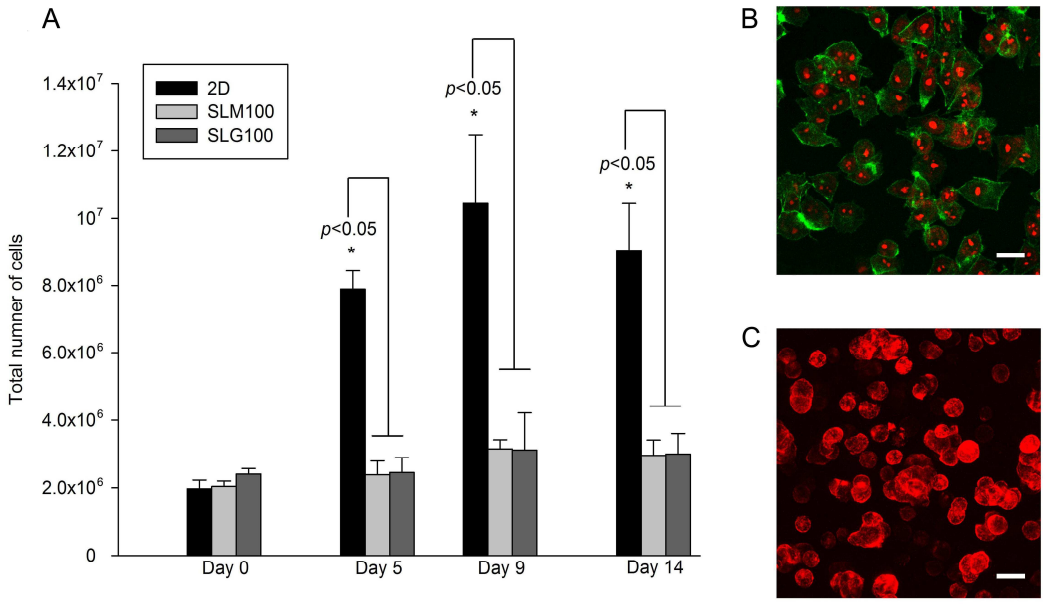


Figure 5. Schematic of cell proliferation between 2D and 3D. (A)The number of HepG2 cells grown on 2-D (collagen coated well plate) and 3D (HepG2 cells encapsulated with SLM100 and SLG100 alginate gels). Data represent the mean ±STDEV for three independent repeats. The (*) indicates statistical 2D culture significance relative to 3D culture at the respective time points, $p < 0.05$(t-test). (B) Confocal micrographs of HepG2 cells seeded on 2D surface showing F-actin distribution and nuclei. HepG2 cells were stained with DAPI and phalloidin. Scale bar 20μm. (C) Confocal micrographs of HepG2 Cells encapsulated in SLG100, HepG2 cells were stained with phalloidin. Scale bar 20μm.

2.3.3 Cell viability and SEM morphology during time

When measuring the cellular viability using the trypan blue assay after trypsinization and de-crosslinking, a 90 ± 1.7% viability was maintained after 2 weeks for the flat substrate culture while over 81 ± 2.3% and 74 ± 3.8% viability was maintained for cells cultures encapsulated within the SLM100 and SLG100 gel (Figure 6A). There is no significant difference in the viability between the two different alginate gel structures - SLM100 and SLG100 ($p > 0.05$). These results simply indicate that

without any modification to the alginate structure, cells remain entrapped within the gel and have limited proliferation activity. The cells do not adhere to the alginate molecular chains due to the negatively charged residues of guluronate and mannuronate molecules. However, these results suggest that the encapsulated HepG2 remain sufficiently viable during 14 days of static culture (>80% at day 9).

As observed in Figure 6, cell proliferation is limited within the alginate matrix. This is primarily attributed to the lack of adhesion between the HepG2 cells and the alginate structure. This is confirmed by SEM images which indicate an aggregation of cells encapsulated within the alginate. As shown in Figure 6B, the cell surface is covered with microvilli which interact with the alginate structure at day 1. Even after 2 weeks of culture, the cells do not significantly change the composition of the surrounding alginate matrix (picture not shown). The cells appear to remain viable while being encapsulated within the gel. Figure 6C shows instances of ‘cell blebbing’ within the gel matrix at day 14. Blebbing is an indication of cell injury or death, which translates to cells cultured within the alginate matrix, continues on with their entire life cycle *in vitro*.

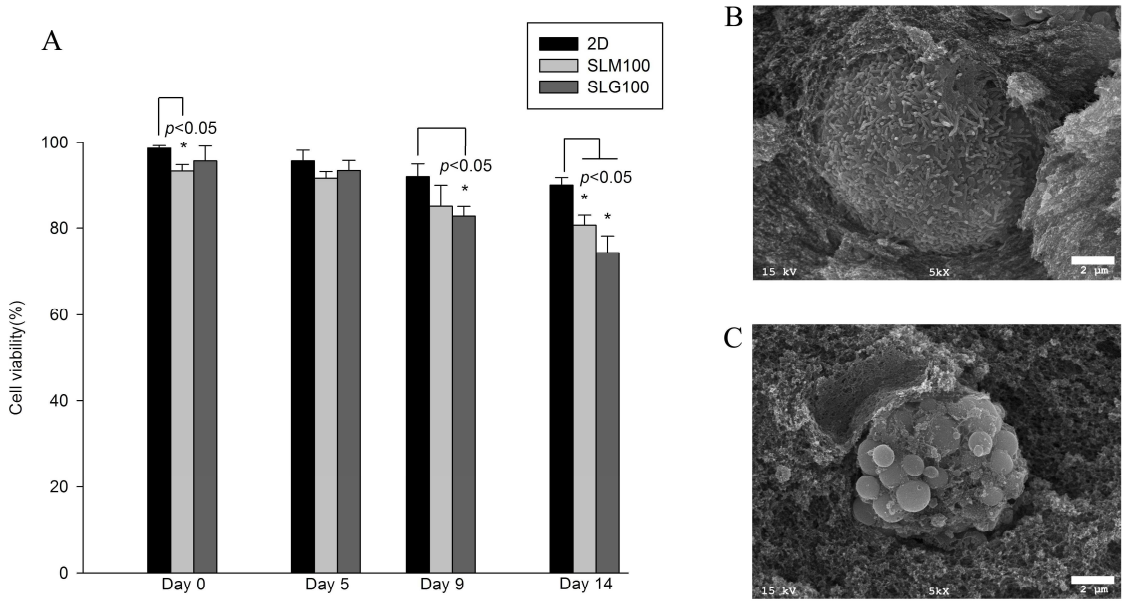


Figure 6. Schematic of viability between 2D and 3D. (A)The cell viability of HepG2 cells grown on 2-D (collagen coated well plate) and 3D (HepG2 cells encapsulated with SLM100 and SLG100). Data represent the mean ±STDEV for three independent repeats. The (*) indicates statistical 3D culture significance relative to 2D culture at the respective time points, $p < 0.05$(*t*-test). (B) Morphology of a HepG2 cell at day 1, the picture represented a healthy liver cell which is covered with microvillus and entrapped by the alginate hydrogel. Scale bar 2µm (C) Morphology of a HepG2 cell at day 14, the image shows an unhealthy cell undergoing blebbing. Scale bar 2µm.

2.3.4 Quantification of cell viability in encapsulated 3D HepG2 SLM100 and SLG100 hydrogels

Live/dead assay was also used to qualitatively and quantitatively indicate the viability of the cells within the gel. Since diffusion of nutrients into the 3D gel is a significant challenge, we have chosen to use confocal microscopy to determine the viability through the thickness of the 3D hydrogel. Live/dead assay results mirror the

results obtained from the direct counting of the live cells using the hemocytometer. As shown in Figure 7, as the days progress, the amount of dead cells within the gel increase, dropping the viability down to 78 ± 5% by day 14.

To determine the distribution of cells within the 3D gel, serial optical cross-sections of the gels were optically imaged using the confocal microsope. The designed 3D hydrogels were about 10-12 mm in diameter with an approximate thickness of 0.6-0.85mm. SLG100 hydrogel was optically sectioned to 100 layers and each slice was observed for cell viability and cell distribution. We found that dead cells were observed uniformly distributed in the SLG100 hydrogel. It was determined that the diffusion of nutrients from the cell culture medium into the alginate gel did not present a problem since there wasn't any apparent loss of cell viability within the central mid-plane of the gel.

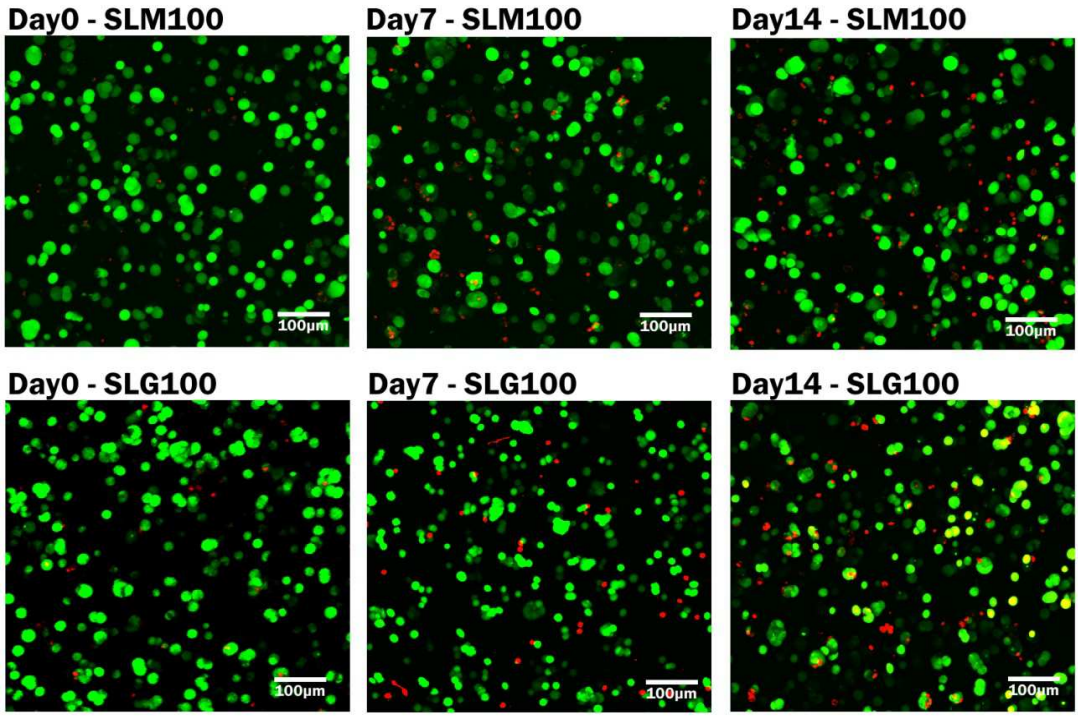


Figure 7. Encapsulated HepG2 cells were encapsulated in SLM100 and SLG100 and stained with Calcein AM and EthD-1 at day 0, day 7 and day 14. Live cells are green, dead cells are red. At day 0, the viability of HepG2 cells was higher than day 7 and day 14. After 14 days in culture, the viability dropped to 78±5%. The confocal images show staining for both Calcein AM and EthD-1 dyes in the hydrogel with a sample thickness of 500µm. Scale bars are 100 µm.

2.3.5 Phase-I Cytochrome P-450(CYP450) metabolism

Drug metabolism is a necessary function within the human body to transform hydrophobic drugs to hydrophilic which are then easily soluble and excreted away through the urinary system. Drug metabolism in the liver involves Phase-I/II reactions that affect the toxicity of a drug and are primarily facilitated by oxidation and conjugation mechanism (Sivaraman *et al.* 2005). It is understood that within the CYP450 enzymes, three families of CYP1, CYP2 and CYP3 account for almost 90% of

drug metabolism occurring in the body. In this study we have chosen to examine the production of CYP1A1 and CYP3A4 from cells cultured in the two different substrates – flat substrate (2D) and the alginate hydrogel.

To determine the stability of HepG2 CYP450 activity, CYP1A1 and CYP3A4 activity were measured for cells encapsulated in SLM100 and SLG100 samples. CYP450 activity in 2D and 3D culture were tested during 14 days of culture by using a specific CYP1A1 substrate – P-450-Glo™ luciferin-6' chloroethyl ether and a specific CYP3A4 substrate – P-450-Glo™ luciferin-6' pentafluorobenzyl ether and. The results are shown in Figure 8A and B where the data from the flat substrate culture is set to 1, and CYP450 activity was calculated by CYP activity/1000 cells. We found that activities of CYP1A1 and CYP3A4 were retained for 14 days across all sets of samples. Compared to CYP1A1 activity for 2D (Figure 8A), the CYP1A1 activity for the 3D gels were at similar levels and in some cases higher than the 2D. In contrast, the CYP3A4 activity for 3D samples (particularly for cells grown in SLM100) was higher than 2D samples (Figure 8B). Overall, these results indicate a healthy maintenance of CYP1A1 and CYP3A4 expression in HepG2 encapsulated in both SLM100 and SLG100 samples over a 14 day period. This activity levels are observed in spite of the HepG2 cells being encapsulated within the 3D gels.

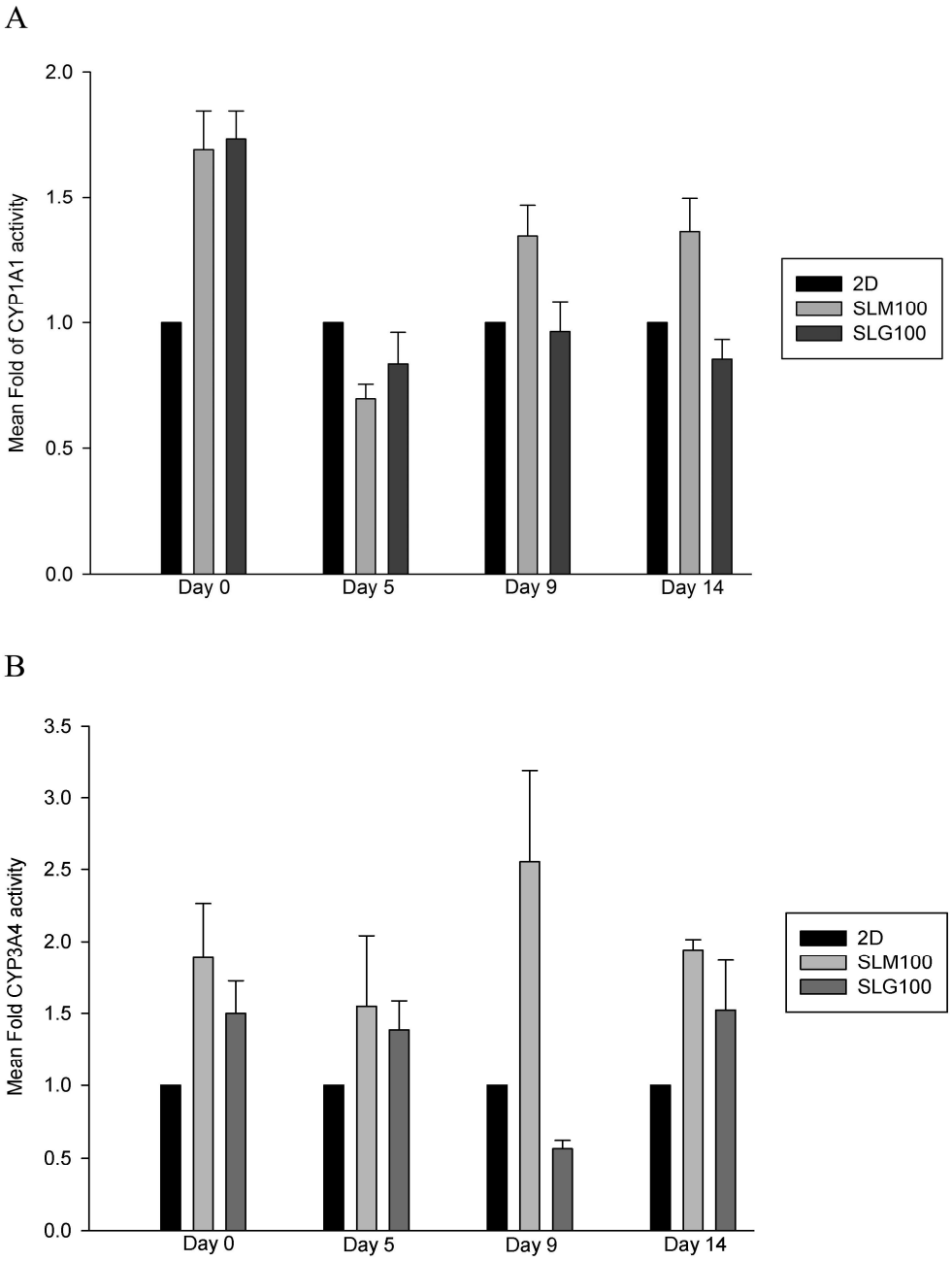


Figure 8. CYP450 activity of HepG2 cells grown on 2-D (collagen coated well plate) and 3D (HepG2 cells encapsulated with SLM100 and SLG100). (A) Determination of HepG2 CYP1A1 activity by measurement of Luciferin secretion into the medium. (B) Initial substrate concentration is Luciferin-PFBE 50µM for CYP3A4 testing and activity was calculated by CYP activity/1000 cells. Data represent the mean ±STDEV for three independent repeats.

2.3.6 Induction and inhibition experiment for CYP450

Frequently, drug candidates are also used as an inducer or an inhibitor for CYP gene expression, which provides a mechanism for amplifying or reducing the detoxification rate in the human body. After the drug is eliminated from the body, the CYP expression should return to normal levels. We have used known inducers and inhibitors to determine the CYP gene expression levels for 2D and 3D hydrogel culture environments. In this study, we have used omeprazole and dexamethasone as CYP1A1 and CYP3A4 inducers, while SB203580 (p38 MAP kinase inhibitor) and actinomycin D as inhibitors. The data from the flat substrate culture is set to 1, and CYP450 activity was calculated by CYP activity/1000 cells. For CYP1A1, omeprazole could induce CYP1A1 activity over 1.5 to 2-fold for all culture environments as shown in Figure 9A. For CYP3A4, dexamethasone could induce approximately 3-fold for all culture environments (Figure 9B). For inhibition, SB203580 could inhibit 85% of CYP1A1 expression, while actinomycin D could inhibit 80% of CYP3A4 expression on all sets of samples. These results indicate the encapsulated HepG2 cells respond positively when exposed to the induction and inhibition agents and also showed induction and inhibition fold have a similar phase between 2D and 3D culture.

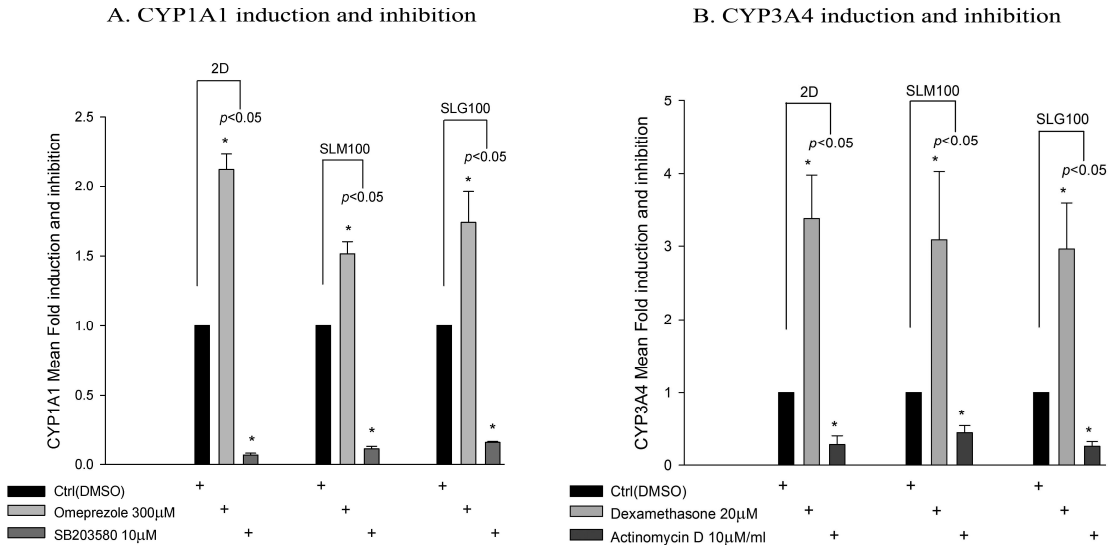


Figure 9. Induction and inhibition of CYP450 isoforms in HepG2 cells. (A) Induction and inhibition of CYP1A1 activity in 2D and 3D cultures (SLM100 and SLG100) (B) Induction and inhibition of CYP3A4 activity in 2D and 3D cultures. Data represent the mean ±STDEV for three independent repeats. The (*) indicates statistical induction/inhibition samples significance different to 2D culture, $p < 0.05$(*t*-test).

