

# **Encapsulation of Hepatic Progenitor Cells for Liver Tissue Engineering**

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## **DEDICATION**

To my dear parents for all the sacrifices they have made and hardships they have endured in raising me to this level. I will be forever indebted to them.

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## LIST OF ABBREVIATIONS AND SYMBOLS

CaCl <sub>2</sub>	Calcium Chloride
DMSO	Di-Methyl Sulfoxide
ECM	Extra-cellular Matrix
EGF	Endothelial Cell Growth Factor
ELISA	Enzyme Linked Immunosorbent Assay
EROD	Ethoxy Resorufin O-dealkylase
FBS	Fetal Bovine Serum
HPCs	Hepatic Progenitor Cells
ITS	Insulin-Transferrin-Selenium
ml	milliliter
mM	millimolar
PDMS	Poly-Di-Methyl-Siloxane
PAS	Periodic Acid Schiff's
PBS	Phosphate Buffered Saline
PROD	Pentoxyl Resorufin O-dealkylase
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy

### Greek Symbols

μl	microliter
μm	micrometer

# **1 Introduction and Study Goals**

## **1.1 Background**

Wang et al. have characterized a hepatic progenitor cell population isolated from uninjured adult mouse liver that phenotypically differentiates into both hepatocytic and biliary cell populations [50]. It was observed in subsequent transplant experiments that direct injection of this cell population (into the spleen or liver) is associated with a high incidence of pulmonary embolism rather than cellular engraftment. This suggests that despite direct tissue injection the cells rapidly enter the vasculature of the recipient and subsequently embed within the pulmonary vascular system (Data not shown). These initial findings are critical in understanding the potential utility of the progenitor cell population in clinical therapies. Unfortunately the efficacy of cellular transplantation with somatic-derived progenitor cell populations (e.g. hepatic progenitor cells) is limited by the inherent requirement for the cell population to exist in an environment that supports cell viability and function [59-61].

Based on the previously described cell transplant experiments it was hypothesized that creating a cellular niche is a critical step to support progenitor cell proliferation and differentiation. The goal of the dissertation research was to develop a three-dimensional environment for the hepatic progenitor cells that would optimize their proliferative potential and their ability to differentiate.

It was theorized that the creation of an ideal three dimensional model where the cells can be cultured at higher densities (due to the physical characteristics and volume of the biohybrids) would circumvent the physical constraints of standard two-dimensional culture systems. In addition, utilizing these methods for creating a neo-microenvironment for the progenitor cells allows further ex-vivo manipulation to optimize cell proliferation, function and the potential to deliver the cells in-vivo.

## **1.2 Project Goals**

The main goals of this dissertation research were as follows:

1. To create a milieu wherein hepatic progenitor cells (HPCs) can be provided an ex-vivo 3-dimensional culture condition to proliferate and differentiate into mature phenotype.
2. To analyze the effect of such biohybrids on HPCs including their bi-potency and sustenance of function.
3. To establish a procedure for transplantation of HPCs integrated on resorbable biohybrids.
4. To provide HPCs with an environment to protect them from the recipient's immune system.
5. To develop systems for cellular encapsulation to facilitate cell transplant.
6. To translate experience gained with encapsulation techniques to the MEMS/microfluidics platform for purposes of cell delivery and analysis of drug/cytokine metabolites.

### 1.3 Manuscript Preparation

The findings of this study were presented at recognized scientific conferences and one manuscript has been previously published in the “Tissue Engineering” journal. Additional manuscripts are currently being prepared for submission. The list of presentations and publications is listed below.

- **Chandrasekaran P**, Neill ATO, Walker GM, Gerber DA - “Microfluidic Approach to Collagen Microencapsulation” – *Article in Preparation*.
- **Chandrasekaran P**, Neill ATO, Walker GM, Gerber DA - “Microfluidic Culture of Hepatic Progenitor Cells in Collagen Microspheres” – *Article in Preparation*
- **Chandrasekaran P**, Neill ATO, Walker GM, Gerber DA - “Microfluidic System for Collagen Encapsulation of Cells” – *Biophysical Society Meeting, Baltimore, MD – March 2007*.
- **Chandrasekaran P**, Seagle C, Rice L, Macdonald J, Gerber DA - “Functional analysis of encapsulated hepatic progenitor cells.” – *Tissue Eng.* 2006 Jul; 12(7):2001-8.
- Wright N, Rice LL, Walkup M, **Chandrasekaran P**, Gerber DA - “Enrichment of a Bipotent Hepatic Progenitor Cell from Naïve Adult Liver Tissue” – (*Submitted for peer review*).
- **Chandrasekaran P**, Walkup M, Wright N, Seagle C, Macdonald J, Gerber DA - “Novel technique for co-culture of Sca-1<sup>+</sup> hepatic progenitor cells and hepatic non-parenchymal cells.” – North Carolina Tissue Engineering and Regenerative Medicine Meeting, Aug 2006.
- **Chandrasekaran P.**, Wang J., Sadiq T., Gerber DA - “Development of a Bio-hybrid liver using 3D Biodegradable Scaffolds and Hepatic Progenitor Cells”- North Carolina Tissue Engineering Interest Group Meeting, Research Triangle Park, NC, June 2003.

## **2 Literature Review**

### **2.1 Tissue Engineering for Organ Replacement**

Tissue engineering is the science of using living cells to restore and rebuild tissues and organs. Engineered tissues could greatly reduce the need for organ replacement and could also help in the development of new drugs [1]. Age related organ degeneration is another reason for the increased demand for engineered tissues. Unlike some pathological conditions from which the body can recover, organ degeneration can be permanent requiring immediate whole organ transplants. Due to the long waiting period for these procedures, engineered tissues are being used to maintain temporary function in the diseased organs until a suitable replacement can be found. Examples of such maladies are end stage renal or liver disease and macular degeneration.

The conventional approaches for tissue or organ replacement [2] are:

#### **Autografts**

This is the process referred to as “*creating a wound to fix a wound*”. Tissue from one location in the patient is harvested and transplanted to another location. The main advantage of this procedure is it produces almost no immune response since the tissue is not recognized as foreign. The main disadvantage is risk of pain and infection at the tissue harvest site. An example of an autograft is

a coronary bypass procedure where veins are harvested from the patient's leg and used to replace blocked arteries in the heart.