2.3.7 Phase-II Cytochrome P-450 (CYP450) metabolism

Glutathione (GSH) plays an important role in conjugation reactions and reduction reactions catalyzed by glutathione S-transferase enzymes in cytosol and enables drugs to be removed from the body. Measuring GSH levels is important for determining cells toxicological responses and is also proved to be indicator of cell viability and functionality. To determine GST activity, we used GSH-Glo™ assay (Promega) to detect and quantify GSH in 2D and 3D samples. The GSH-Glo™ assay is a luminescence-based assay and based on the conversion of a luciferin derivative into luciferin which is catalyzed by GST. The luminescent signal is proportional to the amount of reduced glutathione present in the sample. The data from the flat substrate

culture is set to 1, and GSH activity was calculated by GSH activity/1000 cells. Our results show the GSH levels were decreased after day 4 of culture compared to 2D culture (Figure 10). GSH activity in both 3D hydrogels (SLM100 and SLG100) decreased with time but had sufficient levels of activity necessary to perform as a drug screening model system. The decreasing levels could be attributed to the lack of cellular adhesion for encapsulated cells within the 3D hydrogel.

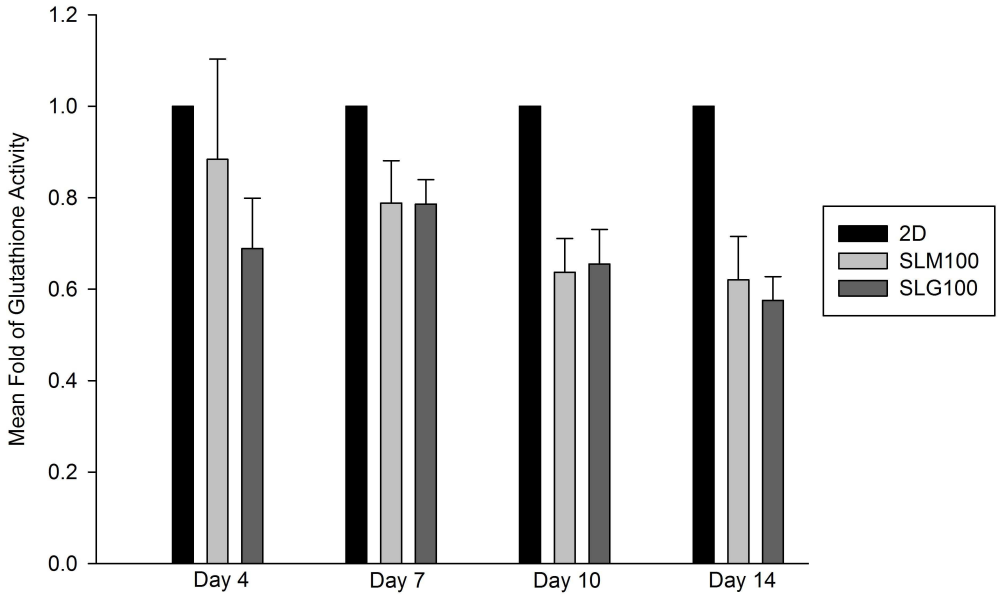


Figure 10. GSH status in HepG2 cells maintained in 2D and 3D culture conditions. For each experiment, cells were seeded at $2x10^6$ cells/ml and activity was calculated by GSH activity/1000cells. Data represent the mean ±STDEV for three independent repeats.

2.3.8 Drug metabolism by the *in vitro* models

Drug elimination experiments can determine the drug residence time using liver cells encapsulated in the alginate hydrogels. The CYP450 enzyme plays an important role in the metabolism of several pro-drugs such as 7-ethoxy-4-trifluoromethyl

coumarin to 7-hydroxy-4-trifluoromethyl coumarin (HFC). The enzymatic product, HFC can be detected by fluorescence using an excitation wavelength of 360 nm and an emission wavelength of 520 nm. For this experiment, we examined the concentration of HFC at 3 different time points during the 14 days of culture. In this experiment, stability of HFC emission peak was shown during three rounds of screening at day 1, day 7 and day 14 and the results are shown in Figure 11. At day 1, concentration of HFC was measured after EFC treatment at 4, 8, 12, 16, 20, 28 and 40h. The cell density for 2D and 3D was 2 x 10^6 cells/ml. For the 2D samples, at day 1, the concentration of HFC increased until 28 h and then reached a saturation level where no more of the EFC was converted to HFC. After second and third repeated exposure to EFC at day 7 and day 14, the result shows the conversion amount decreased steadily from days 1 through day 14. As shown by the results, the drug HFC emission peak in SLG100 was also lower than in the 2D samples. However there was a near steady conversion rate for the 3D samples across multiple time study period.

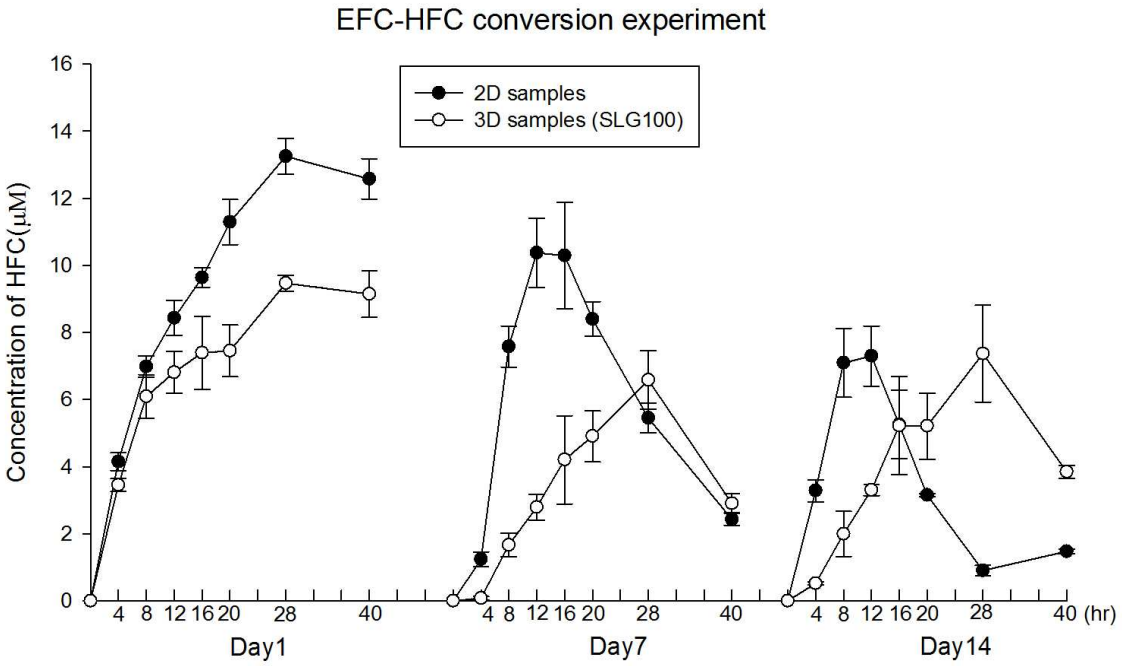


Figure. 11. Drug response rate in 2D and 3D. The concentration of HFC was measured every 4 hours and the cell density was 2 x 10^6 cells/ml for each sample.

2.4 Discussion

The liver in the human body contains a variety of enzymes that are involved in the drug metabolism process. Consequently, cell-based assays involve the use of hepatocytes to predict the toxicity effects and remedial investigation of drug candidate compounds. For decades, toxicology studies have used 2D cell based testing as a widely accepted initial screening platform to screen drug compounds. However, several instances of inadequacies of 2D *in vitro* screening have led to developments of platforms that incorporate 3D environments and dynamic flow to simulate *in vivo* like environments. Failures to obtain FDA approval to potentially new drug compounds are traced back to the Phase-I (safety) and Phase-II (efficacy) laboratory and clinical investigations. The laboratory failures can be attributed to the loss of *in vivo*-like

behavior, when the same cells are cultured using *in vitro* conditions (Bhadriraju and Chen 2002).

Our study is focused on the development of new high throughput screening (HTS) platform which specifically incorporates the use of cells within 3D hydrogels. The research community has described the variety of cellular response differences between 2D culture as opposed to 3D culture which include morphology changes, biological activity levels, growth factor expression and other specific functions (Haramaki 1993 ; Zvibel *et al.* 1998). In conventional two-dimensional cell culture, cells grow until confluency, while encapsulation of cells within 3D gels allow prescribed locations for the cells and limited proliferation within the microenvironment. Control of cell density is an issue for *in vitro* drug screening testing as is the clogging of microfabricated channels within *in vitro* 2D based HTS platforms. Three-dimensional cultures have the potential to provide *in vivo* like environments for cell differentiation, proliferation and development of specific functions (Heppner and Miller 1998), in addition to providing a stable platform for dynamic flow experiments when compared to 2D culture. This paper investigates the behavior of a human cancerous liver cell line (HepG2) when encapsulated in two different alginate molecular structures (SLM100 and SLG100) for possible applications in HTS platforms.

2.4.1 Alginate-based cell encapsulation

Alginates have unique properties which enable their use as a biomaterial base to provide a conducive microenvironment for optimal cellular function and behavior. Alginates are natural materials, derived from seaweed and have been widely used to mimic ECM structure for 3D culture (Smetana 1993). Alginates are formed by cross-

linking polymer chains of ionic bridges between divalent cations to form a water-insoluble polymer. Cells may be encapsulated during the cross-linking process to create cells-hydrogel constructs for drug delivery and tissue engineering applications. As seen in the SEM images, cells encapsulated within the matrix did not adhere to the surrounding molecules primarily due to the lack of adhesion molecules available within the alginate matrix. This has significantly influenced the behavior of the encapsulated cells, especially in terms of cell proliferation. Higher cell density and agglomeration of HepG2 cells proves to be beneficial for cell function and viability. These results mirror the results obtained by Surapaneni et al. 1997. The same result has been reported by others within the research community. The inclusion of adhesion molecules within the alginate structure and higher cell density will significantly enhance cell viability and function (Glicklis et al. 2000).

2.4.2 Cellular viability and proliferation

Our results indicate a very slow growth of encapsulated cells within the SLG100 and SLM100 matrices during the 2 week period of study. Although the alginate structures are highly porous, cells are most likely entrapped within the matrix and do not adhere to the alginate molecular chains. As HepG2 cells are anchorage-dependent, the initial cell adhesion to the matrix structure is a critical stage because it precedes cell spreading and proliferation. The lack of adhesion molecules prevents them from proliferating within the matrix. This lack of proliferation is not necessarily detrimental in using encapsulated cells for HTS applications since the total number of cells within the matrix can be kept relatively constant throughout the period of the drug screening study. This characteristic helps to reduce the variability between sample sets. As

expected, HepG2 cells grown on the flat substrate (2D culture) have shown a steady proliferation growth until confluency within the well plate. The cell numbers available at each time point within the study was determined by de-crosslinking the gel using EDTA and then counting the cells using a regular hemocytometer. This method proved to be a better estimate than standard viability assays such as Alamar blue and MTT assays since the dyes would have to penetrate the 3D gel and this may limit the assay efficacy. Our Alamar blue assay results were erratic and inconsistent throughout the time period, attributed mainly to the diffusion limitations of the Alamar blue dye and the incubation time as defined by the protocol (data not published). Even after 14 days of culture, we have been able to successfully maintain >70% viability observed for both SLM100 and SLG100 alginate gels. These results were further corroborated by our live/dead assay. The LSCM investigation allowed us to obtain spatial information on the distribution of the cells within the gel. We have observed a fairly uniform distribution of the encapsulated cells within the gel matrix. More importantly, cells within the interior of the gels did not show any effects due to diffusion limits of nutrients into the matrix. The open network structure of the alginate hydrogel aids in the diffusion of nutrients within the gel.

2.4.3 Phase-I/II metabolic capacities

Most drugs are metabolized in the liver by the Cytochrome P-450 (CYP) enzymes and other Phase-II enzymes, particularly glutathione (GSH). It is important to establish metabolism related and drug-liver interactions where one drug may affect the metabolic capacities of the liver, leading to possible toxic effects. The relatively stable amount of encapsulated cells within the 3D matrix during the period of study is

beneficial for future pharmacokinetic based time studies. Most papers have described the expression levels of CYP450 enzymes in 3D gels and not much have been said of the Phase-II enzyme production for the encapsulated cells. Some forms of toxicity are directly attributed to the induction of Phase-II enzymes such as UDP-glucuronosyl and glutathione S-transferases (Cantelli-Forti *et al.* 1998).

Time courses of CYP450 activity over 14 days showed that CYP1A1 and CYP3A4 activity varied for both culture environments. In general, the expression level of CYP450 in HepG2 is lower than in primary hepatocytes (Wilkening *et al.* 2003), but primary hepatocytes maintain their function for only a few days and therefore are functionally unstable (Guillouzo *et al.* 1993). The expression of CYP1A1 and CYP3A4 are slightly higher than 2D in SLM100 during 2 weeks and slightly decreased when cultured within SLG100 gels. For Phase-II enzyme testing, glutathione S-transferases appear to be slightly lower than 2D. Our experiments have employed a protein based detection analysis for CYP1A1 and CYP3A4 activity. It is possible that a cross-reaction between CYP3A5 and CYP1B1 may have influenced our results (Madan *et al.* 1999). This can be corrected by using a gene-expression based analysis for detecting specific families of the CYP450 class of enzymes. Since our experiments are targeted towards understanding the response of the encapsulated cells, the fact that the CYP450 activity is sufficiently expressed allows us to use such 3D gels for high throughput drug screening applications.

When liver cells are cultured under conditions that represent the normal function and morphology, CYP450 can be induced or inhibited *in vitro* that reflect the *in vivo* induction and inhibition phase (Runge *et al.* 2000). Understanding the induction and

inhibition of CYP450 has been shown to provide important information to predict drug interaction (Allen *et al.* 2001). If 3D gels can represent the normal induction and inhibition phase, they can potentially be used to provide *in vitro* screening at pharmacological level. Further we demonstrated CYP1A1 and CYP3A4 induction and inhibition profiles were very similar between 2D and 3D sample sets. Compared with previous studies which cultured primary liver cells with fibroblasts (Hewitt *et al.* 2007; Khetani and Bhatia 2008), CYP1A1 and CYP3A4 induced fold were lower than those obtained when cultured with fibroblasts. The fibroblasts provide the liver cells with specific cell interactions which enables them to perform better. Hepatocyte spheroids could extent viability and maintain high level of liver-specific functions, including albumin and urea content (Bokhari *et al.* 2007). In our experiments, HepG2 spheroids were maintained in a relatively stable cell number with a basic level of CYP450 activity levels throughout the 3D culture. Further improvements in our matrix structure can include the optimal ratio of fibroblasts to hepatocytes within the 3D gels to enhance cellular interaction. Previous reports have shown that GSH synthesis is in direct correlation to cell attachment (Morrison *et al.* 1985). Papers have also reported that GSH levels were increased when hepatocytes were cultured on collagen surface due to the improved cell attachment (Moghe *et al.* 1997; Richert *et al.* 2002). In this experiment no proteins were added to the matrix to improve the cell attachment ability. This might explain the lower GSH activity in the 3D samples when compared to the 2D samples. However future improvements of the matrix structure can include collagen and adhesion molecules which may increase the GSH activity levels due to increased matrix-cell interactions (Richert *et al.* 2002).

2.4.4 Drug metabolism by encapsulated cells

To characterize the metabolic clearance rate of a drug candidate through our samples, we have used the pro-drug EFC. In our study we measured the fluorescence intensity of HFC production after treatment with 120 μM of EFC in the medium. All samples were able to convert EFC to HFC over the studied time period. However the amount of HFC content within any sample did not peak more than 14 μM. After further treatment of EFC during days 7 and day 14, the peak HFC content decreased indicating the conversion efficiency dropping as time progresses. It is also noticed that after a peak is reached, HFC content goes down, possibly due to the degradation of the HFC content within the medium. However as pointed out before, gels can provide a stable microenvironment for dynamic culture studies and hence provide advantages for a 3D culture assay system.

In conclusion, our results suggest that pre-screening of drugs using a HepG2 cell line encapsulated within 3D alginate systems is possible. This chapter has shown that some important drug metabolism functions of hepatocytes such as CYP450 and GSH can be maintained at significant levels *in vitro* for 2 weeks. The ability to transform EFC to HFC also provides further proof on the potential application of encapsulated cell lines. Detailed investigations have been conducted to study the viability and proliferation rate of cells within the gel matrix. The study also used SEM and fluorescence microscopy to identify the morphology and structure of cells encapsulated within the 3D matrix. For the next chapter, further improvements are included development of a polycarbonate disc for hydrogel formation and co-culture with two types of cells for drug drugs effect and toxicity studies.

Chapter 3: Alginate based 3D hydrogels as an *in vitro* co-culture model platform for the toxicity screening of new chemical entities

3.1 Introduction

In order to reduce animal testing in drug development process and toxicity studies, *in vitro* techniques have been described for various applications of pre-clinical drug evaluation. Many scientists are involving in developing new models for drug screening are not only for ethical concerns but at the same time can be motivated by reducing cost. In the human body, primarily the liver, plays an important role in biotransformation and the elimination of toxic compounds from human body. Therefore, hepatocytes based studies have been used extensively for drug metabolism studies *in vitro*. In broad terms, hepatocyte based methods can be divided into two categories. The first of category is composed of cellular system such as liver slices (Onderwater *et al.* 2004), primary hepatocytes (Hewitt *et al.* 2007; McGinnity *et al.* 2004) and tumorigenic human hepatoma cell lines such as HepG2 and HepaRG (Hewitt and Hewitt 2004; Josse *et al.* 2008). The second category is composed of hepatocytes-metabolism enzymes, such as human liver microsomes and isolated recombinant CYP450s (Hariparsad *et al.* 2006; Lee *et al.* 2008b). The information obtained from *in vitro* models can be used to apply in identification human drug candidate or drug-drug interaction, furthermore the phase I/II enzymes responsible for the drug treatment can be determined.

Many hepatocytes culture techniques have been widely used for mimicking *in vitro* hepatocytes functionality. The various cellular systems include such as 2-

dimensional, 3-dinmensional cell culture or integrate with a microfluidic network co-culture system. Cell-based assays usually involve culturing cells as monolayer cells on two-dimensional (2D) surfaces. Schuler and coworkers have developed a cell based analog chip system to predict the human response to potential therapeutic drugs (Viravaidya and Shuler 2004). Briefly, the device contained two multi-chambers in which hepatocytes and lung cells to simulate dose dynamics and drug metabolite-cell interactions, naphthalene toxicity can be monitored by H_2O_2 accumulation and glutathione depletion when liver cells were cultured in the chamber. One limitation of this system is that a 2-D monolayer culture was used which may not represent the physiological functions *in vivo*. Despite of the result, this work was still validation study of concept of "cell on chip". In 2009, they improved the original device to a 3-D culture system with multiple cell type for drug testing (Sung and Shuler 2009a). In this study, they used Matrigel as an encapsulating matrix for colon tumor and liver cells, and encapsulated myeloblasts with alginate. The toxicity of Tegafur and 5-fluoroural can be examined using this system. In addition, the viability and toxicity in the 3-D microfluidic device can be monitored real-time using a portable fluorescence optical detection system (Choi *et al.* 2010). A serious problem of microfluidic system bubbles accumulation, especially for long term cultivation. A bubble trap made of PDMS (polydimethyldisiloxane) was designed to trap air bubbles of up to 10 μl volume (Sung and Shuler 2009). A microfluidic device for primary liver cell culture was also established by (Lee *et al.* 2007), wherein a endothelial-like barrier was created to control mass transport. The unit was cultured with primary hepatocytes within microfabricated channels. This microscale culture device mimics cell-cell contact and

nutrients transport across the endothelial cell barrier in liver sinusoids (Zhang *et al.* 2008). Static flat substrates (2D) microscale culture has been developed for hepatotoxicity screening applications (Khetani and Bhatia 2008). Researchers cultured primary rat hepatocytes within a miniaturized system with tiny wells (100μm-1mm) and 2-D multi-well culture to mimic liver-like tissue for drug screening testing; also liver cells phenotypic functions can be maintained for several weeks. Some novel microfluidic devices have been designed not only for drug toxicity but for other functionality testing. Scientists also want to study the relation between the concentration of oxygen and cells functionality (Allen *et al.* 2005). Microfluidic channels are integrated into a bioreactor to perfuse rat liver cells with medium and a gradient of oxygen concentration. Different locations were examined for cells viability, functionality and toxicity. This example demonstrates the power of fluidic system in controlling the transport process for more authentic cell function. In recent years, many dynamic culture systems have been developed where liver cells were perfused within a microbioreactor. A continuous flow was maintained within this system which ensure physiological shear stress levels experienced by *in vivo* liver tissue (Hwa *et al.* 2007; Powers *et al.* 2002).