### **Allografts**

These are organs or tissue obtained from an allogeneic donor and then transplanted into a recipient. The donor may be dead or alive depending on the organ donated. The challenge with allografts is that the patient's immune system will recognize the tissue as "foreign" and initiate an immune response to reject the tissue. Tremendous advances in drugs that prevent or minimize rejection (immunosuppressive agents) have been developed so that the problem of rejection is currently more manageable. Unfortunately, the shortage of organ donors remains a significant limitation in transplantation.

### **Artificial Materials and Devices**

The field of Biomedical Engineering has brought together the fields of medicine and engineering which has enabled the creation of devices and materials that mimic biological systems [3]. These devices and materials can be used to augment or even replicate functions performed by natural organs. These devices do not behave physiologically like true organs or tissues. Thus, these devices are best suited as temporary therapies until a donor organ becomes available.

Other available technologies include obtaining tissues and organs by cloning and gene therapies. While these two have received much publicity, many problems will need to be overcome before they become clinically available.



## **2.2 Stem Cells in Tissue Engineering**

One important criterion in tissue engineering is the choice of cell type. The cells are expected to take up residence in the implant material, proliferate, form colonies and populate the tissue. It is thus important that we choose a cell type that has good proliferation characteristics. Typical tissue engineering applications have employed mature cell populations to populate tissue constructs with limited success. This is because mature cell types have low proliferative capabilities.

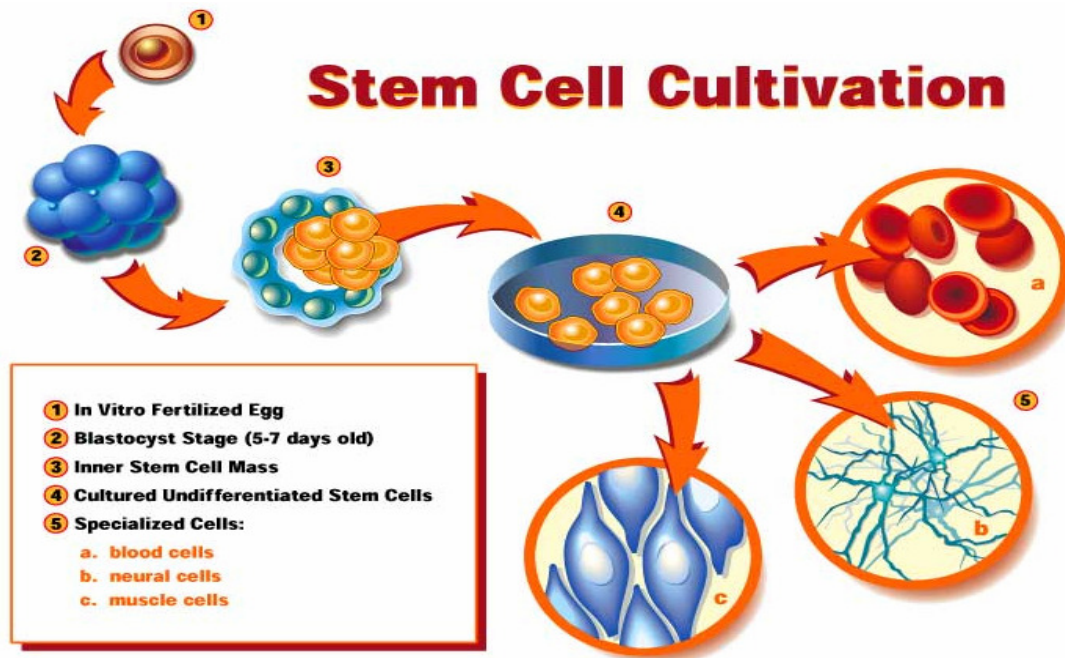
Proliferative potential however, is not the only decisive factor in the choice of cell type. The grafted cell should also be capable of responding to external stimuli and respond by differentiating into a mature cell type which forms the bulk of the tissue/organ.

The availability and use of stem cells has revolutionized the field of tissue engineering [4]. Stem cells were initially thought to exist only in the bone marrow but recent studies have proven that many tissues in the body have their own stem cell compartment. The field of embryonic stem cell research is also gaining importance.

Stem cells can be broadly classified into two major types:

### **Embryonic Stem Cells**

These cells are obtained from very early stages of the embryo called the blastocyst. The inside of the blastocyst called the inner cell mass is harvested and cultured to obtain embryonic stem cells [5].



**Figure 2-1: Schematic for isolation of Embryonic Stem Cells.**  
 (Image Source: <http://stemcells.nih.gov> )

Since embryonic stem cells are undifferentiated, they can differentiate to form any cell in the body. This totipotent characteristic of these cells makes them important in regenerative medicine. They also possess excellent proliferation characteristics as they are at the beginning of their differentiation cycle. However, since these cells are obtained from the embryo, there are ethical issues involved with their usage.

### **Adult Stem Cells**

Adult or somatic stem cells are isolated from mature tissues and are believed to be involved in normal regeneration of the tissue upon injury or disease. These cells are “undifferentiated” and are found scattered within the tissue or organ. The cells are said to be “committed” and can only form the type of tissue from which they are harvested. They form a basis for the tissue or

organ's response to injury. Upon injury or cell loss, they can divide and differentiate into the major cell types of that tissue or organ. As a result, they can be potentially used to treat tissue loss.

These cells do not pose ethical and moral issues as they are isolated from adult tissue. Adult stem cell research has gained immense importance because of the huge controversy surrounding embryonic stem cell research. Recent research has also shown that some adult stem cells are capable of trans-differentiation to cells of other tissue types.

### **2.3 Adult Stem Cell Research – Past and Present**

In the 1960's it was found that bone marrow contains two kinds of stem cells, the hematopoietic stem cells and the bone marrow stromal cells. The hematopoietic stem cells are known to form all the blood cell types in the body. The stromal cells differentiate to form bone, cartilage, fat and other fibrous connective tissue. At the same time, scientists also discovered that the rat brain has resident cells that can differentiate to form the major cell types of the brain (astrocytes, oligodendrocytes and the neurons). Scientists all across the world have since then discovered stem cells in all major organs of the body. Adult stem cells compartments have been discovered in the brain, heart, bone marrow, blood vessels, muscle and liver [5].