Recently, the advantages of microfluidic device including induced reagent consumption and can provide an alternative drug pharmacokinetics platform *in vitro* (Ma *et al.* 2009), but the size of chamber also limited cell culture area and long-term cell culture is hard to reach for 1 or 2 weeks. Due to these problems, new approaches have been developed as an improvement for microfluidic device. When considering high-density cell cultures, adequate delivery of oxygen to the cells appears a crucial

problem especially for hepatocytes (Griffith and Swartz 2006). An oxygen supply system has been setup for improving oxygen perfusion in microfluidic device (Nishikawa *et al.* 2008). High cell density can also be reached by stacking many PDMS layers (Leclerc *et al.* 2004). In the present bioreactor, the cell density can be reached around 4 x 10^7cells/cm^3 and monitor 12 days. However, the cell density can be achieved by stacking method, but the cellular interaction didn't enhance. Tan et al (Tan and Desai 2004) immobilize cell-collagen matrixes inside microfluidic devices. By repeating this procedure, different types of cells can be stacked on the matrixes. Cytochrome P450 (CYP) is one of the most important enzymes which involved in biotransformation or detoxification of xenobiotics. The transformation process can increase the solubility of drugs and in further eliminate easily from human body. However, in some cases, some prodrugs (i.e. cyclophosphamide) can be bioactivated by CYPs or procarcinogens (i.e. aflatoxin B1 and sterigmatocystin) can become toxic to target cells through Phase I/II reaction. The CYP enzymes can be obtained from liver cells and appropriate tissues (Wrighton *et al.* 1993). Some microsomes also separate and purify from complementary DNA expression system (Langenbach *et al.* 1992). A simple testing method was developed in 1980 by Spielberg et al., who developed a method for examining acetaminophen toxicity by using an in vitro system for examining acetaminophen toxicity has been developed by using human lymphocytes and mouse microsomes (Spielberg 1980). A similar experiment was also established for testing cytotoxicity of antiepileptic drugs. Rabbit microsomes were prepared for lymphocytes viability study. Recently, high-throughput screening (HTS) technique has widely used in pharmaceutical industry field. The HTS techniques are focused on biotransformation

testing of a variety compounds. One distinguished example was reported by using array system for drug metabolism testing (Lee *et al.* 2005). They have developed a miniaturized three-dimensional (3D) cell-culture array (datachip) for HTS. CYP450 were encapsulated with sol-gel and several prodrugs (i.e. cyclophophamide and Tegafur) were added onto each spot and cytotoxicity of target cells was tested by overlapping with"data chip and metachip". This system was further improved with hydrogel-encapsulated cells, the results were obtained from datachip and metachip system which are also comparable to conventional 96-well plate assay. The CYP enzymes are easily manipulated for the metabolic clearance of a drug. The disadvantage of microsomes system is that all cofactors required for the CYP enzymes must be added during incubation, and also the viability of liver cells can not be detected. Hepatotoxicity is one of the most common adverse drug reactions during drug induced process (Kaplowitz 2005). A variety of drugs may be transformed and bio-activated by liver cells, and drug metabolism is thought to be involved in the toxicity of many target cells.

When the cells are placed in the monolayer condition, cells toxicity could be more sensitive to a small amount of drugs. Due to human tissue and cell morphology is three dimensional and spheroids, monolayer culture condition is not authentic of the hepatocyte toxicity test (Dhiman *et al.* 2005). Some papers have demonstrated that cells growing in 3D culture to form spheroids culture are more resistant cytotoxic agents than cells in monolayer cell (Hoffman 1991). Two dimensional data might not provide enough information about viability and toxicity, which are important for cell LD_{50} (lethal dose, 50%) evaluation.

Isolated hepatocytes especially primary liver cells start losing their functions and viability within 3-4 days (Chia *et al.* 2000). To maintain viable and functional hepatocytes *in vitro*, several sophisticated culture systems have been used for cytotoxicity study and long-term cell toxicity screening. Some results have been shown that the function of hepatocytes can be increased when co-culture with nonparenchymal cells (Bhatia *et al.* 1998). A dual-compartment perfusion bioreactor was design in co-culture with hepatocytes and stellate cells (Wen *et al.* 2008). Hepatocytes and sellate cells were seeded in separate compartments of perfusion bioreactor. The functionality of hepatocytes was maintained at higher level such as albumin secretion and glucose consumption. In general, long-term metabolism promotion can be induced by co-culture. Another co-culture system could be carried out *in vitro* using mixed cultured of hepatocytes and nonhepatic target cells. The principle of this device is that metabolites formed by liver cells would be toxic for other target cells. These culture systems could be tested in static or perfusion conditions (Gebhardt *et al.* 1996).

So far, a variety of applications for drug screening, degradation ability and liver cells activity has been introduced. According to our chapter 2 results, we proved that pre-screening drugs using a HepG2 cell line encapsulated within 3D alginate systems is possible. The metabolism activity of CYP450 and GSH can be maintained at significant levels *in vitro* for 2 weeks. In this chapter, we have established an *in-vitro* 3D culture system that enables the culture of cells in relevant tissue-like cell densities (10^7~10^8 cells/cm^3), thickness of hydrogel is lower 0.8mm and also able to become a 2D-3D co-culture system for assessing potential cytotoxic effects of drugs and their metabolites toxicity *in vitro*. A biocompatible polycarbonate disk was designed and applied to a

mold and support for 3D culture. The EFC-HFC metabolism was examined by cell densities of 10^5~10^8 cells/cm^3 and cell viability was also monitored for three days. Different drugs of CT_{50} value such as diclofenac and acetaminophen (N-Acetyl-p-Aminophenol ; APAP) can be provided by encapsulated cells on a 10^7 cells/cm^3 cell density. For co-culture design, bio-activated drugs can be applied as a hepatotoxicity assay and target cells viability test. High cell density of HepG2 cells were encapsulated with alginate and cultured in cell medium with monolayer MCF-7 breast cancer cells in order to test drug hepatotoxicity and bioactivated activity simultaneously. A MCF-7 cell was cultured as indicator for cytotoxic effects of cyclophosphamide and acetaminophen. A range of drug concentrations were tested and the viability of MCF-7 was determined. The co-culture system uses liver cells as a drug activated platform and clearly has considerable potential for examining the effects of drugs and their metabolites on indicator cells derived from a tissue of choice. This co-culture design may be particularly useful in the assessment of metabolism and toxicity of new drugs intended for human use. This method not only improves our encapsulation process, but also offers the possibility of the testing of toxicity of liver cells/target cells and metabolites toward specific cell type at a very early stage of drug development.

3.2 Materials and methods

3.2.1 Drug compounds

Acetaminophen, rifampin, quinidine, cyclophosphamide and diclofenac were purchased from Sigma Aldrich (St. Louis, MO). Stock concentrations for drugs used were as follows: acetaminophen (40mM), cyclophosphamide (40mM), diclofenac (20mM), rifampin (100mM) and quinidine (60mM).

3.2.2 Culture of HepG2 and MCF-7 cells

HepG2 were obtained from ATCC (American Type Culture Collection ATCC, Manassas, VA), passage 77. Hepatocytes were harvested between passage numbers 77 to 80 for all experiments. DMEM medium and fetal bovine serum (FBS) was purchased from Invitrogen (Carlsbad, CA). The cells were maintained in standard Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen Co., Carlsbad, Calif., USA) and 1% penicillin G and streptomycin (Invitrogen). The cells were grown in 75cm^2 tissue culture flasks at 37°C in a 5% CO_2 humidified environment. At confluence, cells were washed with phosphate buffered saline (PBS), treated with 0.25% Trypsin/EDTA (Invitrogen) for 5 min to release cells from the flask, pelleted by centrifugation at 1500 rpm for 5 min and finally re-suspended with fresh medium to the desired cell density. MCF-7 human breast cancer cells (ATCC, Manassas, VA), passage 147 were grown in DMEM supplemented with 10% fetal bovine serum, 1% penicillin G and streptomycin and 0.01mg/ml bovine insulin (Sigma, I-1882). After trypsinization, the cell suspension was then transferred to 12-well plates (BD Falcon., Bedford, MA, USA) with cell density on each well was around 10^5 cells/ml, the plate was incubated for 1 day in CO_2 incubator for further co-culture study.

3.2.3 Support disc design and fabrication

A porous polycarbonate disc was designed to fit within a standard 12 well plate (Figure 1). The disc (15 mm diameter disc with circular pores approximately 600 µm wide) was fabricated using a Fused Deposition Modeling (FDM) system (Stratasys, Inc, Minneapolis, MN). The FDM system extrudes the desired material based on a digital

3D model of the disc. Several publications describe the FDM process in detail and will not be covered here (Centola *et al.* 2010). The porosity of the disc is attributed to two factors, one being the intended designed circular pores and the inherent porosity (<1µm pore dimensions) within the material due to the additive nature of the FDM fabrication process. The base is elevated at a height of 1 mm away using support legs, which separates the disc from the monolayer culture of cells (MCF-7) at the bottom of the well plate. The alginate gel was designed to fit in the disc for 3D culture and 2D-3D co-culture studies. The support disc is autoclavable and reusable continuously for repeated experiments.

3.2.4 Alginate preparation and encapsulation of HepG2 cells preformed on the support disc

The hydrogel was prepared using Pronova SLG100 (NovaMatrix Co., Sandvika, Norway), mixed at a final concentration of 1% (w/v) with DMEM medium. HepG2 cells were cultured as described above, trypsinized, counted by a hemocytometer and resuspended in DMEM. A desired concentration of cells (10^5-10^8cells/ml) with the alginate solution (1%w/v) at a concentration of 1:1(v/v) is prepared. The procedure for the 3D hydrogel fabrication is described in Figure 2A. Initially, the support discs are sterilized using an autoclave and placed at the bottom of the 12 well-plate. Then, the cells-alginate solution (200µl) was pipetted onto the disc platform which contains a thin layer of 2.5% $CaCl_2$ cross-linking solution (Sigma. After incubation at 37°C for 5 min, a soft cross-linked alginate gel encapsulated with the desired concentration of HepG2 cells was formed. Each hydrogel is approximately 15mm diameter and 700±100µm thickness. After the crosslinking reaction, the alginate-disc was washed with PBS

(Invitrogen) for the removal of any un-crosslinked solution. About 1.5ml of cell culture medium was dispensed into the well plate which resulted in the medium to be slightly above the hydrogel.

(A) (B)

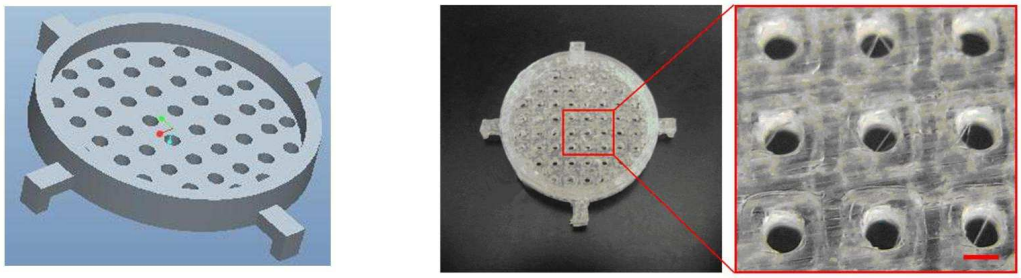


Figure. 1. Schematic of disc design and fabrication. (A) The porous support disc. (B) A magnified view of the poly-carbonate disc. Scale bar is 600 µm. The porosity of the disc is attributed to the designed pores within the base and the inherent porosity due to the additive nature of the FDM process.

3.2.5 De-crosslinking the alginate hydrogel

To de-crosslink the alginate hydrogel, samples were immersed in DMEM (containg 10mM EDTA) and incubated for 5 minutes at 37°C. The gel de-crosslinks back to the solution state and then spun down to retrieve the encapsulated cells. The cell pellets were then re-suspended in cell culture medium for analysis and characterization. Cells were counted by a hemocytometer with trypan blue staining (Invitrogen) because it is a direct method for measuring cell viability. Indirect methods such as LDH leakage and MTT reduction assays are based on metabolic activity and therefore may not authentically represent cell necrosis since low metabolic activity can contribute to higher toxicity.

3.2.6 Live/dead fluorescence viability testing

The viability of encapsulated HepG2 cells was quantified using the Live/Dead viability assay (Invitrogen) consisting of calcein-AM and ethidium homodimer. Hydrogels (SLG100) were formed as described above. On different time point, cell medium was aspirated from the well and the hydrogels were removed from the discs and washed with PBS twice for removing FBS in the hydrogel. Samples were protected from light and stained with 2μg ethidium homodimer and 0.5μg calcein-AM in 1ml PBS solution and incubated at room temperature for 45 min. After multiple washing with PBS, the viability of encapsulated cells was quantified from the z-series projections taken every 10μm and counted as the percentage of dead cells compared to the total number of cells in encapsulated samples, sections were taken using an Olympus BX61WI confocal microscope and software.

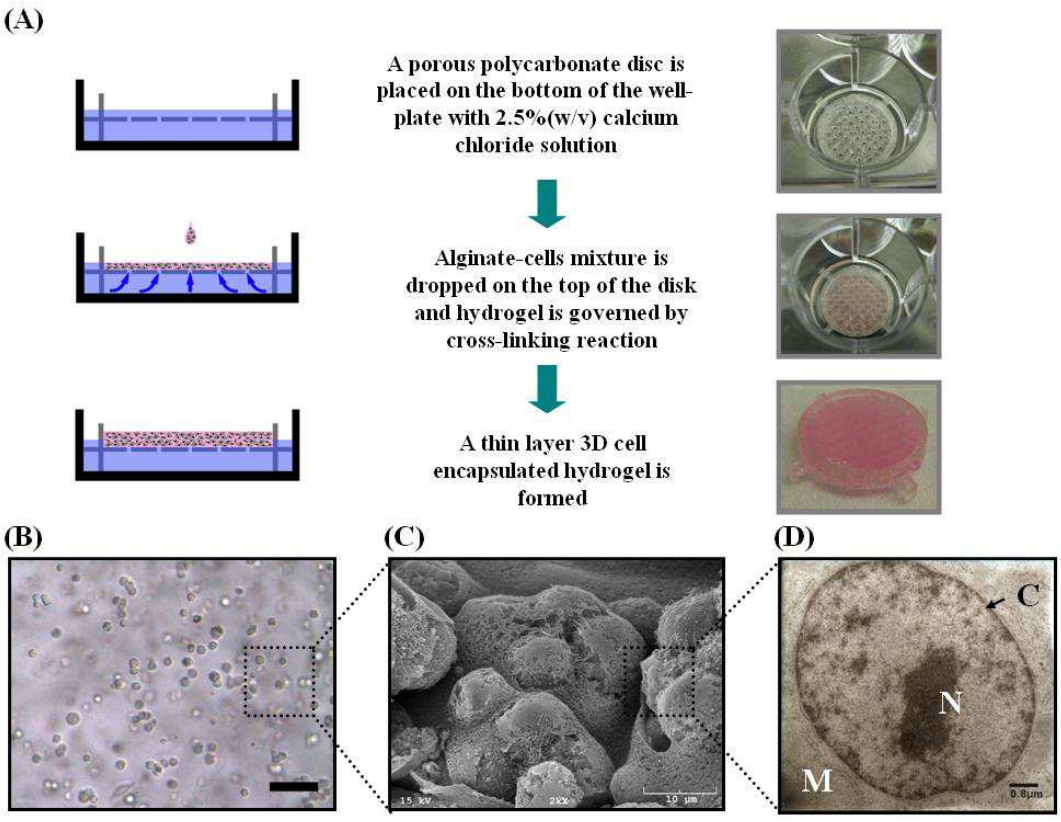


Figure. 2. Schematic of encapsulation method with support disc. (A) Encapsulated HepG2 cells within alginate hydrogels pre-formed on a support disc mold. (B) Phase-contrast micrographs of encapsulated cells. Scale bar 200µm. (C) SEM images of encapsulated cells. HepG2 cells were entrapped within the alginate matrix. (D) TEM images showing HepG2 cells encapsulated in SLG100 alginate. Typical cellular structure such as nuclei (N), cell membrane (C), encapsulated in alginate material (M) are shown. Scale bar 0.8µm.

3.2.7 Sample preparation for scanning and transmission electron microscopy

HepG2 cells encapsulated in alginate were fixed with 2.5% glutaraldehyde in coagulation buffer (0.1M) for 2 hours at 4°C. After washing in PBS, samples were immersed in 1% OsO_4 (aq.) solution for 60min. Samples were dehydrated in ethanol (30%, 50%, 70%, 90%, 100%, 100%, 100%) for 20 min, three times for each respective

ethanol change. For SEM preparation, samples were dried using critical point CO_2 (Tousimis Autosamdri-814). Dried samples were sputter-coated with gold/palladium for 15min using a sputter coater (Anatech Ltd Hummer VI) and viewed in a JEOL JSM-880 scanning electron microscope. For TEM preparation, the dehydrated samples were then transferred to in 25%, 50%, 75%, 100% in Epon-812 resin (Electron Microscopy Sciences, PA, USA) anhydrous alcohol for 1 day for each concentration. When solidified, ultrathin (70nm) sections of the resin embedded and stained with uranyl acetate (UA) for 20min and lead citrate for 5 minutes. Samples were analyzed using a Zeiss 10A TEM.

3.2.8 Pro-drug metabolism by HepG2 liver cells encapsulated within alginate

Non-fluorescent pro-drug EFC (7-ethoxy-4-trifluoromethyl coumarin, Invitrogen) was mixed with DMSO to create a 10mM stock solution of EFC. Drug substrate HFC (7-hydroxy-4-trifluoromethyl coumarin, Sigma Aldrich) was also mixed with DMSO to create a 10mM stock solution of HFC. HFC standard curve range was prepared from 1 – 64μM. Alginate hydrogel encapsulated with liver cells were incubated on the top of support discs with 120μM EFC. At each time point, the concentration of HFC was monitored with a fluorescence reader (FX800, Biotek) using an excitation wavelength of 360nm and an emission wavelength of 520nm.

3.2.9 Hepatotoxicity testing with 2D and 3D

Acute exposure (24hr) hepatotoxicity between 3D and 2D was tested using 4 model drugs: acetaminophen, diclofenac, rifampin and quinidine. For 3D samples, encapsulated HepG2 cells were seeded on the support discs as described above. For 2D monolayer culture, cells were routinely seeded in 24 well-plates at a density of 10^5 cells

in 0.5 ml medium per well and used 24 h later (75% monolayer confluence). Stock solutions of the drugs were diluted in culture medium for various concentrations. DMSO vehicle concentration was kept below 1% to prevent cell damage. The controls were prepared by diluting corresponding amounts of DMSO in culture medium without drugs. After drugs treatment for 24 h, the 2D and 3D samples cells were detached or de-crosslinked from monolayer and encapsulation samples by trypsinization or EDTA treatment, the cytotoxicity 50% values (the concentration at which produces 50% lethal effect on cells viability) were assessed using hemocytometer with trypan blue staining (Invitrogen). For the cytotoxicity 50% data, a fourth order regression analysis was carried out using Sigmaplot 10.0 (Chicago, IL, USA).

3.2.10 2D-3D hybrid co-culture method and drug effect test

In the case of hybrid 2D-3D co-culture, the second type of cell (MCF-7) was seeded at the bottom of well before the support disc containing the HepG2 alginate gels was placed. The 2D-3D co-culture process of the cells is presented on Figure 3A. For our study, the target MCF-7 cells were seeded on the bottom of the 12 well-plate as target cells (2D cell culture) and incubated for 1 day. Cell culture medium was added into each well and incubated at 37°C under a 5% CO_2 humidified environment. The co-culture system was periodically observed using a laboratory microscope to follow growth morphology. Both hydrogel and MCF-7 culture can be observed simultaneously at different focal planes without having to disturb the hydrogels (Figure 3B and 3C). To study the drug concentration effect and the MCF-7 toxicity for different hydrogel cell density, we developed two experiments for our study. For the first phase of the experiment, the 3D hydrogels were plated onto support discs with MCF-7 cells cultured

previously at the bottom of the well plates. Then, 4mM and 12mM acetaminophen were mixed into each well, followed by testing for the viability of hepatocytes and MCF-7 cells. Control samples were designed as 2D-3D co-culture without acetaminophen and MCF-7 was cultured alone with acetaminophen for the control tests of metabolized-acetaminophen effect. The viability of encapsulated HepG2 and MCF-7 was quantitatively determined by trypan blue staining method after incubation for 1 day. For the second phase, three different cell densities were used in this co-culture design. To study the relationship between MCF-7 viability and the cell density of encapsulated HepG2, three cell densities 10^7cells/ml, 10^6cells/ml and 10^5cells/ml were encapsulated within alginate and co-cultured with a mono-layer of MCF-7 cells. The viability of encapsulated HepG2 and MCF-7 was quantitatively determined by trypan blue staining method after incubation for 1 day. For cyclophosphamide testing, 4mM and 12mM cyclophosphamide were also mixed into each well, followed by testing for the viability of hepatocytes and MCF-7 cells.

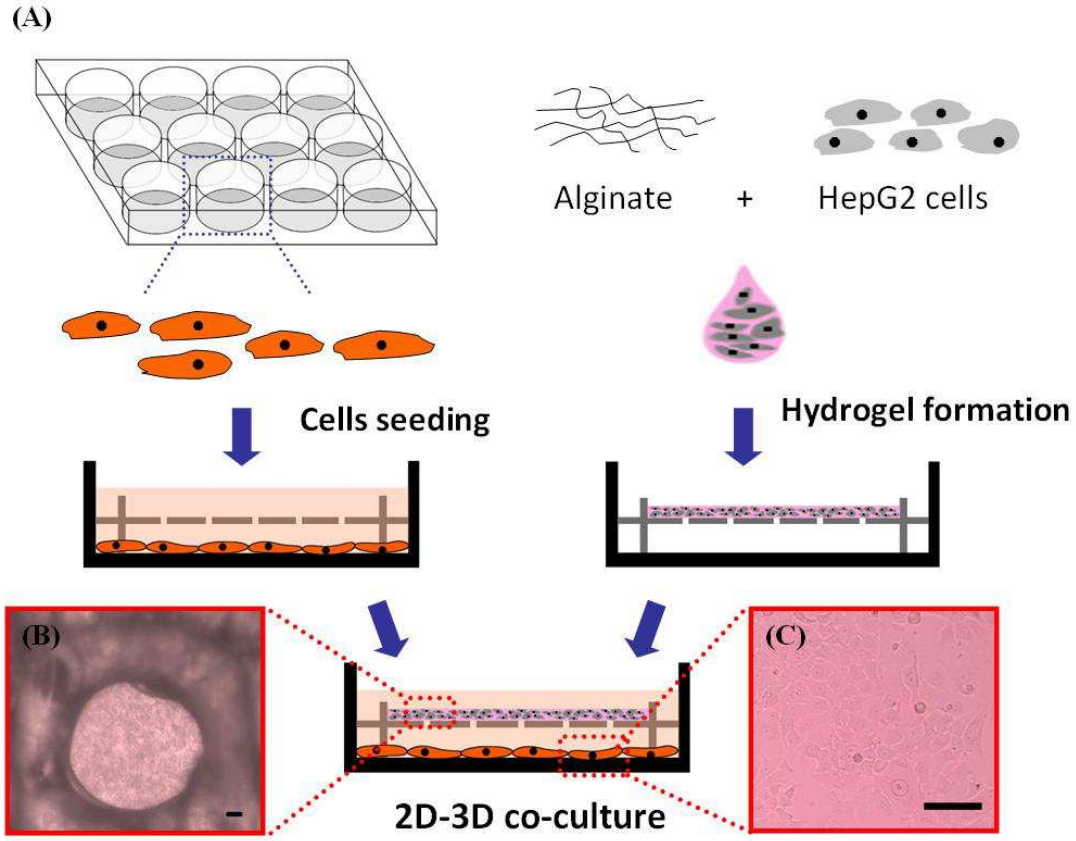


Figure 3. (A) The 3D hydrogels with disc were plated onto culture dishes where MCF-7 cells have been previously cultured. In this type of co-culture, the hepatocytes and MCF-7 have no cell-to-cell contact. (B) The morphology of HepG2 which can be observed from phase-contrast microscope. Scale bar 100µm. (C) The morphology of MCF-7 cells seeded on the bottom of the well plate. Scale bar 100µm.

3.3 Results

3.3.1 Cell viability during 3 day incubation study period

Stable hydrogels were prepared within the support disc with calcium chloride as the crosslinking agent. The morphology of the encapsulated cells is shown in Figure 2B-D. Cells encapsulated in the hydrogel display a spherical shape configuration with pockets of HepG2 aggregation and cell isolation within the gel. SEM image (Figure 2C)

indicates that cells were entrapped within the highly porous alginate and the morphology in the spheroids was similar that seen in *in vivo*. TEM (Figure 2D) picture also shows that cell membrane were totally covered by alginate matrix and still maintain the spheroid morphology.

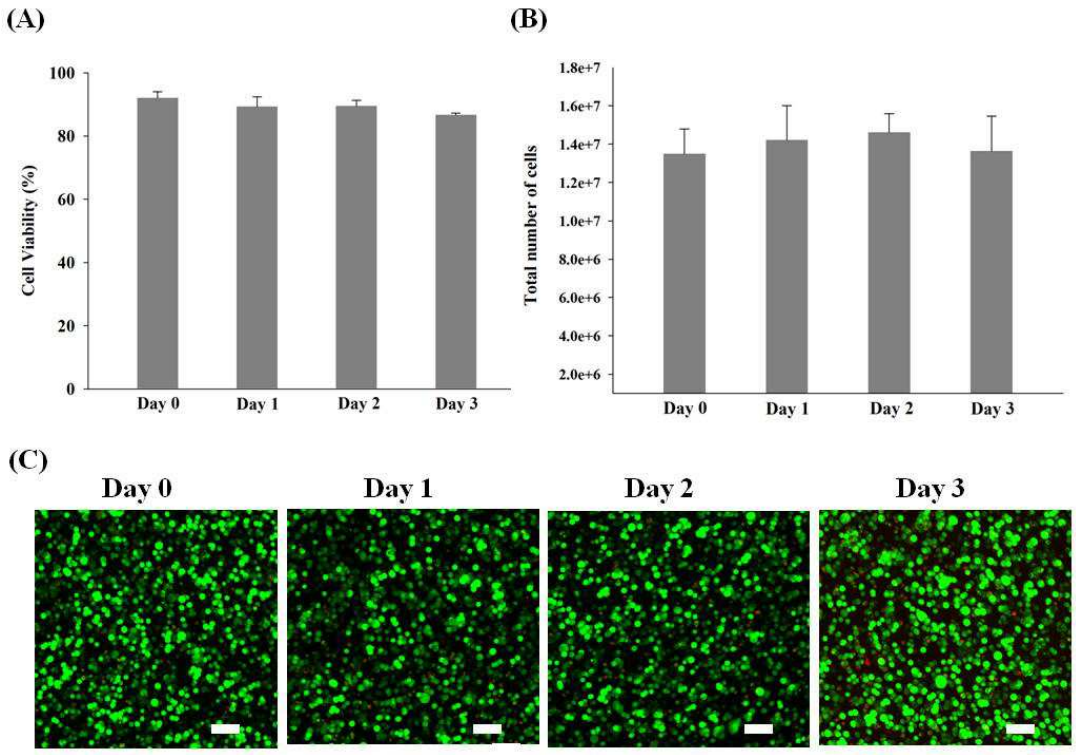


Figure. 4. (A) Cell viability of HepG2 cells grown on 3D for 3 days (Initial cell density: 10^7cells/ml). Encapsulated HepG2 viability was maintained over 80 ± 4% for three days. (B) The total number of live HepG2 cells for 3 days. (C) Encapsulated HepG2 cells were stained with Calcein AM and EthD-1 at Day 0 to Day 3 in 3D hydrogels with an imaged central thickness section of 500μm. Scale bar 100μm.

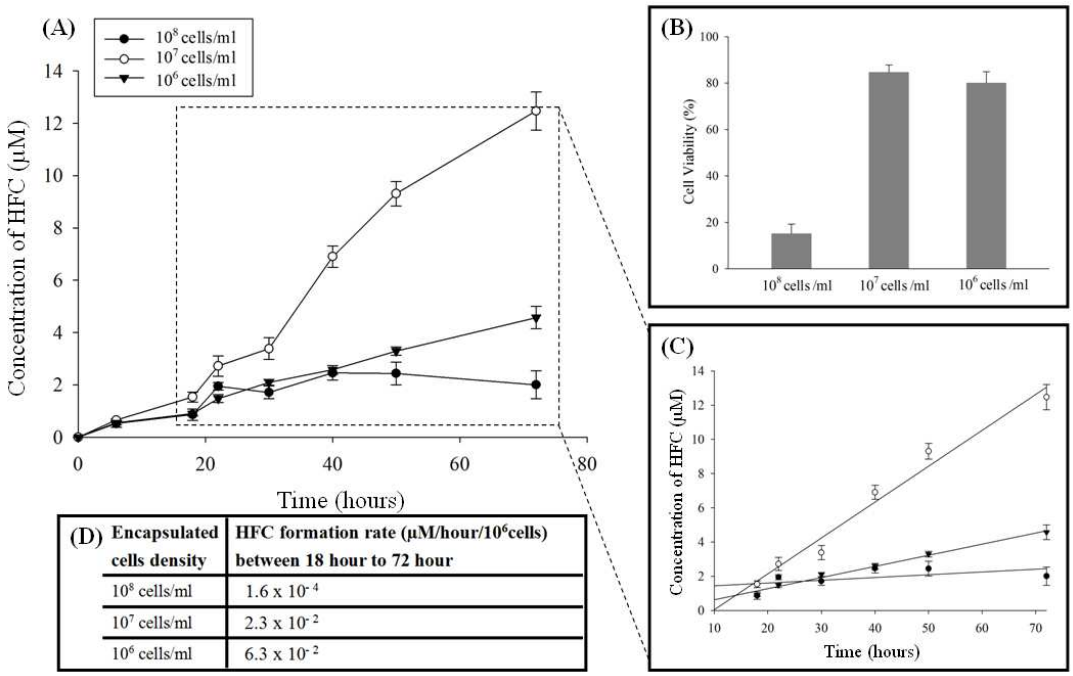
To determine the cell viability and proliferation activity in hydrogels, the cell viability of HepG2 (10^7cells/ml) encapsulated in the alginate hydrogels was studied over a 72hr period using the test platform. Cell culture medium was not refreshed (1.5

ml per well) during this period to ensure that enough cellular viability was maintained during the drug metabolism and toxicity study. As shown in Figure 4A, around 80% cells remained viable indicating that the culture conditions supported the highly dense culture of HepG2 cells within the 3D hydrogel. Live/dead assay was also used to qualitatively and quantitatively indicate the viability of the cells within the gel. Figure 4C shows the live/dead confocal projected images within a central section of 500μm thickness to test for nutrient diffusion limitations. The 700μm thick hydrogel was optically sectioned by 100 layers and each slice was observed for cell viability and distribution. The images indicate over 80% viability of cells over the 3day period and these results mirror data shown in Figure 4A-B. Dead cells were uniformly distributed throughout the sectional slices and were not significantly higher in the mid-section of the hydrogel as compared to other areas of the gel. This data indicates that diffusion of nutrients did not play a major role in cellular death. Data in Figure 4B also indicates that there is limited proliferation of HepG2 cells within the alginate hydrogel matrix.

3.3.2 Drug metabolism by the in vitro models for different cell densities

To determine the toxic effects of the drug and its metabolic products on the cells, a 72hr study period was selected. In the first set of drug elimination experiments, clearance rates of the pro-drug 7-ethoxy-4-trifluoromethyl coumarin (EFC) to 7-hydroxy-4-trifluoromethyl coumarin (HFC) was studied for three different HepG2 cell densities. An initial concentration of 120μM of EFC was mixed with the cell culture medium and the concentration of the metabolic byproduct HFC and cell viability was recorded over the study period of 3 days (Figure 5A). Regression curve of HFC formation rate was calculated from 18 to 72 hours (Figure 5C). Figure 5D shows the

HFC formation rate for the 3 alginate gels with different cell densities. As shown, the rate of HFC formation ($\mu M/hr/10^6$ cells) was the highest for alginate with the HepG2 cell density at 10^6cells/ml, but the final concentration of HFC at Day 3 (Figure. 5A) for samples with 10^7cells/ml cell density was much higher than other two cell densities (10^6cells/ml and 10^8cells/ml). Figure 5B indicates that the rate of HFC formation at 10^8cells/ml cell density drops down due to the corresponding loss of cell viability during the 72hr period. This is expected since at such super high density numbers, a static system may be inadequate in providing sufficient nutrient diffusion for the current hydrogel slice disc. A dynamic culture system with continuous closed recycling of medium is necessary for cell densities that mimic *in vivo* environment. In consideration of cell viability, HFC formation rate and the final concentration of HFC formation during time, 10^7cells/ml cell density is better than other two cell densities for *in vitro* testing under static condition due to high cell viability (~80%) and an adequate response time during three days.



(D)

Encapsulated cells density	HFC formation rate (µM/hour/10^6cells) between 18 hour to 72 hour
10^8 cells/ml	1.6×10^{-4}
10^7 cells/ml	2.3×10^{-2}
10^6 cells/ml	6.3×10^{-2}

Figure. 5. (A) Drug response rate in 3D samples for three different cell densities (10^6 cells/ml, 10^7 cells/ml and 10^8 cells /ml). (B) The cell viability of HepG2 cells grown on 3D for three different cell densities at day 3. (C) HFC concentration curve from 18 to 72 hours with linear regression analysis. (D) The HFC formation rate for three cell densities.

3.3.3 Hepatotoxicity testing with 3D culture design

The liver plays a major role in transforming and clearing chemicals within the body and is therefore susceptible to the toxicity from chemical compounds. We tested our *in vitro* model platform and traditional mono-layer cell culture for hepatotoxicity results with four known commercially available drugs. The cell viability was observed in 3D platform cell culture system as compared to those grown in the monolayer system. Since our previous results have indicated an optimal 10^7cells/ml density, this has been used for all subsequent studies. HepG2 cells were encapsulated and cultured

on the porous support disc for 24 hours prior to treatment at different concentration of drugs. Serial concentrations of drugs were prepared in culture medium and treated with 3D hydrogel samples encapsulated with HepG2 cells and monolayer system. Their hepatotoxic effect was evaluated by quantifying cell viability (Figure 6). In the *in vitro* toxicity data collected, the CT_{50} for the four drugs in 2D and 3D system are indicated in Table 1 and compared with *in vivo* LD_{50} results from rats. The CT_{50} values in 2D culture system were higher than 3D samples when treated with acetaminophen and diclofenac. These results indicate that HepG2 cells encapsulated in 3D hydrogel shows increased sensitive to the model compound of acetaminophen and diclofenac. Similar dose dependent responses and CT_{50} values were obtained in 2D and 3D using rifampin and quinidine. Two linear regression curves between 2D/3D CT_{50} values and LD_{50} values were obtained (Figure 7). The correlation between CT_{50} values derived using 3D platform system correlated well with the reported in vivo LD_{50} values (Paillard *et al.* 1999; Toh *et al.* 2009; Wishart *et al.* 2008), on 3D culture ($R^2 > 0.97$) was better than that obtained with 2D mono-layer cell culture ($R^2 < 0.86$). These results indicate the encapsulated HepG2 cells toxicity respond appropriately when exposed to the drug compounds and also can be correlated to *in vivo* toxicity.

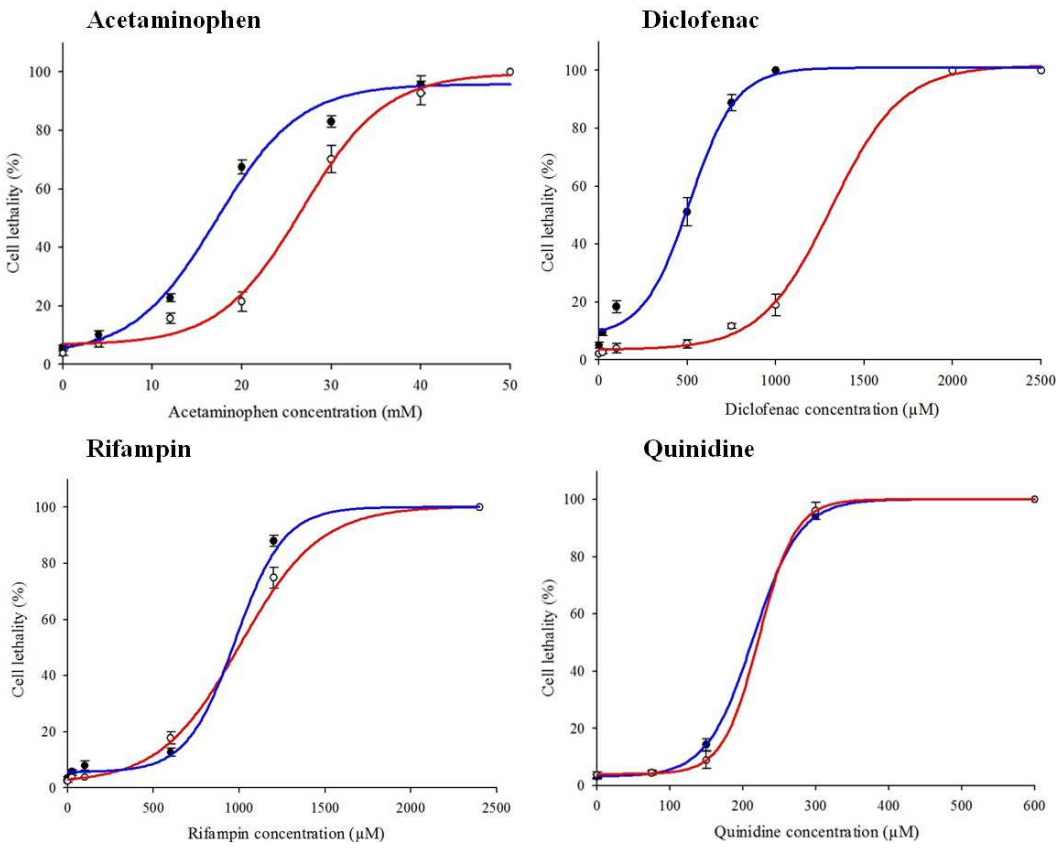


Figure 6. Concentration–response HepG2 cell cytotoxicity curves for the acetaminophen, diclofenac, rifampin and quinidine. Blue lines show the cytotoxicity regression curve of HepG2 encapsulated 3D alginate hydrogels (HepG2 Cell density: 10^7cells/ml). Red lines show the cytotoxicity regression curve of 2D samples. See Table 1 for CT_{50} dose values of the regression analysis for each curve.

Table 1. Cytotoxicity 50% dose values (CT_{50} dose) calculated from the 3D culture and published LD_{50} values for 4 model drugs.

Drugs	**3D culture CT_{50} dose (mM)**	**2D culture CT_{50} dose (mM)**	**LD_{50}** (mmol/kg) (Paillard *et al.* 1999; Toh *et al.* 2009; Wishart *et al.* 2008)
Acetaminophen	17.3	26.1	14.01
Diclofenac	0.48	1.3	0.33
Rifampin	0.97	1.01	1.4
Quinidine	0.21	0.22	0.24

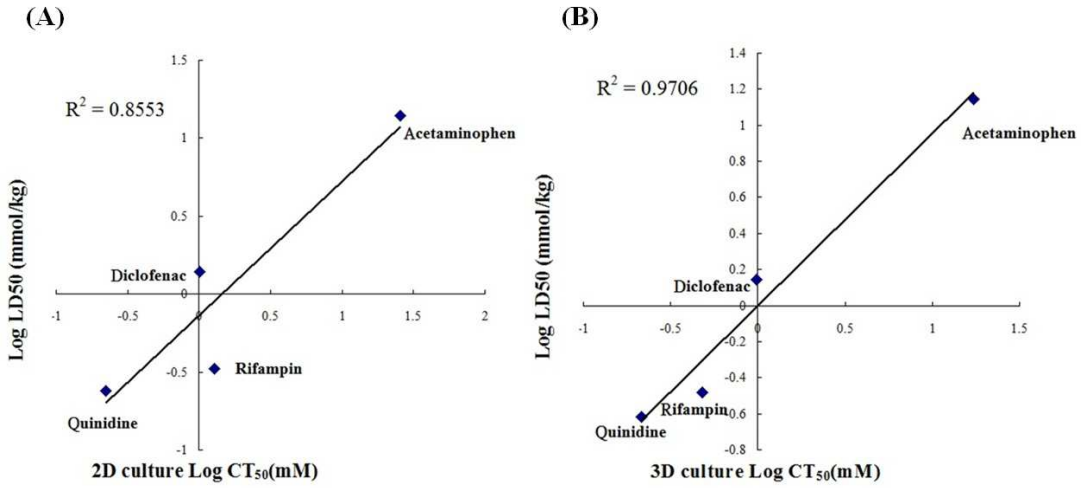


Figure 7. Correlation of CT_{50} values calculated from the 2D mono-layer cell culture and 3D encapsulated cells to reported LD_{50} values in rats. (A) A linear correlation between the 2D culture CT_{50} and LD_{50} values (R^2 = 0.8553). (B) A linear correlation between the 3D culture CT_{50} and LD_{50} values (R^2 = 0.9706).