The problem with adult stem cells is that they constitute a very small portion of the cell mass. They remain dormant until they are activated by conditions such as disease or physical injury. Scientists are now working on mechanisms to isolate, culture and expand this stem cell population ex-vivo with

the aim of transplanting these cells into injured tissue to enable recovery. The issue of identifying and isolating these stem cells from the myriad of cells in every tissue/organ has perplexed researchers.

Research is ongoing to develop methods to successfully identify these cells from the heterogeneous cell population in the tissue. The most popular method to isolate these stem cells has been to select a molecular marker that is expressed on the cell of interest and use techniques like flow cytometry to sort the cell population that is positive for that marker. Research is also underway to find molecular markers globally expressed on such adult stem cells. There is no agreement on the choice of cell type among researchers yet.

Graft rejection is a difficult problem in transplant medicine that can only be circumvented with immunosuppressive drugs. Adult stem cells can be isolated from a patient's own cells, expanded and preserved and then transplanted back into the patient. Use of adult stem cells would mean that the cells would not be rejected by the immune system. This represents a significant advantage as the difference between donor demand and supply widens.

The discovery of adult stem cells in many tissues has created opportunities for researchers to find answers that have previously eluded them. Since stem cells have the capacity to self renew they can create genetically identical copies of themselves called clones. Research is ongoing to produce adult stem cell lines that are “undifferentiated” and can be coaxed with external stimuli to differentiate into a mature cell type of that tissue when needed. This has a huge demand in the field of cell transplant therapy where the clones can be

injected into the damaged tissue, engraft, differentiate and replace the tissue's lost cell mass.

## **2.4 Biohybrids for Tissue Regeneration**

The discipline of tissue engineering has gained importance in recent years. To engineer living tissues in vitro, cultured cells must be coaxed to grow on bioactive degradable scaffolds that guide their differentiation and assembly into three-dimensional (3D) tissues [6]. These bio-hybrids are then cultured and transplanted to induce the growth of new tissue. The goal is to induce cell attachment, proliferation and differentiation into normal healthy tissue as the bio-hybrid construct degrades. Skin and bone replacement have been the best success stories so far in tissue engineering. Many issues need to be resolved in order to enable creation of more complex tissues like heart and liver.

## **2.5 Micro-Encapsulation for Tissue Regeneration**

The basic principle of micro-encapsulation is to provide the encapsulated cells with a protective microenvironment. The cells are protected from external insults like shear and macrophages (upon transplantation). Lim et al. encapsulated Islets of Langerhans [7] using a protocol first developed by Chang in 1964 [8].

Encapsulation techniques have been used for the treatment of Parkinson's disease [9], hepatocytes [10] and hypoparathyroidism [11]. Since encapsulation is not cell type restrictive, it has also been used to encapsulate genetically modified cells and even hormones [12, 13]. The most published application of

this method to date has been encapsulation of Islets from the pancreas for treatment of diabetes [14-16].

## **2.6 The Liver**

### **2.6.1 Liver Structure**

The liver is the largest internal organ in the human body [17] and an average human liver weighs about 1-2.5 kilograms. The liver is located below the diaphragm on the right upper side of the abdomen. It has a dual blood supply of which 80% is nutrient-rich blood from the portal vein arising from the stomach, intestines and spleen and 20% is oxygen-rich blood from the hepatic artery. At a microscopic level, the liver is made up of individual units called lobules. Each lobule is surrounded by four to five portal triads and has a central terminal hepatic venule. Hepatocytes are the major cell type in the liver. They are arranged in plates that branch out from the portal triads toward the central veins. The bile canaliculus is found on the contact surface of adjacent liver cells. Bile is produced in these canaliculi and drains into the duodenum through ductules, interlobular bile ducts and then larger hepatic ducts.

Sinusoidal lining cells are another important cell group consisting of endothelial cells, Kupffer's cells, fat-storing cells and pit cells. The hepatic endothelial cells lack a basement membrane unlike the vascular endothelium found everywhere else in the body. They contain numerous fenestrae that enable hepatocytes to access nutrients and macromolecules in the blood plasma. The Kupffer's cells constitute the liver macrophage compartment. The major functions of this cell type are phagocytosis of foreign objects, removal of harmful toxins

and the release of agents that modulate the tissue's immune response. Fat-storing cells (Ito cells) are the storehouse for Vitamin A. They are converted into fibroblasts upon liver injury, causing hepatic fibrosis. Pit cells, the least common sinusoidal cell type, are lymphocytes, which function as natural killer cells (NK cells).

Another cell type that is normally in the quiescent state is the hepatic stellate cell. These cells are activated during liver cirrhosis when they overproduce certain proteins, such as type I collagen. The extra-cellular matrix of the liver includes its reticulin framework and several molecular forms of collagen, laminin, fibronectin and other extra-cellular glycoproteins.

### **2.6.2 Liver Functions**

The liver plays an important role in the body's metabolic process [18] and also fulfils functions such as detoxification, glycogen storage and synthesis of coagulation factors in the plasma. A majority of the liver functions are carried out by the liver cells also called hepatocytes.

Some of the important functions of the liver are:

- Secretion of bile
- Metabolism of carbohydrates (Includes gluconeogenesis and glycogen synthesis)
- Lipid metabolism (synthesis of cholesterol)
- Breakdown of hormones (insulin)
- Production of blood coagulation factors (fibrinogen etc.)
- Detoxification of blood and neutralization of drugs

- Conversion of ammonia to urea
- Other functions include storage of glycogen, Vit.B12 etc.

### **2.6.3 Rationale behind Cellular Encapsulation**

Clinical advances in surgical technique and immunosuppression have made liver transplantation the primary clinical option for patients with end-stage liver disease [41, 42]. Despite optimized use of donor livers through split liver transplantation and living-donor liver transplantation; organ needs do not meet the increased demand by patients diagnosed with advanced liver disease [43, 44].