3.3.4 Drug effect study on 2D-3D co-culture

The previous studies showcase that the encapsulated HepG2 cells within alginate at certain cell density responded to the serial concentration of commercial drugs. The proposed design of the disc platform enables the simultaneous study of drug compounds on target cells within a co-culture setting. In this experiment, we have studied the effect of acetaminophen on MCF-7 cellular viability. Specifically, this model was used to reveal that liver metabolized drug such as metabolized acetaminophen stimulates target cells viability. Acetaminophen is a common analgesic drug which is oxidized to the toxic N-acetyl-p-benzoquinone-imine (NAPQI) by cytochrome P450, and this compound results in detrimental effects on liver cells and on other cell types (Bender *et al.* 2004; Hazai *et al.* 2002). In general, MCF-7 has resistance to acetaminophen treatment because it does not have caspase-3 protein that triggers acetaminophen-induced apoptosis (Boulares and Ren 2004), but MCF-7 can be damaged under metabolized-acetaminophen compounds such as NAPQI (Lee *et al.* 2005).

To study the drug concentration effect and the MCF-7 toxicity for different cell density, we developed two experiments for the study. From Figure 8A, when MCF-7 cells were cultured in the absence of encapsulated HepG2 cells with 4mM and 12mM concentration of acetaminophen, the viability was over 92% which indicate that MCF-7 has a resistance to acetaminophen treatment. After 4mM and 12mM drug treatment with 2D-3D co-culture samples that includes encapsulated HepG2, the viability of MCF-7 dropped to around 71%. These results indicate that our co-culture platform with encapsulated HepG2 cells metabolized acetaminophen leading to MCF-7 loss of cell

viability. The viability of MCF-7 was not dependent on the concentration of acetaminophen due to a significant loss in HepG2 cell viability (from 87% to 62%) at higher acetaminophen concentrations. Both concentrations at 4mM and 12mM led to similar loss of MCF-7 viability. This result is similar to the CT_{50} dose values seen in Figure 6.

For the second phase experiment, three different cell densities were used in this co-culture design. To study the relation between MCF-7 viability and the cell density of encapsulated HepG2, three cell densities 10^7cells/ml, 10^6cells/ml and 10^5cells/ml were encapsulated within alginate and co-cultured with a mono-layer of MCF-7 cells. Figure 8B revealed that when MCF-7 cells are co-cultured with higher cell density of HepG2, this leads to higher toxic effects on MCF-7. Control samples did not show significant loss of viability, thus indicating the combined need for higher cell density and presence of HepG2 cells to render acetaminophen effective against MCF-7 cells. These results also indicate that the drug interaction between two types of cells can be studied using the co-culture system.

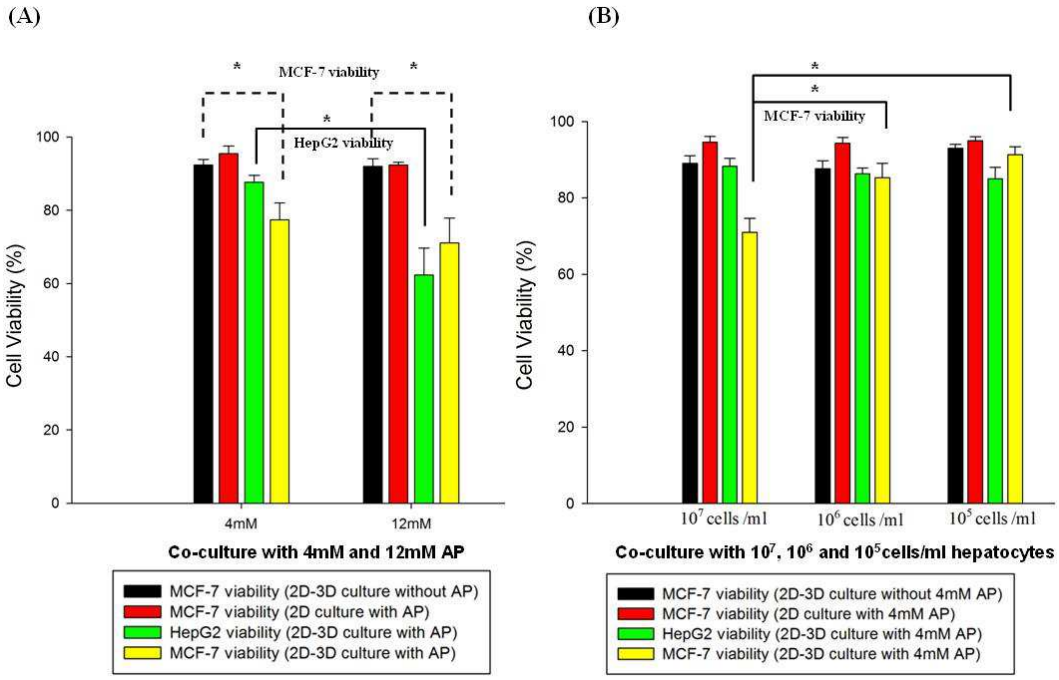


Figure 8. Results from operation of 2D-3D co-culture with monolayer MCF-7 and encapsulated HepG2. (A) Comparison of viability response after 4mM and 12mM acetaminophen incubation for 1 day. Dashed line (*) shows the comparison of MCF-7 viability. Solid line (*) showed the comparison of HepG2 viability. (B) Comparison of viability response with co-culture with different HepG2 cell density. MCF-7 viability decreased when encapsulated HepG2 cell density increased. Solid line (*) showed the comparison of MCF-7 viability. Data represent the mean ±STDEV for three independent repeats. The (*) indicates $p < 0.05$.

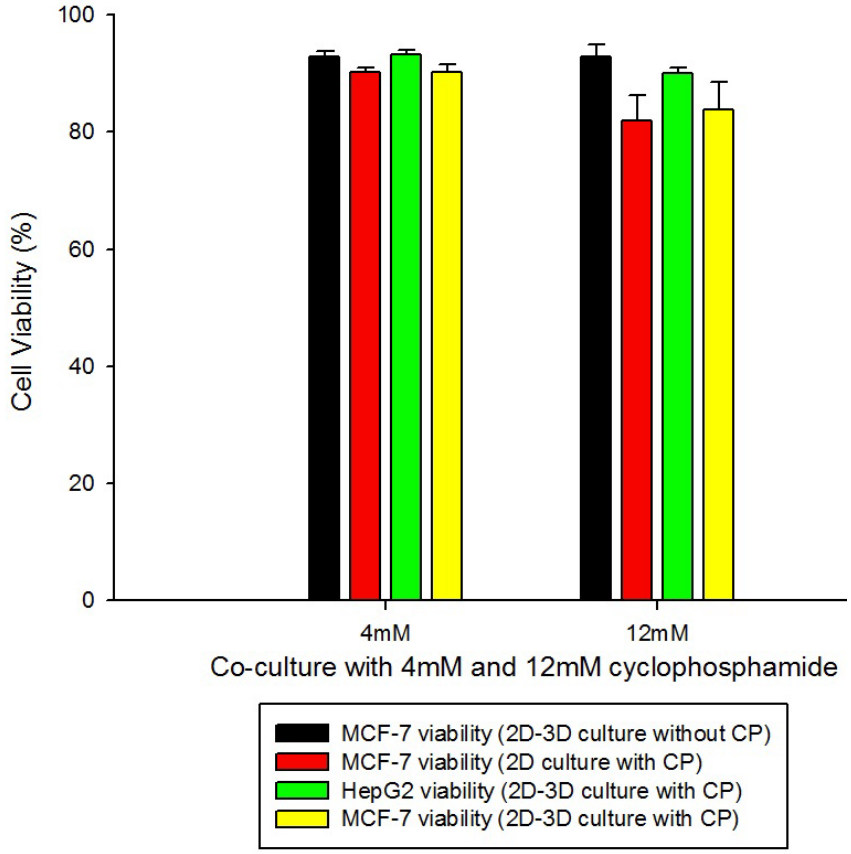


Figure 9. Results from operation of 2D-3D co-culture with monolayer MCF-7 and encapsulated HepG2. Comparison of viability response after 4mM and 12mM cyclophosphamide incubation for 1 day.

After 4mM and 12mM cyclophosphamide treatment with 2D-3D co-culture samples that includes encapsulated HepG2, the viability of MCF-7 was no significant drop when compared with the control (Figure. 9). These results indicate that our encapsulated HepG2 don't highly metabolized cyclophosphamide and kill MCF-7 breast cancer cells. The viability of MCF-7 was also not dependent on the concentration of cyclophosphamide due to a minor loss in HepG2 cell viability (from 92% to 81%) at higher cyclophosphamide concentrations and low metabolism rate of cyclophosphamide.

3.4 Discussion

Compared to traditional 2D cell culture, 3D culture can provides culture environment that is more physiologically similar to native tissue (Vukasinovic *et al.* 2009). Recent research has demonstrated that 3D culture show different metabolic activities and expression profiles compared to mono-layer culture. This expression profiles and more authentic culture condition can provide better reflect cells behavior such as toxicity in their native environment (Fischbach *et al.* 2007). Our study is focused on the development of new platform for 3D culture which can also specifically incorporate the 2D cell culture for drug candidate compounds screening and testing. Many novel designs have been published from recently years but unfortunately tedious process and expensive equipments still limits the practicality. In this chapter, our design greatly simplifies the handling process and also provides a method which is compatible with existing well-plate system. This study confirmed the following: (a) 3D culture combines a support disk can provide a well *correlate hepatotoxicity data with the reported* in vivo LD_{50} values and (b) Metabolized drug effect can be studied and examined under 2D-3D co-culture.

3.4.1 Fabrication of 3D hydrogel with support disc

Co-cultures with cell culture inserts have been widely used to study two types of cells interactions such as local proliferation response or tumoral development (Gache *et al.* 1998; Uyama *et al.* 2002). In this study we have developed a unique porous support disc which is similar to cell culture inserts, but has more potential applications. To the best of our knowledge, none of the cell culture inserts are reusable and customized (e.g. Millipore or BD^{TM} culture insert). Our design can be easily designed and modified by

Pro Engineer or other software and manufactured by biocompatible polycarbonate material. This final product can be sterile by autoclave and alcohol solution for experiment usage. The disc design can be placed into regular well-plate and provide a solid support for 3D cell culture. Spin coating apparatus have been widely used for a ring-shaped or a thin layer of hydrogel preparation (Jacchetti *et al.* 2008). For 3D hydrogels preparation, spin coating requires a clean room to prevent contamination of the samples that might not be available for every lab. For our fabrication process, porous support disks were placed in well-plate with calcium chloride solution and alginate-cells mixture was added on the top of the disk to form 3D hydrogels. During this crosslinking reaction calcium chloride can be evenly placed on the disk and diffuse uniformly from the porous surface to hydrogels. A thin and evenly hydrogel was made on the top of support disc without using spin coater or custom made cutter.

3.4.2 Comparison with other cell-based 3D culture

Methods for 3D hydrogel fabrication often involve specialized equipment and not likely to be widely used in the biological community, such as lithography equipment for photopatterning (Liu, V *et al.* 2007) and microarray systems for high-throughput testing (Lee *et al.* 2005). In this paper, we developed an accessible and standardized tool for making 3D cultures for drug testing. Photolithography is standard equipment for labs but when this technique is used for making 3D cultures, especially with cells, access to clean room or compatible bio-hood is necessary and limits the handling. For our study we provide an alternative way of standard fabrication process that is capable to generate a thin hydrogel for drug testing. The fabrication of 3D cultures on support disk is convenient for handing and easy to transfer to other well-plates for co-culture

experiment. A wide variety of disc can be customized and scaled down for 24 or 48 well-plate. The manufacturing process and material prices cost less than $30 for each disk. Versatility of cell types and material (e.g. Matrigel ^{TM}, PEG) also can be mixed and deposited on this disc, depends on the research material. The ability to produce large quantities of samples and can be utilized by liquid handing machine for automatic screens (Tung *et al.* 2011) or cell printing for making pattern hydrogel (Varghese *et al.* 2005).

3.4.3 3D culturing

Large sized hydrogels are not suitable for 3D culture because (a) large size hydrogels need more cells and materials for preparation, (b) not easily integrate with common biology products, and (c) contribute to oxygen or nutrients diffusion problems. Unlike native tissues have vasculature to support nutrients and remove waste, 3D hydrogels only allow medium and oxygen diffuse into hydrogels for cells surviving. Many papers have calculated that if oxygen is a limiting factor for growing cells in 3D culture, then the size of fabricated modules with a thickness lower than 200μm will not be limited by nutrient transport, under this condition the cells can be maintained around 10^8-10^9 cells/cm^2 (McGuigan *et al.* 2008b; Nomi *et al.* 2002). For our 10^7 cells/ml 3D culture samples, not many dead cells were observed in hydrogel with thickness larger than 200μm.

Oxygen and nutrients are important for cell survival in thick tissues, but in most cases, cell death within the thick hydrogel is caused by hypoxia rather than lack of nutrients(Choi *et al.* 2007). In order to improve the oxygen supply to our hydrogel, we generated a porous and relative thin alginate for our 3D culture. According to our

viability results by confocal microscopy, we don't find any significant dead cells distributed in top, bottom or middle hydrogel. Yanagawa et al, have established a equation which can evaluated the maximum alginate-based hydrogel thickness for cell survival (Yanagawa *et al.* 2011). For our condition we estimated the diffusion of oxygen in the hydrogels based on Fick's diffusion laws. Confocal results showed that encapsulated cells were distributed uniformly within hydrogel, so a spatially uniform oxygen concentration gradient was assumed. In addition, we also assumed a constant external concentration of oxygen, constant oxygen consumption rate (OCR) and a steady-state system with diffusion. Calculation was done based on the assumption that cells would die at zero oxygen concentration at the bottom of hydrogel.

The maximum hydrogel thickness for cell survival (A [cm]) can be given as follows:

$$A = \sqrt{\frac{Do_2 \cdot Co_2}{n \cdot OCR}} \qquad (1)$$

Nomenclature:

Do_2 : Diffusion coefficient of oxygen in the alginate (cm^2/s)

Co_2 : Concentration of oxygen in the medium (mol/cm^3)

n : Density of cells ($cells/cm^3$).

OCR : Oxygen consumption rate (mol/cell/sec)

The thickness of the hydrogels packed with HepG2 cells was estimated to be 0.6~0.85mm at $2x10^6$ cells/ml cell density (chapter 2 static culture) and 0.7mm at 10^7 cells/ml cell density (chapter 3 static culture with support disc). From eq. (1), $Do_2 = 1.5x10^{-5}$ cm^2/s (Hulst *et al.* 1989), $Co_2 = 2.14x10^{-7}$ mol/ cm^2 (Provin *et al.* 2009), OCR values of encapsulated HepG2 is $2x10^{-16}$mol/s/ cell (Mishra and Starly 2009).

From previous results, there were no significant differences in the viability of encapsulated cells among the top, middle, and bottom layers. For above prediction, alginate thickness 2400 µm at the cell density of 2 x10^6 cells/mL and 1100µm at the cell density of 10^7 cells/mL. This result indicated that our hydrogel can maintain the viability of encapsulated cells under high cell density for different thickness. HepG2 cells is a high oxygen tolerance hepatoma cell line, 50% viability can be maintained under anaerobic condition during 3 days (Kim *et al.* 2007) and even during 6 hr of anaerobic incubation without additional substrate,viability of HepG2 cells was not significantly affected(Hugo-Wissemann *et al.* 1991). Although this thickness of the hydrogel construct seems to be much less than prediction, thicker hydrogels may still cause encapsulated cells under hypoxia condition. Normally, the oxygen uptake rate (OCR) of encapsulated cells decreases with increasing cell concentration (Mishra and Starly 2009), also the decline of OUR can be attributed to the higher cell density because of the reduction in diffusive flux (Provin *et al.* 2009). The OCR value for encapsulated cells was measured under 10^5 cells/ml cell density, so we can assume that the OCR for our higher cell density will be lower than this value, although the thickness used here is not very thin (>200µm) to eliminate oxygen diffusion problem, but viability of encapsulated cells can be maintained without apoptotic cell death induced by hypoxia.

We also observed that alginate material has limited encapsulated HepG2 proliferation activity, the total number of cells was almost the same as initial. Some possible explanations of is that (1) Encapsulated cells were entrapped in alginate and didn't proliferate, so low oxygen and nutrients still can provide enough support that

enables cells to remain viable; (2) 3D cultures were placed on the support disks and close to the medium level, so the nutrients and oxygen can diffuse into hydrogels from each direction; (3) During 3D cultures cultivation we did not refresh the medium during three days, we put 1.5ml volume of medium into each well to substitute refreshing. Large amount of medium has more serum for cells extent their viability. (4) The concentration of our alginate hydrogel is 0.5% which is relative soft compared to other papers material such as PEG or fiber scaffold (Sumaru and kanamori 2004). (5) Encapsulated cells density was lower than 10^8/ml. In order to prove the influence of different cell density, we made 10^8/ml 3D cultures and monitored the viability for three days, the viability dropped to 15% compared to initial (data not shown). This experiment proved that diffusion is still a problem when you culture over a critical number.

3.4.4. Drug metabolism by different density of encapsulated cells

To characterize the metabolic clearance rate of a drug candidate through our samples, we have used the pro-drug EFC for testing 10^8/ml, 10^7/ml and 10^6/ml cell density of 3D cultures. In our study we measured the fluorescence intensity of HFC production after treatment with 120μM of EFC in the medium. All samples for each cell density were able to convert EFC to HFC over 3 days and also the HFC emission peak was quite linear from 18-72 hours time period. Interestingly, the HFC formation rate in 10^6/ml samples was larger for the 10^8/ml and 10^5/ml samples. When compared with the viability of each sample after three days, 10^8/ml samples dropped to 15% after three days but 10^7/ml and 10^8/ml samples still remained viable for over 80%. That result can provide an explanation as to why HFC formation rate in 10^7/ml and 10^6/ml samples was

much higher than 10^8/ml, which was possibly due to the reduction of the viability within the 3D cultures. On the other hand, 10^7/ml samples HFC formation rate was also higher than 10^8/ml possibly due to the higher cell viability. High drug conversion rates can be achieve by high encapsulated cell density has been proofed (Chang *et al.* 2008a). However as pointed out before, cell viability, metabolic activity and nutrient diffusion problems should be also considered for a 3D cultures assay system.

3.4.5. Hepatotoxicity study in 3D cultures

3D cultures are being used in the prediction for hepatotoxicity study (Yamada and Cukierman 2007). Several research papers have shown that culturing cells in 3D environment may increase the drug resistance ability (David *et al.* 2008; Horning *et al.* 2008) or increase the sensitive of drugs compared in 2D culture (Nakamura *et al.* 2011). In addition, Gurski (Gurski *et al.* 2009) has also pointed out that culturing cells in 3D matrices for anti-cancer drugs testing was superior to traditional 2-D culture due to tumor morphology can be represented in 3D culture.

These sensitivity differences in 3D cultures may be representative of drug treatment in *in vivo* conditions. For our results, cell viabilities of HepG2 cells grown in 3D culture systems and exposed to different concentrations of acetaminophen and diclofenac were significantly lower than those of cells grown in monolayer culture and exposed to the same concentrations. Acetaminophen, a commonly used analgesic, is known to cause hepatotoxicity when ingested in large quantities in humans. Acetaminophen can be biotransformed by cytochrome P450 (P450) enzymes, that are known to such as CYP1A, CYP2E and CYP3A, and cause cellular necrosis (Zhang *et al.* 2004). High CYP450 activity results in increased acetaminophen toxicity

(Kostrubsky *et al.* 1997). In Chapter 2, we have demonstrated CYP450 activity of HepG2 cells grown on 2-D and 3D. The main CYP450 enzyme, CYP3A4, on 3D was almost higher than 2D during 2 weeks (Chaper 2, Figure 8). HepG2 cultured on the 3D alginate show more sensitivity towards acetaminophen-treated hepatotoxicity than hepatocytes cultured on 2D cell culture. The 'amplified effect of hepatotoxicity' of the acetaminophen treatment on 3D might be due to the higher enzymatic activity of CYP 450 enzymes.

The differences in diclofenac toxicity observed in 2D and 3D culture could reflect the different sensitivity of hepatocytes. Diclofenac (0.75mM) for 24 h was almost low-toxic to the 2D monolayer (survival ratio of 88%) but highly toxic to the 3D spheroids (survival ratio of 11%). Liver spheroid culture has been widely used for cytotoxicity evaluation due to maintenance of native morphology. For 3D culture, the hepatocyte structural and enzymatic functions are similarity to the *in vivo* conditions(Xu *et al.* 2003). The trend between CT_{50} values obtained using the 3D cultures was similar to *in vivo* LD_{50} values and also correlated better than 2D mono-layer cell culture. A larger dataset of drugs for testing by our 3D culture system will be required in the future as a fully validated *in vitro* prediction model. This indicates the applicability of the 3D construct as a hepatotoxicity test platform and also showed good predictions in hepatic cytotoxicity. The platform can enable the encapsulation of any relevant cell line besides HepG2, since each cell type is configured to express certain protein levels. This can include primary cell lines and variations of the cancerous cell lines (HepG3A, HepLiu, HepRG) for incorporation into the alginate matrix, depending on the specific study.

3.4.6. 2D-3D co-culture for drug effect testing

The efficacy of our 2D-3D co-culture system to test the drug effect was methodologically evaluated in two steps, dose and cell density dependent interaction studies. As designed, the acetaminophen dose dependent interaction experiment was determined by MCF-7 viability. N-acetyl-p-benzoquinone imine (NAPQI) has been investigated a toxic byproduct produced during the xenobiotic metabolism of the acetaminophen (Dahlin *et al.* 1984). In order to study the acetaminophen toxicity and byproduct effect for liver or other type of cells, normally acetaminophen and NAPQI were directly added into the cell medium and treated with cells for different concentration or time period (Albano *et al.* 1985; Manov *et al.* 2004; Roe *et al.* 1993). But using *in vivo* condition, the half-life of NAPQI in the presence of tissue is just seconds, which means it is very hard to predict and mimic the treatment time for liver or target cells viability testing (Burcham and Harman 1991). In this paper we announced a new design for testing hepatotpxicity and drug effect simultaneously by using our 2D-3D cultures design. After acetaminophen treatment for 1 day, encapsulated HepG2 viability dropped from 85% to 55% when acetaminophen concentration increased. However MCF-7 viability didn't significant decrease from low to high concentration of acetaminophen due to the HepG2 viability lost. For the second cell density experiment, when co-cultured 10^7/ml density HepG2 with MCF-7 the MCF-7 viability significant decreased to 71% and 90% of control. From these results, we utilized or co-culture system that models *in vivo* situation, in which the prepared liver cells were placed in a support disk and the target cells were cultivated on the bottom of the well plate. It can be imaged that in clinical experiment, the drugs would be pretreated with liver cells and

the drugs can be metabolized by liver cells and the drug effect can be determined or show from target cells. Normally co-culture systems have been shown for localized proliferation activity testing (Wang *et al.* 2009) and stimulation cells differentiation (Heneweer *et al.* 2005). As we have known, this 2D-3D co-culture designed has not been widely used for hepatocytotoxicity and drug effect testing. Lee *et al* has published by using microsomes (Lee *et al.* 2005) for drug effect testing. Although microsomes can provide a relative low experiment error and standardized procedure for high-throughput screening, but microsomes still not sufficient to replace hepatocytes-based study (Lam and Benet 2004), many compounds still failed to predict by microsomes due hepatic transporters lost (Naritomi *et al.* 2001). Our purpose is that provide a more authentic *in vitro* platform which is truly reflective of these exist *in vivo*; provide a better model for what happens in human body.

Cyclophosphamide (CPA) is currently used to treat a variety of tumor cells such as breast cancer cells and also for its immunosuppressive properties in organ transplantation. CPA is a prodrug bioactivated in human liver by several CYP isoforms including CYP2B6, 3A4/5 and 2C8/9/18/19 (Chang *et al.* 1997; Gervot *et al.* 1999). The therapeutic efficacy of this drug is largely dependent on the liver CYP450 enzyme function with respect to prodrug activation and on the target cancer cells (Chen *et al.* 1996). According to our result (Figure 9), cyclophosphamide didn't metabolize by CYP450 and kill breast cancer cells. This is probably due to low levels of major P450s. Previous paper has been showed that the transcript levels of CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4 presented in HepG2 was lower than primary human hepatocytes (Westerink and Schoonen 2007). In previous papers have shown

that the cyclophosphamide was greatly metabolized by a primary hepatocyte culture (Chang *et al.* 1997; Vanaerts *et al.* 1995). The toxicity of MCF-7 with CPA treatment was low probably due to the low CPA biotransformation activity of HepG2. In order to improve this experiment, we can use primary liver cells or stem cells for our cells-based testing platform in the future.

In this chapter, our approach for realizing a high cell density 3D culture model which has utility *in vitro* drug testing, involves 3D that is conducive for the maintenance of hepatocyte functions and heaptotoxicity testing, and extending the design of the 3D hepatocyte culture system to enable 2D-3D co-culture for drug effect testing. A 3D hydrogel is constructed on a porous biocompatible disk, which provides the benefits of 3D cell culture while allowing more straightforward 2D plating and 3D hydrogel handling. The hydrogel on disk design enables *in vitro* toxicity testing by allowing for the simultaneous, dose-dependent administration of drugs to hepatocytes and target cells. We used our design to assess the hepatotoxicity of four model drugs; acetaminophen, diclofenac, rifampin and quinidine. CT_{50} values that are derived from the dose-response curves are correlated well to the reported *in vivo* LD_{50} values; 2D-3D co-culture system was also established to a platform for testing activity of hepatocyte-activated drugs. These results illustrated the potential predictive value of hydrogel-on-disk design for acute hepatotoxicity. For the next chapter, we integrated 3D culture, support disk and bioreactor to create a dynamic environment for toxicity and drug metabolism study.

Chapter 4: Development of 3D liver cells bioreactor

4.1 Introduction

The overall goal for liver cells bioreactor is to setup an *in vitro* platform for drug metabolism testing and a temporary hepatic support for long term testing applications. The first step in the development of construct for liver cells bioreactor is to seed the liver cells within some sort of a conducive matrix or co-culture with other types of cells inside the chamber. Then the cells have to be provided with adequate amounts of oxygen and nutrients to enable them to survive, proliferate and differentiate (Miki *et al.* 2011). Several types of bioreactors have been developed for specific usages (Figure 1). For example, Spinner-flask bioreactors can be used for increasing the mass transfer to the cells by medium stirring; Rotating-wall vessels provide a dynamic culture environment to the cells with low shear stress; Hollow-fiber bioreactors also enhance mass transfer during the culture. These fibers then create a semi-permeable barrier in which the cells are growing and the medium is flowing in side, also hollow fibers provide a large surface area for cell cultivation; Direct perfusion bioreactors, medium can flow directly through the pores of the scaffold and therefore have the ability to enhance mass transfer (Hutmacher and Singh 2008).

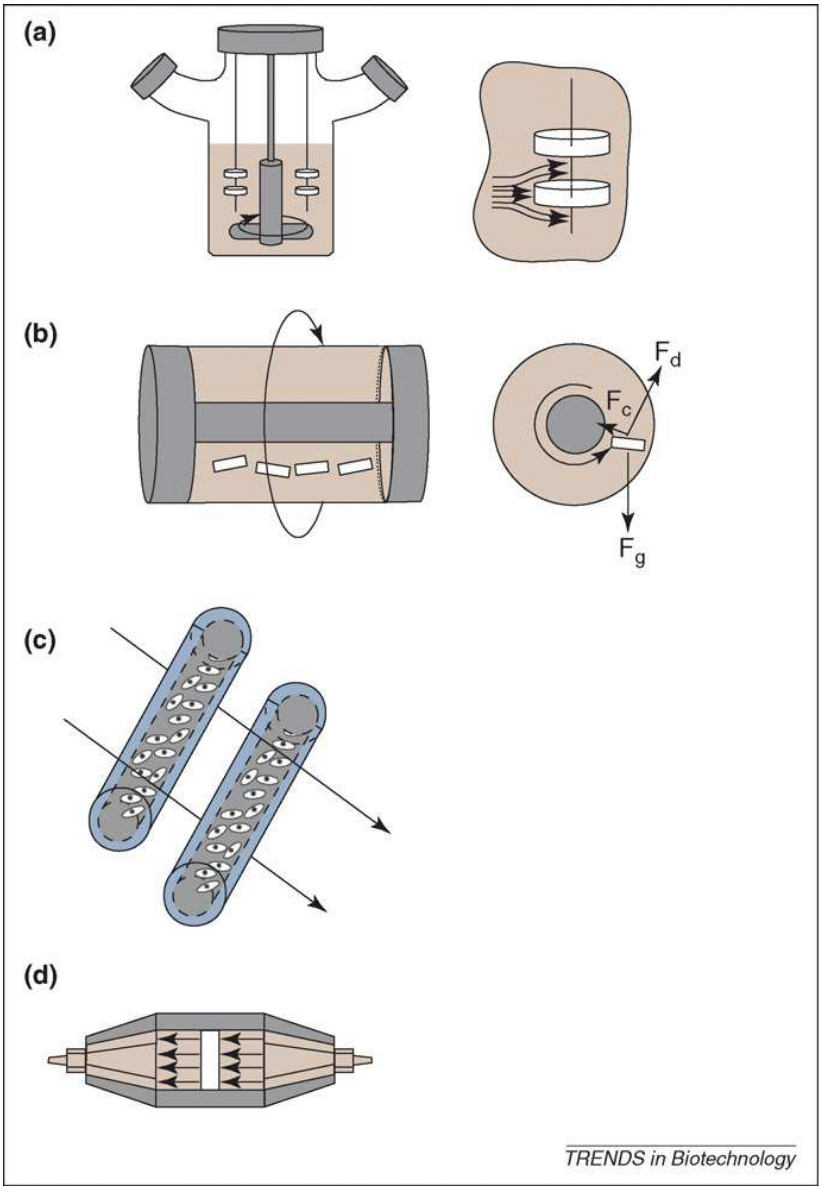


Figure 1. Representative bioreactors for tissue engineering applications (Hutmacher and Singh 2008).

Among several challenges, cell seeding is one of the critical problems for liver cells in dynamic culture. Seeding cells at high cell density (5.73×10^6 cells/cm^3) may favor high viability and metabolism activity such as albumin and urea secretion when compared to low cell density (Dvir-Ginzberg *et al.* 2003). Uneven distribution of cells

in the scaffold or encapsulating hydrogel might lead to variation in oxygen and nutrient concentration gradients that would affect the viability of cells within the scaffold (Lan *et al.* 2010). Growing and mimicking liver cells *in vitro* is challenging because hepatocytes have to be cultured at high cell density and also nutrient requirements are much higher than other cells (Balis *et al.* 1999). *In vivo*, the liver is surrounded by capillaries with nutrients by a high blood flow that reaches the inner cells to get enough nutrients. These capillaries (sinusoids) system can support sufficient nutrients and oxygen for liver cells and they also remove the waste metabolites from liver cells. For the liver cells bioreactor design, it has been well known that the supply of oxygen and nutrients is important for the *in vitro* 3D culture system (Martin *et al.* 2004). Previous study has been showed that cellular spheroids larger than 1 mm in diameter usually suffer from hypoxia and necrosis if the cells aggregate in the center (Sutherland *et al.* 1986). Similar results were reported for other types of cells such as chondrocytes (Ishaug *et al.* 1997). This is a critical problem for most engineered tissue because these constructs usually are at least few mm in size and mass-transfer limitations represent one of the main challenges to be addressed. 3D tissue culture can inhibit mass transport within the tissue construct, resulting in a limited nutrient supply and accumulated metabolic waste. Therefore, the authentic cellular response may be camouflaged by the limitations of a metabolic environment. However, this problem can be solved via a stirred flask (Gooch *et al.* 2001), reduced the thickness of the gel (McGuigan *et al.* 2008a) or increased flow velocity inside the system to get higher oxygen update rates (Nyberg *et al.* 1993). The above methods aim to improve the mass transfer from the surface of the construct into the inner volume. For a regular perfusion system, filtration

methods are widely used for cell culture, but filtration method requires sustained filtering to prevent clogging over the study period. Typically, high flow rate generated by the pump is used to overcome this problem but this method can lead to leakage and damage to the construct structure (Tokashiki and Takamatsu 1993).

Under dynamic bioreactor in which the seeded hepatocytes are in direct contact with the perfusion medium, this condition can result in abnormal wall shear stress at the cellular interface. The mechanical effects of flow-induced shear stress has been reported to alter the morphology, functionality and gene expression for different types of cells such as vascular endothelial cells(Davies 1995) ,bone cells(Owan *et al.* 1997) and hepatocytes PAI-1 gene(Nakatsuka *et al.* 2006). When rat hepatocytes were seeded on the flat surface with flow medium over 5dyn/cm^2 wall shear stress that significantly decreased albumin and urea synthesis rates over 3 days(Tilles *et al.* 2001a). In hepatocytes co-cultured with non-parenchymal cells, the ammonia metabolic rate and urea synthesis rate were both enhanced on day 1 and progressively decreased over 11 days of 1.3 dyn/cm^2 perfusion medium(Kan *et al.* 2004). As a means of reducing the flow-induced effects of shear stress, some papers have shown that microchannel based bioreactor design can protect the seeded hepatocytes from the effects of high shear stresses, resulting in their maintaining stable albumin and urea production(Park *et al.* 2005). Three-dimensional hydrogels can reduce the shear stress on the cultured cells. In these kind of designs, the 3D cultures were placed in the bioreactors and low shear stress was achieved with sufficient mass exchange(Miyoshi *et al.* 2010). Moreover, cells can be seeded on the gas permeable membrane to enable direct oxygenation into the cell compartment(Schmelzer *et al.* 2009). Oxygen level could be a factor for

functional heterogeneity in the liver. In one study where hepatocytes were chronically exposed to increasing oxygen tensions about 5 mm Hg (perivenous) to 85 mm Hg, urea synthesis increased about 10-fold but the activity of P450 and albumin production rate slightly decreased (Chan *et al.* 2004b). These results indicate that by creating different environmental conditions, it is possible to mimic the hepatocyte metabolism in a way that is consistent with *in vivo*.

Perfusion bioreactors have been widely used for many applications such as liver cell transplantation therapies and pharmacologic models (Schmelzer *et al.* 2010). Schmitmeier *et al* have designed a small-scale bioreactor with a gas-permeable membrane and cultured with primary hepatocytes (Schmitmeier *et al.* 2006). The cells could represent their specific functions such as drug detoxification, and Phase-I enzymatic activities when cultured in the bioreactor. Various systems also have been examined for their *in vitro* and traditional culture performance and expect that this small-scale bioreactor system will be applied to drug metabolism studies (Bader *et al.* 1998; Jasmund *et al.* 2002; Langsch *et al.* 2009). Traditional static two-dimensional culture model is inadequate for research tools because two major components are lacking that are required to provide a native *in vivo* environment: a dynamic environmentand a three - dimensional support architecture. Dynamic bioreactor design can provide the information on drug clearance and cytotoxicity that are important for the development of new drugs or for NCE screening. However, similar to previous considered thus far, these bioreactors should provide adequate viability and liver-like metabolism activities as in the native liver (Park and Lee 2005). The metabolism studies can be studied in static condition, such as Petri dishes or well-plates, and these designs

are easy to use and inexpensive. The main drawback for static studies is that the mass transport resistance especially in 3D culture or in culturing in high cell density under a thick layer (Catapano 1996).

Recently some papers have shown that using 3D dynamic culture; primary hepatocytes can be differentiated to liver-specific functional cells (Gerlach 1997; Miki *et al.* 2011). As various authors have mentioned, 3D culture and physical parameters, such as flow improve survival and prolong hepatic functions of primary adult hepatocytes *in vitro* (Fiegel *et al.* 2004; Ring *et al.* 2010).

In an effort to design a liver cells based bioreactor, the hepatocytes functionality must be maintained in an environment that mimics the native liver cells as closely as possible. There are several critical design issues that must be considered when developing a hepatocytes bioreactor. (1) To maximize the long-term functional stability of hepatocytes; (2) to create a liver bioreactor unit that is scalable; and (3) to eliminate transport limitations (Chan *et al.* 2004a). (4) Easy 3D hydrogels/scaffolds insertion and removal processes. (5) Compatible with other scaffold materials and are easily interchangeable.

To our knowledge, the influence of drug toxicity culture with 3D environment under perfusion condition has not been investigated a lot. In the present of study, we applied a meso-scale perfusion bioreactor with stacked 3D hydrogels to demonstrate the application of this device. Encapsulation technique is used to fabricate the cell laden hydrogels onto the support disc. The designed bioreactor contained at least a stack of 3 discs, provide a capability to test multi-samples at the same time. Metabolic activity of the cells inside the bioreactors was quantified on a daily basis by measuring the

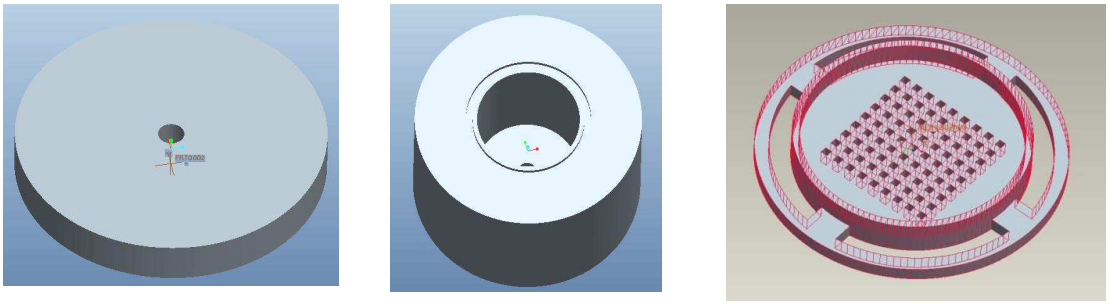
concentrations of HFC in the culture medium with a fluorescence assay. Cells toxicity under static and dynamic condition for 4 model drugs was also tested. This bioreactor can be used in other experiments in which the application of flow to maintain cells, different types of cells can be cultured inside.

4.2 Material and methods

4.2.1 Chamber and disc design

A perfusion chamber and lid were designed and prototyped using a CNC machine with polyetherimide plastic (PEI) as its material with internal slots to support multiple discs within the chamber. A porous polycarbonate disc was designed to fit within a chamber (Figure 2). The disc (15mm diameter disc with circular pores approximately 600µm wide) was fabricated using a Fused Deposition Modeling (FDM) system (Stratasys, Inc, Minneapolis, MN). The disc and chamber were autoclaved to ensure sterility before usage. The support disc and chamber are reusable continuously for repeated experiments. A mesh will allow hydrogels to be placed inside the chamber and allow the medium to pass through the hydrogel. HepG2 cells will be encapsulated by SLG100 alginate on the support as described previously. The support discs with hydrogels were placed carefully inside the chamber. The bottom chamber and the top of the lid will be connected with joints and tubes to allow the cell medium to flow pipes which will allow cell medium flowing from the top to the bottom.

A. Lid **B. Chamber** **C. Disc**



D. Assemble parts

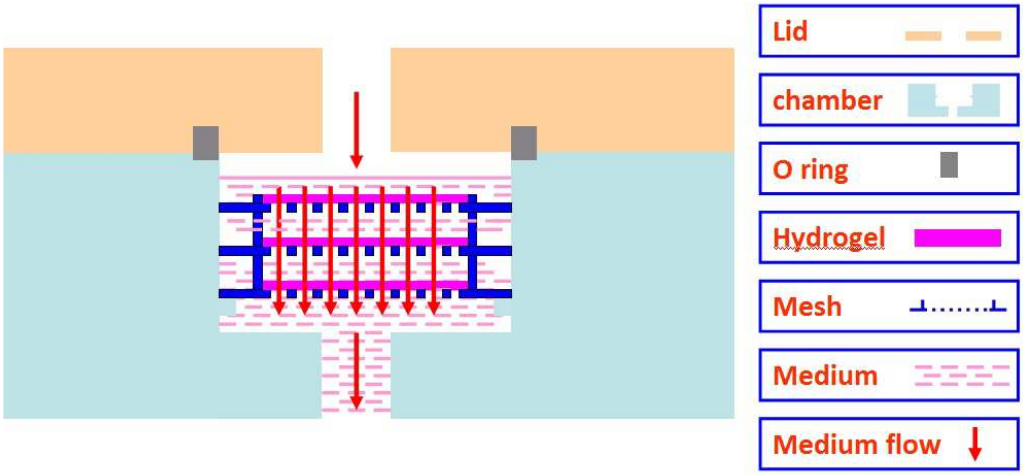


Figure 2. Perfusion bioreactor design and fabrication. (A) Lid device 50mm x 7mm. (B) Chamber device 50mm x 34mm . (C) Mesh device 22.4mm diameter, each hole will be around 0.5mm in dimension with spacing at the middle of mesh. (D) The assemble parts setup, the hydrogel will be seeded inside the chamber.