Several cell-based therapies for liver disease have been proposed because of this severe donor shortage (e.g. extracorporeal bio-artificial liver devices, cell transplantation, and tissue-engineered liver constructs) [45, 46]. These therapies require an adequate cell supply with stability of liver-specific functions. Although extracorporeal cell-based support devices have been studied in clinical trials, the efficacy of engineered cell therapies in the management of liver disease requires further improvements to incorporate approaches for maximizing hepatocyte function. To apply such strategies, we must integrate principles from liver biology as well as the clinical experience for supporting patients with liver failure.

Successful cellular therapies must therefore integrate individual liver-derived cell populations into a microenvironment that supports their potential expansion and maintains liver-specific functions. Unfortunately, the full gamut of cellular functions required to replace the liver and affect clinical outcomes has yet



to be fully determined. Mature hepatocytes have been previously used in cell transplantation but have had minimal success due to their limited engraftment, sensitivity to ischemia and minimal proliferative ability [22, 47-49].

Wang et al. characterized a hepatic progenitor cell population isolated from uninjured adult liver that phenotypically differentiates into both hepatocytic and biliary cell populations [50]. It is theorized that since hepatic progenitor cells have proliferative potential and the ability to differentiate to mature cells, they may be better suited for transplantation than mature hepatocytes [51-58].

Numerous challenges still exist with tissue-derived (or somatic-derived) progenitor cell populations like this hepatic progenitor cell. It is challenging to isolate these cells as they are typically quiescent in their in situ state and the requisite signals (either through paracrine signaling from other cells or the microenvironment) are frequently unknown.

## 3 Hepatic Progenitor Cells on Collagen Biohybrids

### 3.1 Introduction

Tissue engineering techniques to repair or replace failing organs have generated great interest due to the shortage of donor organs [68]. These techniques typically involve culturing specific cells from the tissue to be replaced with support architecture like a porous sponge or scaffold and transplanting them to replace the failing organ or to provide sustenance till a replacement organ can be found.

There are typically two approaches to tissue engineering:

1. A *cell biology approach* where culture conditions are tweaked so as to coax cells to grow and flourish in a 3-dimensional environment. This approach usually requires detailed understanding of the biology and pathophysiology of the organ being replaced. This approach requires knowledge of the different cell types in the organ, their proliferative and differentiative abilities and also control over conditions that can induce or inhibit such growth. This includes cells mixing cells with different media components, cytokines and growth factors.

The other major area of interest involves creation of co-culture systems. Here the proliferation/differentiation cascade of the cell of interest is triggered by culturing it in close proximity with another cell type

normally found in that organ and permitting signaling between them. This signaling could be either by physical contact or by soluble chemical signals in a separated co-culture system. The reasoning behind this method is that cells in the tissue are never in isolation. They always are in contact with other native cell types and there is definite evidence that communication between different cells in the organ/tissue is a critical feature. Because of this, an optimal tissue engineered organ cannot be made using a single cell type but should include the different cells that inhabit that organ.

2. A *matrix biology approach* where the extracellular matrix is assumed to play the most important role in regulating cellular proliferation and differentiation. This involves combining novel biomaterials with cells or using naturally occurring matrices as tissue replacements. The former approach involves adjusting the matrix material to provide the most suitable environment for the cell of choice. The latter approach is done in engineering tissues like skin, tendon and bone. In these cases, it may not be even necessary to include cells in the engineered tissue. The biomaterial is expected to integrate into the recipient as their cells grow into the implant. One example of such a natural matrix is the small intestine submucosa (SIS) that is currently being used as a replacement of skin and even tendon.

### **3.2 Tissue Engineering of the Liver – A Historic Perspective**

A successful tissue engineered liver has been the Holy Grail of the field of tissue engineering. There are a multitude of reasons that stimulate interest in engineering liver tissue and an equivalent number why it has been unsuccessful thus far. As explained in detail in an earlier section, the liver is an extremely complex organ containing its own stem cell compartment, multiple differentiated cell types performing various metabolic functions, and numerous extracellular matrix components all performing synergistically. Engineering the liver is tricky because of its physical complexity, multifunctionality and the vitality of its function for survival. Also troublesome is the fact that differentiated liver cells (hepatocytes) lose their function when cultured in-vitro.

Numerous approaches have been published in the literature for 3-dimensional culture of liver cells that are directed towards studying the physiology, pharmacology and tissue engineering of the liver. They range in complexity from culture on collagen coated polystyrene plates [69] to microfabricated perfusion chambers [70]. Hepatocytes have also been cultured on a plethora of biocompatible materials with a 3-dimensional porous architecture including synthetic materials like PLA/PLGA and natural matrices like collagen, alginate [71] and hyaluronan and blends of natural and synthetic matrices [72]. The majority of the synthetic matrices used in these studies are those approved by the FDA for use as suture material.

The other approach that generated great interest in the 1990s was the construction of Bio-Artificial Livers (BAL). A typical BAL design involves

inoculating a bioreactor with either primary hepatocytes or immortalized cell lines and supplement hepatic function in a patient by processing their blood or plasma through the cultured cells [73].

The majority of the bioreactor designs differ only in two aspects: the variation of extracellular matrix design and the cell source. Initial bioreactor/BAL designs [74, 75] did not possess a special culture chamber/matrix. They consisted of a perfused suspension of hepatocytes immobilized in a bioreactor chamber.

Later modifications included cell carriers [76], natural matrix substrates like collagen [77] and other advanced culture techniques like microencapsulation, porous scaffolds, cell-matrix sandwiches and co-cultures of different cell types [78,79]. The cells used in BAL systems have traditionally been porcine mature hepatocytes due to the shortage of donor organs to obtain human hepatocytes. A reliable source of differentiated hepatocytes is required to inoculate the BALs on demand as bioreactors primarily function as emergency liver supplements, it is required to have. This requires culturing hepatic cells in-vitro and when needed induce differentiation into mature hepatocytes.

The discovery of a stem cell compartment in the liver has revolutionized in-vitro culture of hepatic cells. These include oval cells [80], small hepatocytes [72, 73] and hepatic progenitor cells [50]. It is now possible to culture the committed undifferentiated cells on 3-dimensional matrices in-vitro and cause differentiation using chemical inducers when a BAL is necessary.

The lack of a robust BAL design has led researchers to look for other potential therapies for liver failure. These include delivery of hepatic cells by

direct injection, encapsulating them on 3-dimensional biohybrids or in microcapsules.

### **3.3 Materials and Methods**

#### **3.3.1 Mice**

C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All animals were maintained on a rodent chow under a constant day/night cycle. Six to eight week old mice were used in all experiments. A “GFP tagged” mouse model was used for purposes of visualization of cells on the scaffold.

All care and use of animals was approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

#### **3.3.2 Hepatic Progenitor Cell Isolation**

Liver cells were isolated using a modification of the two-stage liver perfusion technique described by Seglen [62]. The hepatic progenitor cells (HPC) were separated from mature hepatocytes using gravity separation and centrifugation. Cellular analysis by phase contrast, fluorescent and confocal microscopy demonstrated that the hepatic progenitors formed ex vivo colonies with a morphological appearance similar to committed hepatocytic progenitors isolated from embryonic mice and rats. The protocol was slightly modified from the isolation and characterization of HPCs previously established [50]. All initial isolation steps were performed on ice to keep the cells metabolically dormant.

### **3.3.3 Collagen Scaffold Preparation**

BD™ 3D collagen composite scaffolds were sectioned into two pieces with a thickness of about 3-5 mm to achieve an open architecture for cellular ingrowth. The pieces were then soaked in 70% ethanol for a few hours and air dried overnight in a laminar flow hood under ultraviolet light. The sterile scaffolds were transferred into 96 well plates for cell culture.

### **3.3.4 Cell Loading**

Both HPCs and mature hepatocytes were separately loaded at a concentration of about  $4 \times 10^5$  cells per scaffold by slowly pipetting the cells suspended in growth medium over the scaffold in the 96 well plates.

After cell-loading the plates were kept on a rocker to aid uniform diffusion of nutrients through the scaffold. As a control HPCs were also cultured on 35 mm plates with a collagen I coating.

The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) plus 10% fetal bovine serum (FBS), 20mM hepes, 10mM nicotinamide, 1 mM ascorbic acid 2-phosphate, 1  $\mu$ M dexamethasone, 0.5 mg/L ITS (insulin-transferrin-selenium) solution, 30 mg/L proline, 100 mg/L antibiotic solution, and 10 ng/ml epidermal growth factor (EGF).

The plates were kept in a 5% CO<sub>2</sub>/95% room air incubator at 37°C. The medium was replaced after 1 hour of loading to remove unattached cells and subsequently replaced at regular time intervals (3-4 days). The supernatant was collected and frozen at each time point for analysis.

### **3.3.5 Light Microscopy**

The GFP<sup>+</sup> cells were visualized with a Zeiss Axiovert 100 inverted fluorescence microscope at regular time intervals to ascertain lack of contamination and to study cell distribution within the scaffolds and colony formation. Care was taken to check for absence of any reading on the red (Rhodamine) channel as hepatocytes are notorious for auto-fluorescence.

### **3.3.6 Colorimetric Assays for Cellular Proliferation**

As a supplemental test for cellular proliferation and differentiation, urea and protein synthesis by the HPCs were analyzed in the media at specific time points in culture (1, 4, 7, 10 etc.). These assays were performed according to the manufacturer's protocol using commercially available test kits (Sigma-Aldrich Corp.).

### **3.3.7 Transplantation of collagen biohybrids**

The collagen biohybrids loaded with GFP<sup>+</sup> HPCs were transplanted into wild type C57BL/6 mice initially to analyze the effect of the in-vivo environment on the cells in the biohybrid. The host mice were sacrificed 1 week post transplant to isolate transplant biohybrids.

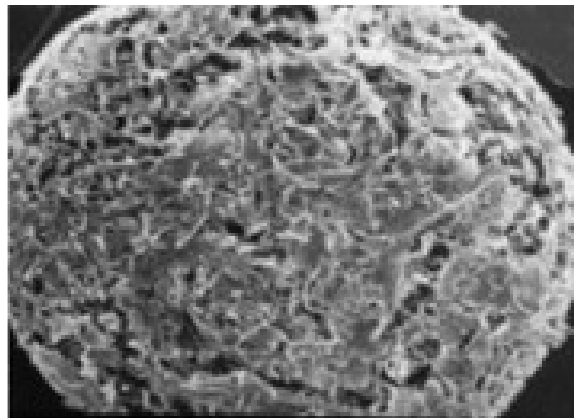


## 3.4 Results

### 3.4.1 Microscopy

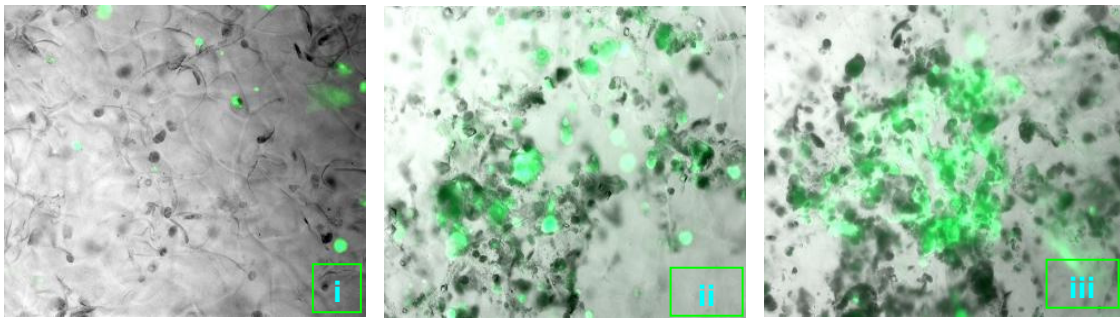
Figure 3-1 shows a SEM image of the BD collagen composite scaffold. The image depicts the highly porous nature of the scaffold and even though the outer surface appears slightly closed, sectioning it into two presents an open architecture for cell penetration into the scaffold center.

Figure 3-2 is a overlay (fluorescence + transmission) image of mature hepatocytes and Figure 3-3 shows fluorescent micrographs of HPCs cultured on the BD™ collagen composite scaffold at specific time points in culture. Viable cells (HPCs and mature hepatocytes) were found to be uniformly distributed through the lumen of the scaffold and are viable even at day 28 in culture. The rhodamine channel registered no auto-fluorescence from either the scaffold material or the cells.