4.2.2 Integration of hydrogel within fluidic bioreactor chamber

When the cell laden hydrogels are placed into the chamber, necessary connections are made to connect the chamber to a peristaltic pump to allow for the cell-encapsulated hydrogels to be perfused within the medium (Figure 3A). A sterile lid is

laid on the top of the chamber and lightly pressed by four screws. To create a sealed space between the lid and the chamber, one O-ring is used to secure the bond to ensure a leakage-free flow system. Four screws are used to seal the lid and the chamber and avoid leaking (Figure 3B). The cell medium *(Dulbecco's Modified Eagle Medium, DMEM*) was introduced through a peristaltic pump (SCI-Q 200 SERIES pump, Watson-Marlow, UK) into a bubble trap (Stovall Life Science, Greensboro, NC) that was placed between the peristaltic pump and the chamber. The bubble trap near the inlet of chamber will prevent the air bubbles from reaching the chamber.

The outlet port of the device is connected to a tube (Silicone Double Manifold Tubing, 2.79 mm, Watson-Marlow, UK) used to collect the medium back and drains itself into the reservoir (Fig. 3A). Cell culture medium was pumped through a peristaltic pump and circulated through the device. The reservoir was placed on the stirrer to increase oxygen infusion from the environment and also allow infusion of the candidate drug compounds (ex pro-drug EFC) into the device system. Adequate amounts of the medium were collected from the bottom of chamber for analysis during different time point (Fig. 3A). An equal amount of medium was recycled to keep the amount of medium constant. The chamber device contains about 7ml of medium and the entire system (tubes and reservoir) held a total of approximately 30ml of culture medium. The entire system was incubated in the 37˚C, 5% CO_2 incubator (Figure 4). The medium will flow at an inlet flow rate of about 300µl/min (3rpm) which is sufficient to ensure adequate tissue perfusion and nutrient availability.

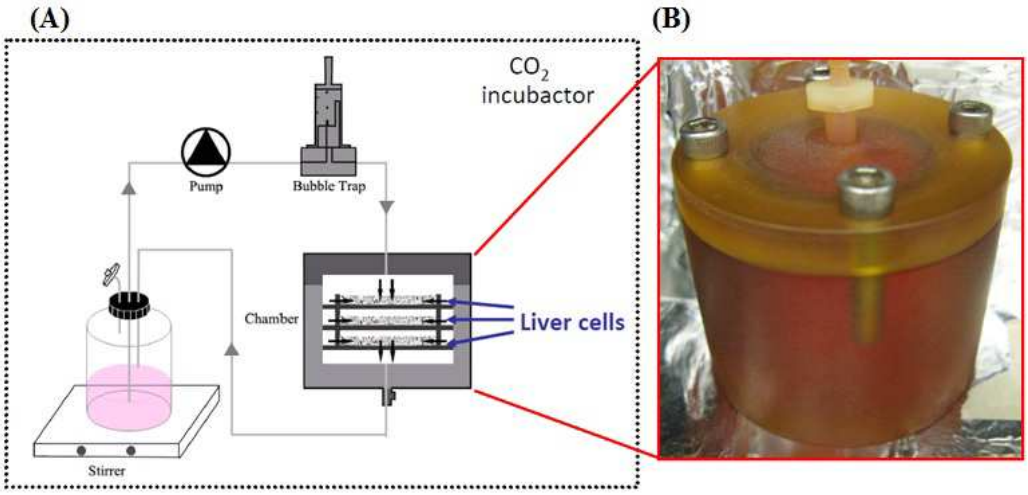


Figure 3. (A) The dynamic culture system used during 3D dynamic culture experiments. The hydrogel is held within a bioreactor and cultured under direct perfusion. (B) The perfusion bioreactor.

Figure 4. Schematic of setup for 3D dynamic culture.

4.2.3 Encapsulated cells viability test

The viability of HepG2 cells in encapsulated was quantified using trypan blue after hydrogels de-crosslinking. On different days, the hydrogels were taken out from the chamber and de-crosslinked by 0.5% EDTA. De-crosslinking process was described on chapter 3. Cells viability was examined using a hemocytometer with trypan blue staining (Invitrogen).

4.2.4 Pro-drug metabolism by encapsulated HepG2 cells within alginate in perfusion system

Non-fluorescent pro-drug EFC (7-ethoxy-4-trifluoromethyl coumarin, Invitrogen) was mixed with DMSO to create a 10mM stock solution of EFC as described before. To determine the EFC-HFC conversion rate under different cell density and flow rate, a period of time was selected for study. In the first set of drug elimination experiments, clearance rates of the pro-drug 7-ethoxy-4-trifluoromethyl coumarin (EFC) to 7-hydroxy-4-trifluoromethyl coumarin (HFC) was studied for 10^7cells/ml, 2.5x10^7cells/ml and 10^8cells/ml cell density under different flow rates. At each time point, the concentration of HFC was monitored with a fluorescence reader (FX800, Biotek) using an excitation wavelength of 360nm and an emission wavelength of 520nm.

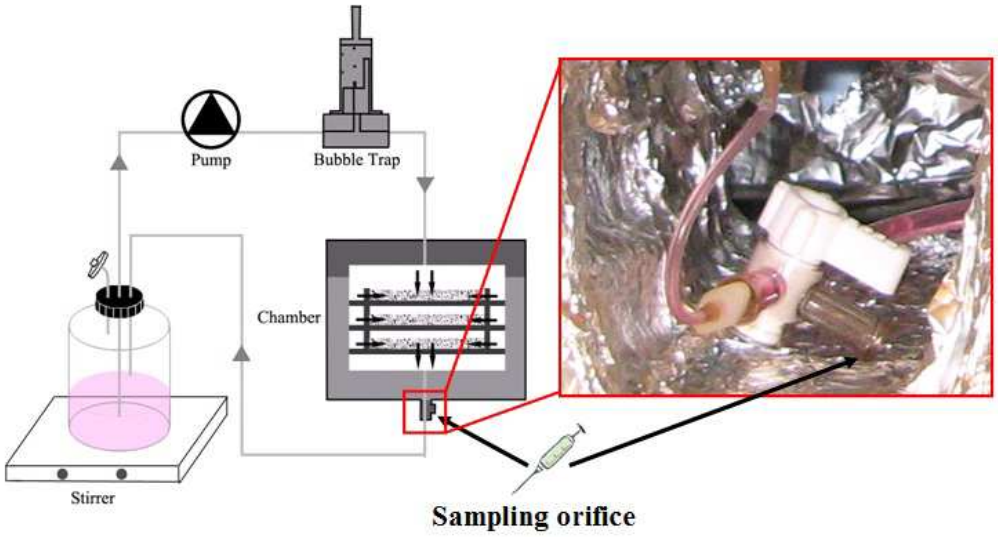


Figure 5. Bioreactor orifice design. Cell medium can be collected from the bottom of the chamber without distributing the system.

4.2.5 3D hydrogels hepato-cytotoxicity testing with static and dynamic condition

Acute exposure (24hr), hepatotoxicity under static and dynamic condition was tested using 4 model drugs: acetaminophen (20mM), diclofenac (0.5mM), rifampin (0.6mM) and quinidine (0.15mM). For static testing, encapsulated HepG2 cells were seeded on the support discs as described above. For dynamic culture, three layers of disc with hydrogels were seeded in a bioreactor chamber and treated with flowing medium with different concentrations of drugs. The controls were prepared by diluting corresponding amounts of DMSO in culture medium without drugs. After drugs treatment for 24 h, the static and dynamic samples cells were de-crosslinked from encapsulation samples by EDTA treatment, the cell viability values were assessed using hemocytometer with trypan blue staining (Invitrogen).

4.3 Results

4.3.1 Effect of cells viability and HFC formation activity under different cell densities and flow rate

Encapsulated cells viability screening of flow medium requires a device that permits development of multiple hydrogels with controlled flow rates and determination of HFC formation activity during time. We constructed such a device with fluidic channels integrated into 3D dynamic culture. The use of a designed support disc format allowed compatibility with static culture and dynamic culture. Encapsulated cells morphology is generally determined using the LIVE/DEAD Back Light stain combined with imaging of green, the morphology of encapsulated cells were no significant different compared to our previous static results (Data not shown).

To test if our dynamic device provides a reliable environment for encapsulated cells viability, we first experimented with 10^8 cells/ml cell density under 3ml/min and 0.3ml/min flow rate. HepG2 cells were encapsulated in hydrogels on three support disc and put into the bioreactor for a period of time. Initial concentration of 120μM EFC was treated inside and a small amount of medium was collected from the orifice for HFC concentration testing. The concentration of HFC was monitored at different time points during 3 days. In this experiment, HFC emission peak under 10^8 cells/ml cell density, 3ml/min flow rate was higher than in the 0.3ml/min flow rate. HFC conversion activity was higher under high flow rate. The cell viability was also tested after finishing the experiment at day 3. The viability of encapsulated cells remained around 25% under 0.3ml/min flow rate, when we increased the flow rate to 3ml/min, the viability was no significant different between 0.3ml/min and 3ml/min. This result indicates that the

higher flow rate did not provide a sufficient oxygenated and nutrition diffusion for sustenance of high cell density culture within the hydrogel

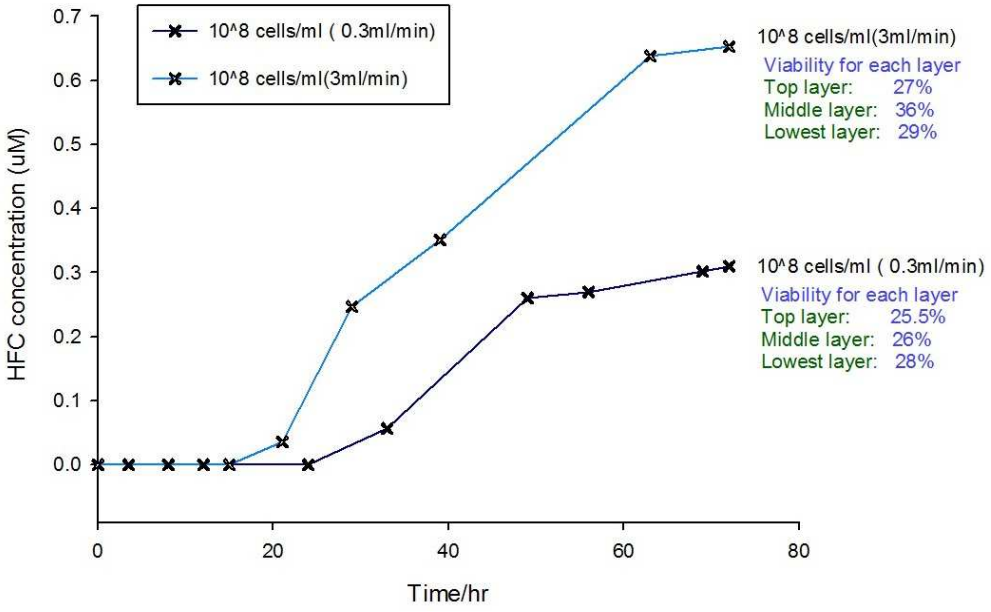


Figure 6. HFC emission peak in 3ml/min and 0.3ml/ml flow rate. The concentration of HFC was measured at different time point and the cell density was 10^8 cells/ml for each sample.

According to the first 10^8 cells/ml experiment, the viability and HFC formation activity was not good. In this experiment we decreased the cell density to 2.5 x 10^7 cells/ml and tested the HFC concentration during time under 3ml/min, 0.3ml/min and 0.05ml/min flow rate. Figure 7 shows the HFC formation peak for the 3 different flow rates. As shown, the concentration of HFC was the highest for alginate with the flow rate at 3ml/min, but the final viability 0.3 ml/min was the highest. Samples with 0.05min/ml flow rate was much lower than other two flow rates (3ml/ml and 0.05ml/min). This result indicates higher flow rate can increase the HFC formation

activity but also may result in loss of cell viability. This is expected since higher flow rate can cause loss of alginate structural integrity due to higher wall shear stresses.

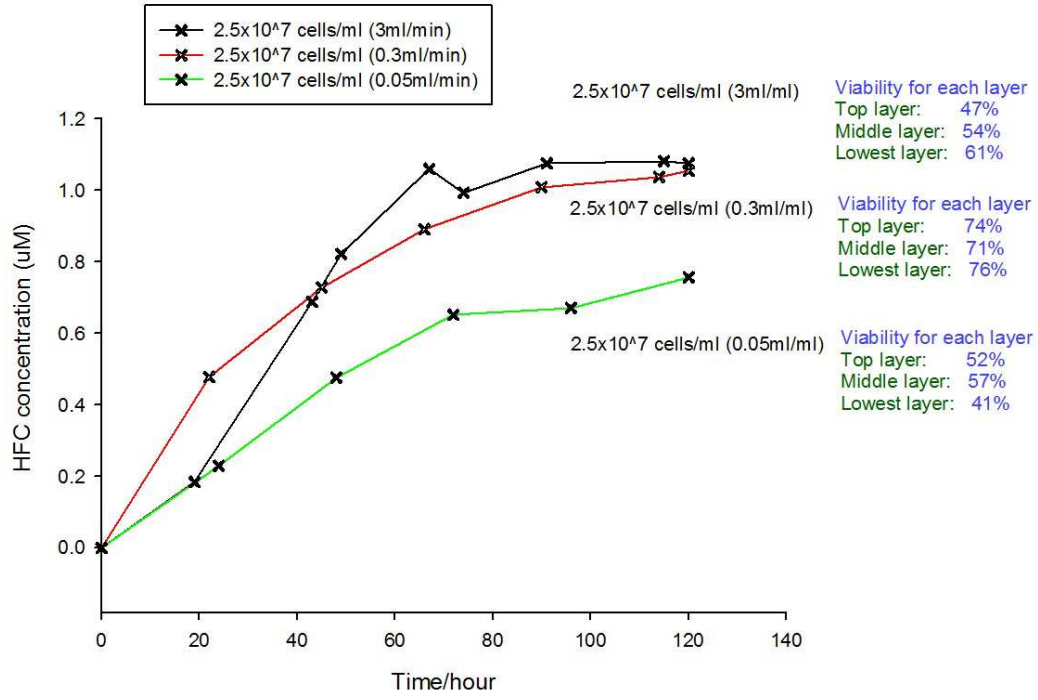


Figure 7. HFC emission peak in 3ml/min, 0.3ml/min and 0.05ml/ml flow rate. The concentration of HFC was measured at different time point and the cell density was $2.5x10^7$ cells/ml for each sample.

For the 10^7 cells/ml cell density, we cultured our samples under 0.3ml/min and 3ml/min different flow rate and the concentration of HFC was monitored during time. Figure 8 shows the HFC formation peak for the 3 different flow rates. As shown, the concentration of HFC was the highest for alginate with the flow rate at 0.3ml/min. For the higher flow rate, the cells viability dropped to 62% compared the viability of 84% under 0.3ml/min flow rate. In consideration of cell viability and the final concentration

of HFC formation during time, 10^7cells/ml cell density and 3ml/min flow rate is better than other two cell densities and flow rates.

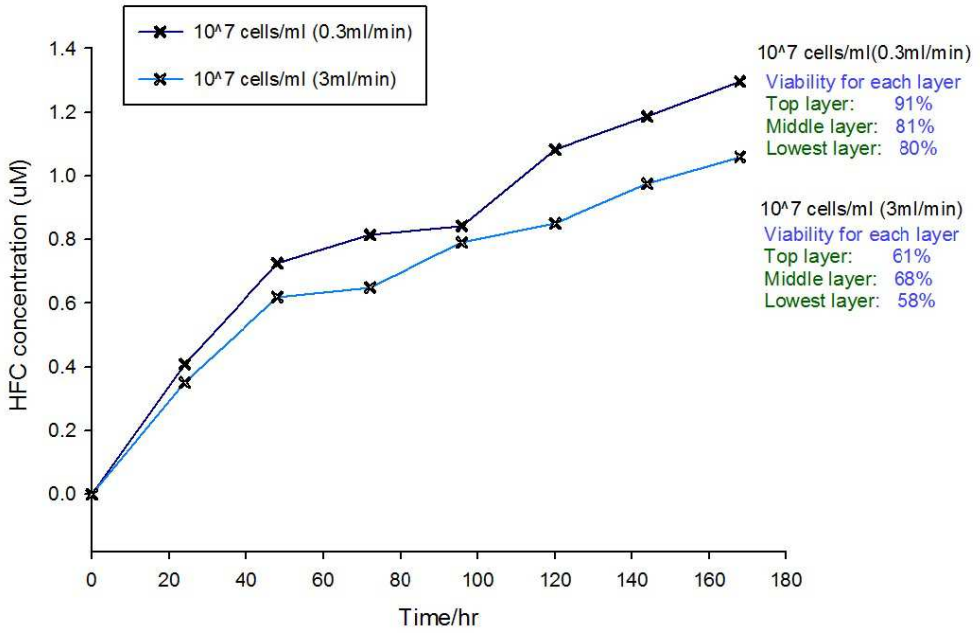


Figure 8. HFC emission peak in 0.3ml/min and 3ml/min. The concentration of HFC was measured at different time point and the cell density was 10^7 cells/ml for each sample.

4.3.2 HFC formation activity between static and dynamic for a long period of time

For this experiment, we examined the HFC concentration on static and dynamic(0.3ml/min) culture condition during 14 days. The cell density for static and dynamic samples was 10^7cells/ml. For static samples, the concentration of HFC increased until day 3 and then reached a saturation level where no more of the EFC was converted to HFC. For the 3D samples, the concentration of HFC increased until day 9 and then reached a saturation level. The maximum concentration of HFC on dynamic culture was higher than static culture. For dynamic culture, the result shows the conversion amount increased steadily from days 1 through day 8. On day 7, the viability

still remained around 84± 6% compared the viability 62±4% under static culture (data not shown). HFC concentration can be produced higher under dynamic culture and viability was also better than static culture. This result indicates that our dynamic system can provide an environment for 3D long-term cell culture, also enhance the HFC formation activity which could be utilized to drug testing platform *in vitro* culture. In consideration of encapsulated cells viability, HFC conversion ability, cell density and flow rate, 10^7 cells/ml cell density, 0.3ml/ml flow rate is better than other parameters for *in vitro* testing under dynamic condition.

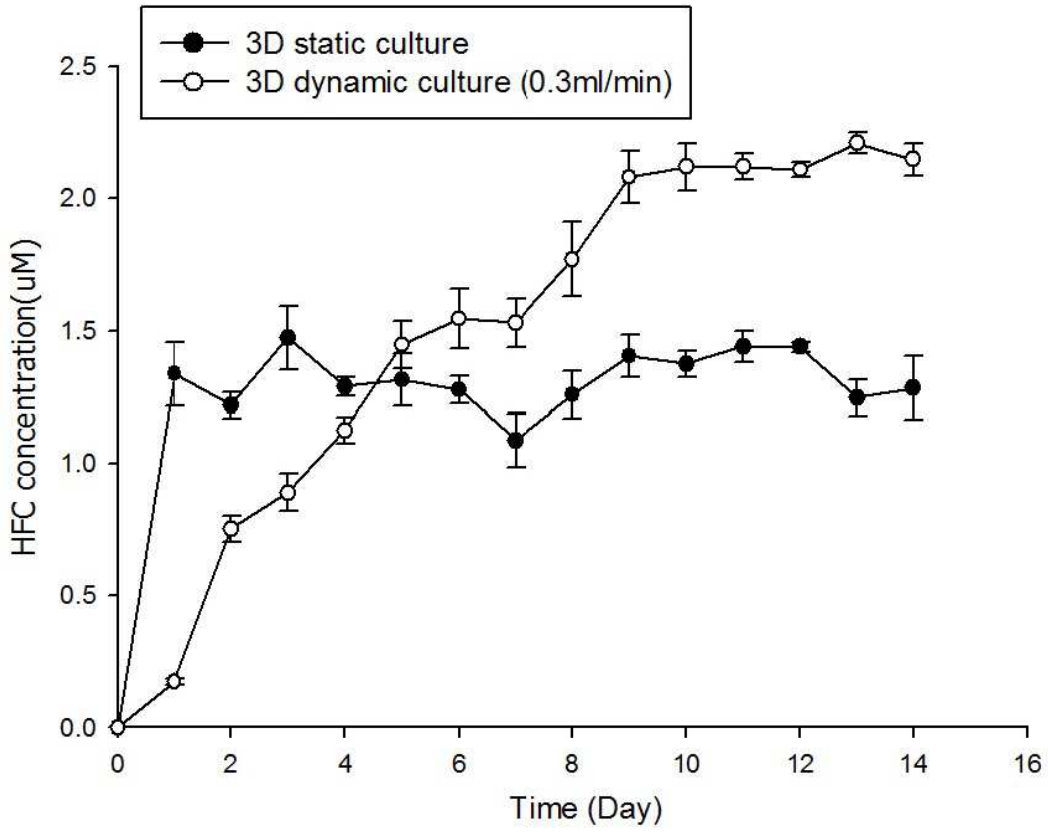


Figure 9. HFC emission peak in static and dynamic (0.3ml/min flow rate) condition. The concentration of HFC was measured at different time point and the cell density was 10^7 cells/ml for each sample.