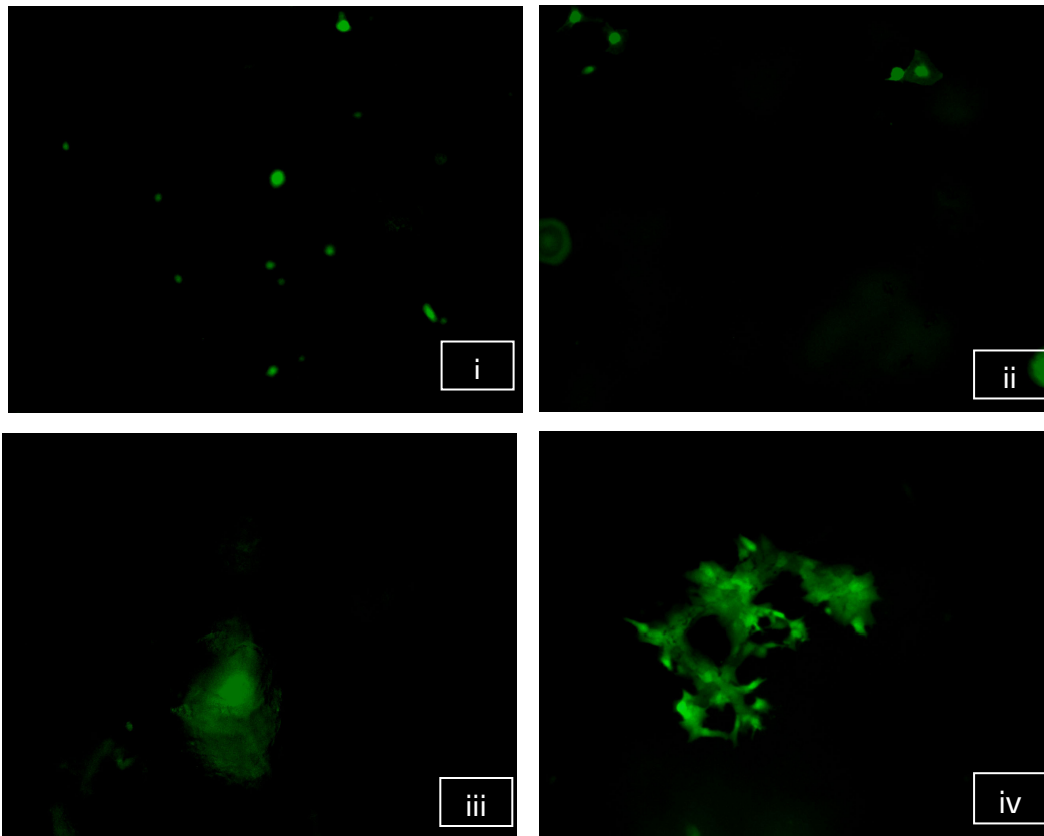


**Figure 3-1: Scanning Electron Micrograph of the BD™ collagen composite scaffold showing a closed pore structure on the exterior.**

Source: B-D Biosciences Catalog



**Figure 3-2: BD™ collagen composite scaffold loaded with GFP+ mature hepatocytes**  
Day 4 (i); Day 21 (ii); Day 28 (iii) in culture. Mag: 10x



**Figure 3-3: Fluorescent micrographs of GFP+ HPCs cultured on the BD™ 3D collagen composite scaffold.**

Day 1 (i), Day 7 (ii), Day 21(iii) and Day 28 (iv). Mag: 10X

### **3.4.2 Colorimetric Assays for Hepatic Function**

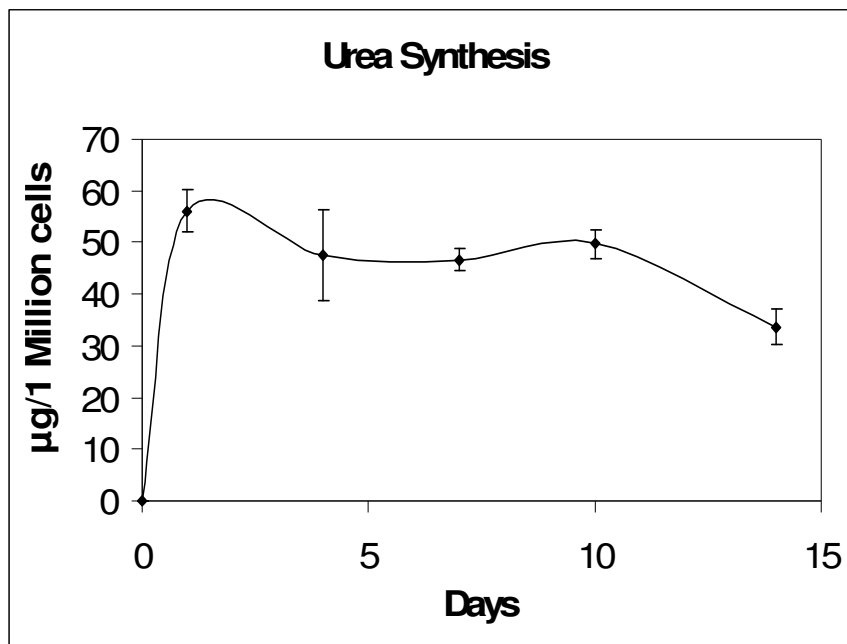
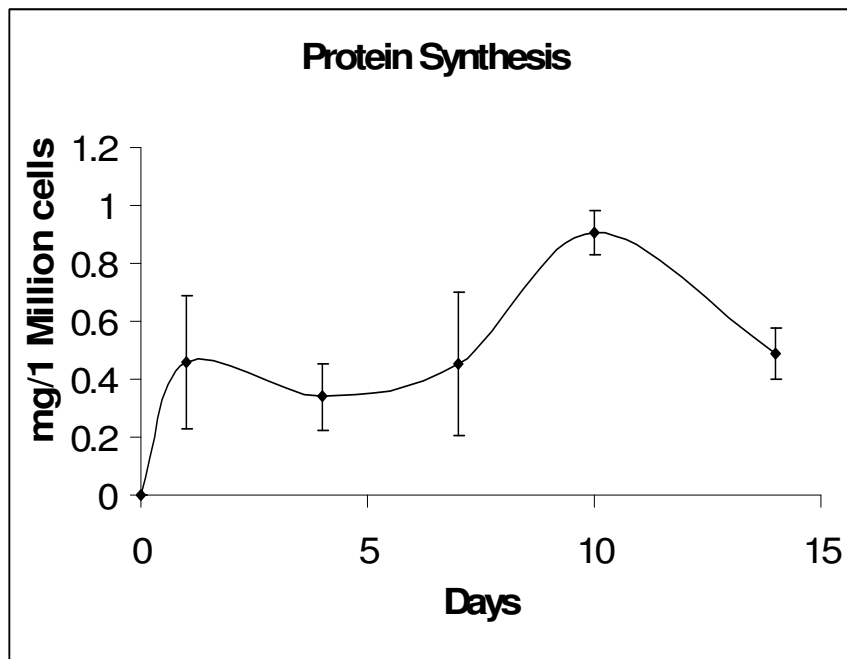
The cells' ability to undergo urea-nitrogen and protein synthesis was studied in order to analyze hepatocellular function of the HPCs cultured on the collagen scaffolds. Figure 3-4 shows the protein and urea synthesis by HPCs cultured on the collagen scaffolds.