4.3.3 The influence of flow rate on the hepatotoxicity of static and dynamic culture

Several factors can affect uptake rate, disposition, and pharmacodynamics of drugs. It has been known for many years that flow rate can have a major influence on the extent of drugs uptake (Horowitz and Powell 1986). For this result, our 3D samples were cultured in bioreactor under 0.3ml/min flow rate. 20mM acetaminophen, 0.5mM diclofenac, 0.6mM rifampin and 150μM quinidine were treated for 3D samples under static culture and dynamic culture. 4 drugs were incubated for 24hr and the cells viability was examined after de-crosslinking process. The results demonstrated that exposure to 4 drugs for 24h resulted in different cytotoxicity response between static and dynamic condition (Figure 10). It was noted that there is a statistically significant difference between static and dynamic culture condition under 150μM quinidine treatment (P value = 0.001<0.05). There was no significant difference when comparing the cytotoxicity effects for acetaminophen and rifampin between static and dynamic treatment (Fig. 10). Cells toxicity are dependent on blood flow rates and drug clearance (Cutler and . 1986). In our human body, the liver blood flow rate is up to 1500ml/min and the major part for the portal vein is around 1000ml/min (Loos *et al.* 1985). Due to the flow rate effect, hepatotoxicity may be different under different flow rate with drug treatment.

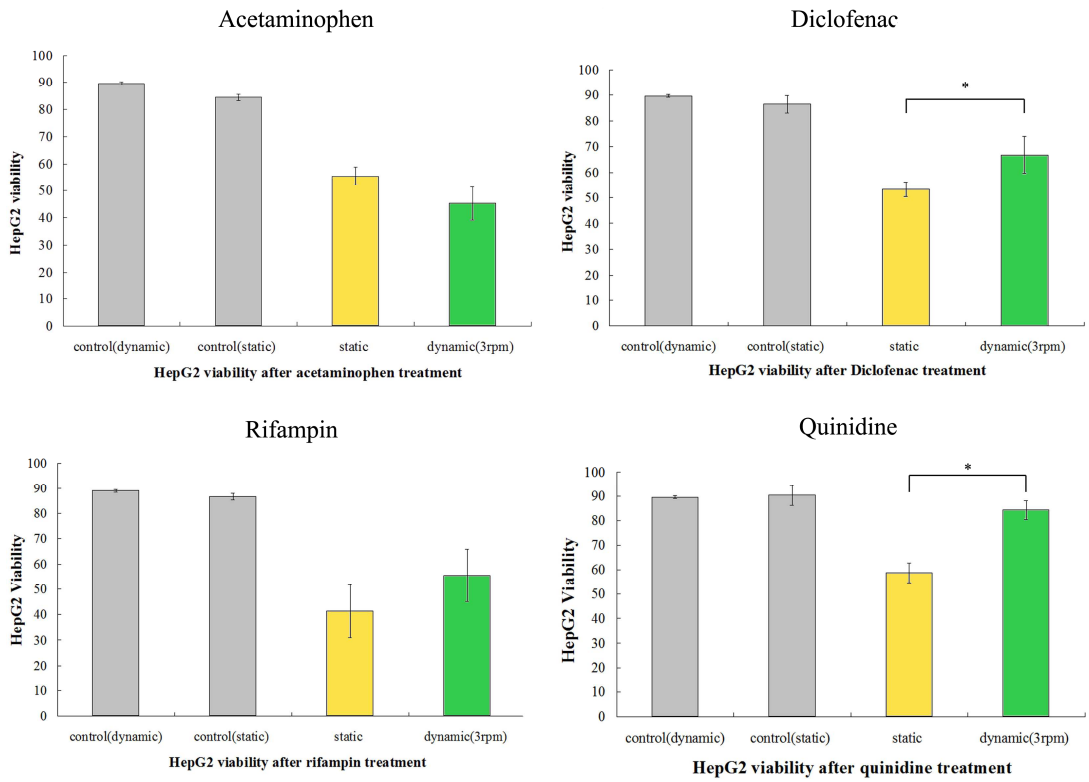


Figure 10. Effect of cytotoxicity on static and dynamic culture condition in encapsulated HepG2 liver cells. 3D samples were treated with different concentrations of drugs for 24 h. Control samples were only treated with culture medium without drugs. (*) indicates a statistically significant difference compared to static and dynamic samples ($p < 0.05$).

4.4 Discussion

4.4.1 High cell density culture under dynamic condition

For the result on Figure 6, the effect of changes in flow rate of the medium on the viability evaluation of 10^8 cells/ml cell was investigated. The results show that there is a correlation between the HFC synthesis and the flow rate. In high cell density condition the increase of the flow rate improve the HFC formation ability, but the cell viability was lower than 30% on low and high flow rate. It has been reported that by

increasing the flow rate to the perfused rat liver, which increased oxygen delivery and drug clearance rate (Cardoso *et al.* 1994). Overcoming diffusion limits for the delivery of essential nutrients and the removal of waste products is a priority issue in the development of 3D cell culture. One possible solution is to create a vascular network in vitro which enhances the diffusion rate of nutrients and waste products (Griffith *et al.* 2005). At very high cell density, oxygen demand is expected to be high and the rate of flow is directly proportional to the oxygen gradient due to increased diffusion limitations(Hay *et al.* 2000). For our 10^8 cells/ml cell density, the viability was low even when we increased the flow rate. Previous papers have shown that the HepG2 oxygen consumption rate under 10^8 cells/ml cell density was around 3.4×10^{-17} mol/s/cell was found, which is much lower than previously reported values for hepatocytes (Provin *et al.* 2009). This is probably because cells surface are diffusion-limited layer, oxygen is very hard to pass through inside the hydrogel due to the high cell density. The increasing cells content in alginate disc increased the diffusion barrier (Hilge-Rotmann and Rehm 1988). For cell densities of approximately 4 x 10^7 /mL, severe oxygen limitation must be expected, whereas for cell densities of 10^8 /mL (a tissue-like cell density) the penetration depth for oxygen is less than 100 μm. As the 1 mm thickness of vessels used for experiment, an appropriate cell density with sufficient oxygen supply is about 10^7 /mL of the matrix (Portner and Giese 2007). For the next experiment, we decreased the cell density to 10^7 cells/ml, and the viability was much better when we compared the result with $2x10^7$ cells/ml and 10^8cells/ml. In order to adverse the oxygen limitation problem, one way is to increase oxygen supply without raising oxygen tension is to enhance the solubility of oxygen in the culture medium by adding an

oxygen carrier. By using this method oxygen can be in direct contact with the cell membrane and resulting in a reduction of the oxygen diffusion resistance in high cell density and 3D material without vascular structure.

For our dynamic culture, three layers of hydrogel were stacked in the bioreactor. According to our results the viability distribution is no significant different between each three layers. For our staking disc, the three-layer stack under dynamic condition that allowed perfusion of the medium into its center from the sides. Encapsulated cells cultured in this"loosely packed layers" didn't show a gradient of viability. Our "loosely packed layers" also provide an alternative way for nonvascularized hydrogel to enhance oxygen diffusion. In further if we want to determine the responses from encapsulated cells under oxygen gradient, analyzing oxygen-responsive genes by destacking the layers and isolating RNA from cells originally located at different layers. Several hypoxic extent and relative genes can be select for testing such as vascular endothelial growth factor (VEGF) gene (Sonna *et al.* 2003). By stacking and destacking the disc with hydrogels in bioreactor which make it possible to test oxygen and nutrient gradients in 3D and analyzed the genes response from encapsulated cells. This method can provide a simple process to use when studying of nutrients and biological responses in these gradients without histological sectioning and cell sorting. Different types of cells can be allowed to create a heterogeneous 3D culture *in vitro*. Multiple discs with hydrogels also can be incorporated into standard well plates, for high throughput screens or simple cells-based assay.

4.4.2 Effects of flow rate on the viability of dynamic culture

In our work, flow rate is an issue for cells viability. To reduce mass transfer limitations, we tested different flow rates under $2.5x10^7$cells/ml and 10^7cells/ml cell density. For $2.5x10^7$cells/ml samples under 0.05ml/min, 0.3ml/min and 3ml/min flow rate. The HFC formation ability and cells viability on 0.3ml/min and 3ml/min were higher than 0.05ml/min. Flowing medium can enhance oxygen diffusion and continuously introduce nutrients and remove wastes, but cells viability and enzyme activity may decreased in accordance with the increase of pressure of flow rate (Tokunaga *et al.* 1988). A more gentle approach may prevent shear–stress induced injury and thus improve cells viability ('t Hart *et al.* 2007). Higher perfusion pressure potentially causes damaging of alginate structure, resulting in leakage of encapsulated cells in the cell medium. A lower perfusion pressure will result in low distribution of the oxygen and nutrients. According to our results, 10^7 cells/ml cell density under 0.3ml/min flow rate has the best result for HFC formation and viability. This study also indicates that tuning of the perfusion rate is crucial to cell viability.

In order to study the homogenous medium perfusion, we placed three discs with three hydrogels inside the bioreactor and three layers of viability were determined separately after finishing experiment. Generally, viability was no significant different between each layer, but the mechanical property of upper layer became softer after incubation compared with middle and lower layer. But 3D culture environment can enable higher cell density and reduce shear stress or perfusion pressure effect under dynamic culture.

4.4.3 Encapsulated HepG2 cells in long term 3D static and dynamic culture

For the ca-alginate material, the oxygen diffusion coefficient was varied between 2.54 x 10^{-5} cm^2/s and 2.58 x 10^{-5} cm^2/s for 1-3% alginate, and the oxygen diffusivity in alginate was medium due to the small pore size and hydrophilic(Mehmetoglu *et al.* 1996). In order to supply more oxygen through this 3D hydrogel, reduce the number of cells that can be seeded in alginate or increase the medium flow rate(Streeter and Cheema 2011). In this study, we designed a perfusion bioreactor for dynamic culture and compared the HFC conversion ability between static and dynamic during 2 weeks. For static culture (Figure 10), the medium was found to be inadequate to maintain 3D samples. HFC formation rate was improved by dynamic culture due to maintenance of higher cell viability. In the bioreactor with the 0.3ml/min flow rate, most cells were viable at all three locations(upper, middle and lower) within the bioreactor. Using hepatocytes entrapped within a biocompatibility material within the dynamic device has been widely used for improvement of oxygen utilization of hepatocytes and waste removal (Tilles *et al.* 2001). It was also determined that the CYP450 catalytic activity for the metabolism of EFC can be maintained around 9 days without stopping the system. In the static 3D culture model, samples have to be refreshed the medium frequently and initial drugs concentration will not be the same if you test it for a long period of time. For our dynamic device, medium is pumped continuously from a medium reservoir through the cultivation unit and back. A very small amount of medium can be collected from the bottom of orifice for drug metbolism testing during time. Although this experiment we put three layers of encapsulated hydrogels inside, with further modifications, the usage of such a 3D co-culture model

can be widely applied in studying the interaction and interplay between different types of cell on different layer.

4.4.4 Encapsulated cells hepatotoxicity under static and dynamic culture

For drug screenings based on cell models, especially for 2D cell-based assays, have been widely used for toxicity testing and high-throughput screening, but for prediction of toxicity profiles in clinical response is limited. The predictability of 2D cell-based assays is attributed to the fact that traditional 2D cultures do not mimic the cellular response in 3D environment and flow rate factor is not also considered. In particular, the level of activity of key detoxification enzymes in the cytochrome P450 (CYP450) family has proven inconsistent and may differ from physiological levels such as oxygen level (Hewitt and Hewitt 2004; Wilkening and Bader 2003) or hepatic extraction ratio of drugs (Yoon *et al.* 2011). A combination of these factors likely resulted in the different toxicity response between static and dynamic culture. To further elucidate that why the viability different between static and dynamic condition under the same concentration of drugs treatment, it would be necessary to evaluate the CYP450 gene expression level and oxygen uptake rate in both static and dynamic condition along culture time and correlate it to the livers specific phenotype obtained and also compare the results with the more established hollow fiber or encapsulated hepatocytes perfusion bioreactor(De *et al.* 2009; Tostoes *et al.* 2011). It is clear that the absence of mass transfer limitations and combination of 3D culture makes the 3D dynamic system described here a better alternative to drug testing and toxicological studies than traditional 2D culture.

Our results demonstrate the efficient maintenance of liver cells using a perfusion bioreactor in 3D alginate hydrogels. Hepatic viability associated pro-drug conversion functions were detected with three-dimensional hydrogel under dynamic condition *in vitro*. This design may provide a new approach for 3D liver cells engineering, critical for drug toxicity testing and drugs discovery. Our design consists of a reusable, inexpensive cultivation units and control temperature and medium supplies. Moreover, this device is also capable of operating automatically and continuously without many manipulations. For our prototype dynamic device, the bioreactor can be an advantageous method in terms of low contamination risk, ease to handling and scalability. In conclusion, our multilayer bioreactor design is not very complex and also on the early stage of development. In the future, cell printing and other co-culture designs can increase fundamental understanding of the complex issues that will impact drug screening testing method in bioreactors.

Chapter 5: Conclusions and Future Directions

5.1 Summary

Developed for a range of tissues where the culture environment takes into account, 3D cell culture models serve to bridge the gap between *in vivo* studies and *in vitro* testing. Encapsulation method is simple three-dimensional models that can be generated from a wide range of cell types and form due to the tendency of adherent cells to aggregate. Our studies have investigated 3D culture using a three-dimensional alginate-based encapsulation method for HepG2 liver cells. Some of the major challenges using this technique are mechanical damage, liver specific enzymes functionality and long-term incubation. To address this issue, we have developed a 3D encapsulation method which has high viability, designed a new support disc for easily hepatotoxicity and drug effect study, and established a bioreactor for long-term 3D culture and dynamic study.

5.2 Research contributions

The contributions of this research are summarized as follows:

(a) The described protocols represent a simple and useful method to encapsulate cells within alginate materials in a cytocompatible manner. Such techniques are especially important since 3D culture have been widely used and also can exhibit different behavior in 2D versus 3D microenvironments.

(b) Development of an inexpensive platform to enable long term high density liver cell culture in combination with another type of cell in static culture environments.

The test platform allowed us to evaluate drug dose concentrations to predict hepatotoxicity and its effect by using 2D-3D culture techniques.

(c) Development of a bioreactor for 3D dynamic culture. The bioreactor has been designed to integrate our disc design which allows multi-layered hydrogels cultured inside. The bioreactor design ensures endless applications and permits to use the same encapsulation method and the same support disc to work with an unlimited number of cells or biomaterials. All bioreactor and disc design are biocompatible and are built with autoclavable materials, perfusion condition can be easily control from a peristaltic pump and the patterned architecture can be made on the disk to achieve complexity from traditional cell culture.

(d) Establish of an easy design process for different users usage. In our case, our support disc was designed for 12-well plate also with the size of bioreactor. For different researchers or experiment purpose, the devices can be scale up or scale down satisfies scientists' requirements. Furthermore, this 3D culture process that is saleable and may be customized for unique needs.

5.3 Future research recommendations

The work presented in this thesis can be improved upon to include more features and alginate can be modified to include several types of adhesion proteins for cell-matrix interaction study. Following research tasks have been outlined and can be undertaken to for future research and development.

5.3.1 Conjugation of adhesion proteins into 3D hydrogel alginate

For our alginate material, cells were entrapped within the gel and have limited proliferation activity due to without any modification to the alginate structure. Recent

reports have revealed that an adhesion protein such as RGD modification can increases cellular proliferation and inhibits chondrogenic differentiation in mesenchymal stem cells(Connelly *et al.* 2007). Scaffold architecture and materials may also modify responses of cells (Pruksakorn *et al.* 2010). 3-D culture using scaffold-based techniques offers advantages in providing a structural support for cellular attachment with a different orientation. In the future we can encapsulate HepG2 cells or other types of cells with modified alginate on the top of our disc design and combine with the dynamic bioreactor for cells specific genes study or tissue regeneration study.

5.3.2 Combination of cell printing technique and support disc to create patterned scaffolds

Our encapsulation technique can provide a quick, simple and fast method for 3D culture. Moreover, simple encapsulation provides little or no control over the cellular organization of the resulting culture. Recently, a variety of rapid prototyping techniques have been developed to make patterned hydrogels by depositing biomaterials, including photolithography and syringe-based gel deposition. These techniques are similar in that the finished printed cell construct would be fabricated from the bottom up (layer-by-layer) and can mix heterogeneous cell and biomaterial in three dimensions. Using our above porous support disc and cells printing technique, patterned hydrogels are capable to be printed on the support disc in a controlled environment. The support disc with the patterned 3D hydrogel can be placed into well-plate or bioreactor. Use of the patterned 3D hydrogel as an *in vitro* drug testing platform showed many additional benefits, (1) scaffold-like structure can be fabricated on the disc to mimic native environment in the

liver, (2) multilayered construct can be fabricated by controlled nozzle, biologically relevant scale or co-culture environment can be reached on the disc.

5.3.3 Determination and validation of drugs degradation products by HPLC system

For our co-culture results, we established that the HepG2 cells actually metabolize acetaminophen and that the toxic metabolite is released into the media and delivered to the MCF7 cells. However, this result is indirect evidence and it is not 100% to conclude that the MCF7 toxicity is due to metabolism of acetaminophen by the HepG2 cells. The only reliable technique to directly measure the amount of toxic metabolite released into the medium is through high-performance liquid chromatograph (HPLC) testing. HPLC analysis of chemical drug degradation is generally more favorable; testing at various points in time using our bioreactor can reveal the appearance of new peaks or significant peak growth for testing drug byproducts. In the future we can treat several drugs in our 3D dynamic culture device and integrate with HPLC system. HPLC can analyze several active components in the present of bioreactor and even degradation products that be present in the system.

5.3.4 Multi-perfusion chamber for metabolism-dependent toxicity study

For our design we only used one perfusion chamber for our study. If we want to test metabolism-dependent toxicity, we need to connect with at least two components, metabolism system and target cells such as kidney or endothelium cells. At first, we will incubate drugs in the presence of HepG2 cells dynamic chamber. The metabolizing medium will be removed after exposure and target cells will be further incubated before cell viability is measured (Figure 1). Alamar blue or MTT assay can be applied for

measuring target cells viability (Al-Nasiry *et al.* 2007). By using this concept, a very simple multi-compartment model can be designed. The drug can be metabolized by "liver" into reactive metabolites, which then circulate to the "target cells" for dynamic drug effect testing. A more complete multi-chamber system can be readily fabricated to provide *in vitro* ADMET studies on new drugs or drug combination.

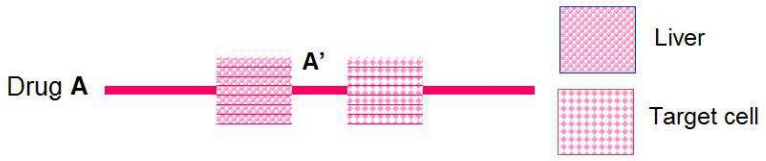


Figure 1. Perfusion system for metabolism-dependent toxicity study (A' is the converted drug by liver metabolism).

5.4 Concluding remarks

3D Cells-based assays *in vitro* require appropriate biomaterial, suitable process and bioreactors that simulate physiological environment for cell growth on 3D. In our study, our design has been meet the requirements of tissue engineering, *in vitro* drug screening and toxicology studies. This study has showed several advantages for researchers such as high versatility, simplicity of use, custom design and affordable instruments. Some similar commercial 3D bioreactor products have been selling on the market recently (3D bioreactor from 3D biotek company, P3D chambers from Eberis company). Although these products have significantly produced better device for 3D culture, they have shortcomings such as limited material selection, co-culture testing, and lack of versatile static and dynamic experiment. Our design concept can provide a

better solution for researchers working on tissue engineering, pharmacology and biology field.

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