These assays were evaluated at select time points during 2 weeks of culture and demonstrate persistence of urea metabolism and protein synthesis during that time. The data from both the urea-nitrogen and protein synthesis assays were normalized for number of cells loaded per scaffold.

### **3.4.3 Transplantation of HPCs on Collagen Biohybrids**

To analyze effect of transplantation on the HPCs, recipient mice were sacrificed one week post-transplant. However, the scaffolds could not be found in the test site. This shows that the scaffolds cannot be used "out of the box" as a transplant vehicle and need to undergo further treatment to increase their longevity in-vivo.

This can be accomplished by treating the collagen scaffolds with glutaraldehyde but the toxic effects on the cells need to be evaluated. The other alternative is to use natural materials like genipin that has excellent crosslinking properties and have been proven to be biocompatible when used in combination with other polymeric materials (73).



**Figure 3-4: Total Protein (a) and Urea-Nitrogen (b) synthesis by the HPCs on the BD™ Collagen composite scaffold.**

### **3.5 Conclusions and Future Work**

Collagen has long been the favored material for building biohybrids for purposes of tissue engineering because of its abundance in all tissues and also due to its great material properties and biocompatibility. The ability to manipulate and mold collagen into desired shapes also leads to its popularity as a tissue engineering substrate.

The BD™ collagen composite scaffold is a natural scaffold manufactured from a proprietary mixture of collagens that are derived from bovine hide. Overall, this material exhibits collagen fibrillar architecture, which is representative of the structure of collagen within the interstitial matrix.

From the results, it is evident that cells undergo proliferation and colony formation on this substrate. The drop in urea and protein synthesis after Day 10 in culture is cause for worry. This loss in hepatic function is indicative of two possible events:

- (a) De-differentiation of the hepatic progenitor cells into non-committed cells or
- (b) Proliferation of non-parenchymal cells like fibroblasts, stellate cells etc. in the isolation matrix that do not express hepatic markers combined with the death of the bi-potential progenitor cells.

These results suggest a need for a further purified progenitor cell population that does not have the mature hepatocyte or non-parenchymal cell constituents. Experiments are ongoing to purify the hepatic progenitor cell population using a magnetic separation process using the antibody Sca-1 (Stem Cell Antibody).

The transplantation study reveals the necessity to crosslink the collagen matrix, usage of other natural matrices that have demonstrated better longevity when used in-vivo or the usage of other strategies for hepatic therapy or cell delivery like encapsulation in matrices like calcium alginate.

## **4 Alginate Micro-spheres for Hepatic Therapy**

### **4.1 Introduction and Literature Review**

The concept of alginate encapsulation has been well described for several decades, but has typically involved encapsulation of mature, differentiated cell populations (e.g. islets, mature hepatocytes or cell lines) [19-21]. Alginate is an excellent encapsulation substrate because it is a hydrogel (preparation entirely aqueous), and it has no detectable adverse effects on cells during preparation or post-cast.

Alginate maintains its material properties for long periods of time in culture [22-33]. The porous properties of sodium alginate also make it an ideal choice for cellular encapsulation. Analysis of calcium alginate gels demonstrated a wide range of pore size in the gels. This typically fosters the secretion of large molecules and growth factors both into and out of the alginate bead. Encapsulating the cells with alginate protects them from physical insults that will occur during direct injection of these cells. These insults include decreased cell viability from shear during injection and minimizing direct cell contact with phagocytic cells of the host upon transplant [37].

In a recent study, Rahman et al. [34] demonstrated the use of human HepG2 cells encapsulated with alginate in plasma perfusion bioreactors. These cells were observed to maintain differentiated liver functions and were able to

reverse the loss of systemic functions after chemically-induced liver injury in a rabbit model including acid–base balance, cerebral oxygen consumption and systemic blood pressure.

Mai G et.al [35] demonstrated that alginate beads can be used to cryo-preserve immortalized hepatocyte cell lines, thus providing an instant supply of cells to populate bioreactors. In previous studies, Aoki et al [38] established that the spleen was a good transplant site for encapsulated hepatocytes due to easy availability of nutrition and showed that intrasplenic transplantation of encapsulated mature hepatocytes into a rat model with 90% partial hepatectomy enabled the animal to survive without the need for immunosuppression [39]. They also observed rapid liver regeneration after the transplant. Minimal fibrotic response but with no immune response was observed from the host despite the fact that alginate was a foreign body.

Alginate encapsulation is not restrictive of cell type and can also be used to develop three dimensional co-culture systems as demonstrated by Gao et.al [40]. In their studies, xenogeneic transplants of a co-culture mix of primary rat hepatocytes and islets into mice were found to functionally out-perform hepatocytes or islets encapsulated separately.

## **4.2 Encapsulation Techniques**

Three different techniques were used to encapsulate the hepatic progenitor cells in alginate:



#### **4.2.1 “Cannon-Ball” Method**

In this method, the alginate solution was drawn up in the syringe and dropped from a height of 2-3 cm into a receiving bath containing 125mM  $\text{CaCl}_2$  solution. The droplets instantly gelled to form 3-5 mm diameter beads. Experiments performed with this method revealed that the encapsulated cells died within several days of encapsulation presumably secondary to a lack of nutrition due to the huge diffusion distances involved. Though this method had the advantage of maximum control and minimum stress on the encapsulated cells, it was abandoned for alternative methods that would produce beads with smaller dimensions.

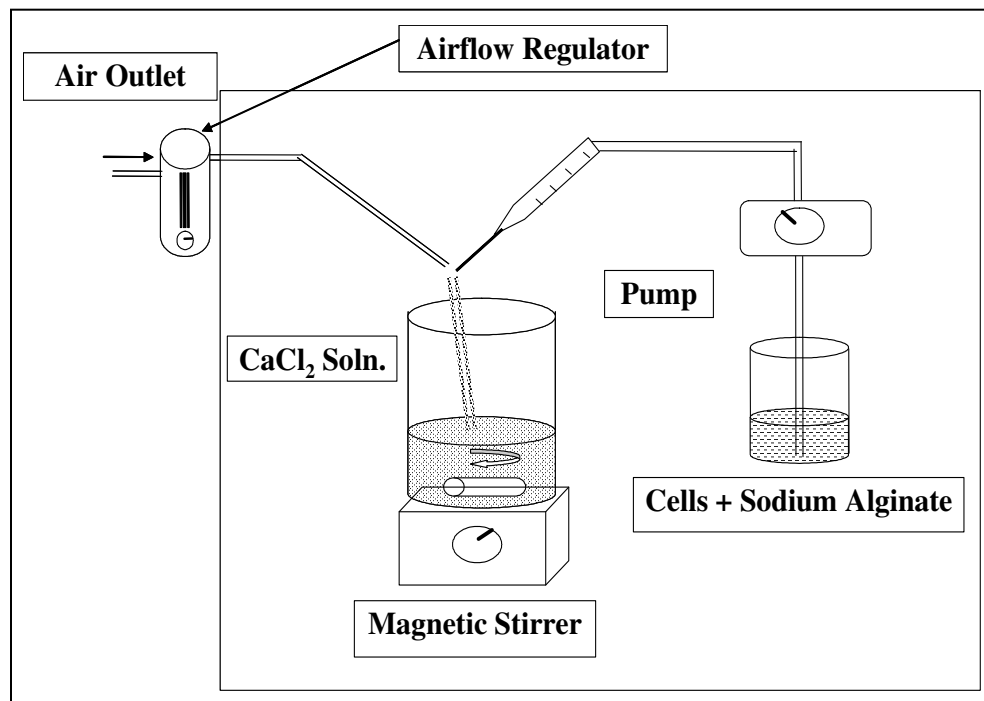
#### **4.2.2 Air-Jet Encapsulation Method**

A controlled air stream was used to make alginate beads in this system. A wall air outlet was fitted with a filter and an air valve with a control mechanism (kindly provided by Dr. Bob Dennis, UNC-Chapel Hill). The syringe filled with cells suspended in 2% alginate solution was connected to a peristaltic pump. (Figure 4-1)

The jet of air at a rate 20L/min was concentrated on the outlet of the syringe such that the expelled solution would be broken into tiny droplets that would fall into a solution of  $\text{CaCl}_2$  held in a beaker below. The system was arranged so that the tip of the syringe was about 2-3 cm above the top level of the  $\text{CaCl}_2$  solution.

The pumping rate was set at 2 ml/min. The sodium alginate droplets from the syringe were reduced to 300-500  $\mu\text{m}$  droplets depending on the pump and air

flow rates. These micro-droplets were instantly converted to solid beads of calcium alginate upon contact with the  $\text{CaCl}_2$  solution in the beaker, thus encapsulating the progenitor cells within them. The calcium chloride solution was constantly stirred to prevent the beads from sticking to each other.

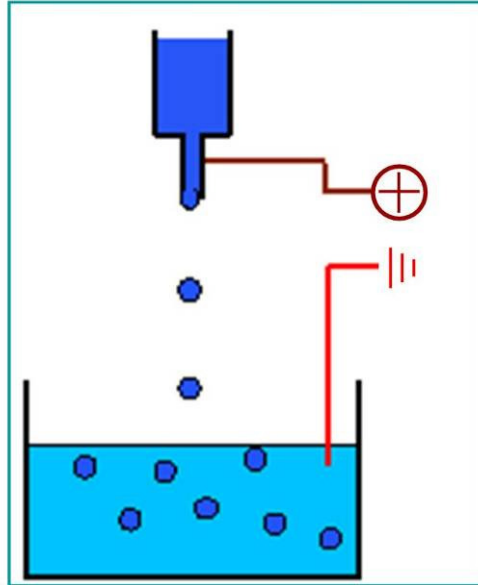


**Figure 4-1: Air-Jet Encapsulation System**

### **4.2.3 Electrostatic Encapsulation Method**

Cell encapsulation was also performed using an electrostatic bead generation apparatus (Figure 4-2) (81). In this setup a high electrostatic potential was used as the operating force to break the flow into tiny droplets. The syringe was loaded onto a syringe pump (Braintree Scientific BS-8000, Braintree, MA)

and arranged such that droplets ejected from the angiocatheter fell orthogonally into the receiving bath.



**Figure 4-2: Electrostatic Encapsulation System**

The receiving bath was a 250mL glass beaker filled with 125mM  $\text{CaCl}_2$  solution. The syringe was fitted with a 24 gauge angio-catheter soft tip. The angiocatheter was pierced at the hub with a 23 gauge needle to serve as the positive electrode in the electrostatic casting process. The distance from the angiocatheter tip to the surface of the  $\text{CaCl}_2$  was fixed at approximately 2.5 cm. Pump flow rates were set within the range of 0.75 to 1.5 ml/min. A grounded electrode was immersed in the  $\text{CaCl}_2$  receiving bath. An electrostatic potential was developed across the angiocatheter tip and  $\text{CaCl}_2$  bath by a high-voltage DC source (Spellman model RHR30PF30, Hauppauge, NY) in the range of 3.8 to 6 kV depending on the bead size required.

Bead size was controlled by adjusting the applied potential and pump flow rate. When the syringe pump was turned on in the presence of the high electrostatic potential, the expressed sodium alginate solution was pulled away as tiny droplets that polymerized into solid calcium alginate immediately upon contact with the calcium chloride solution.

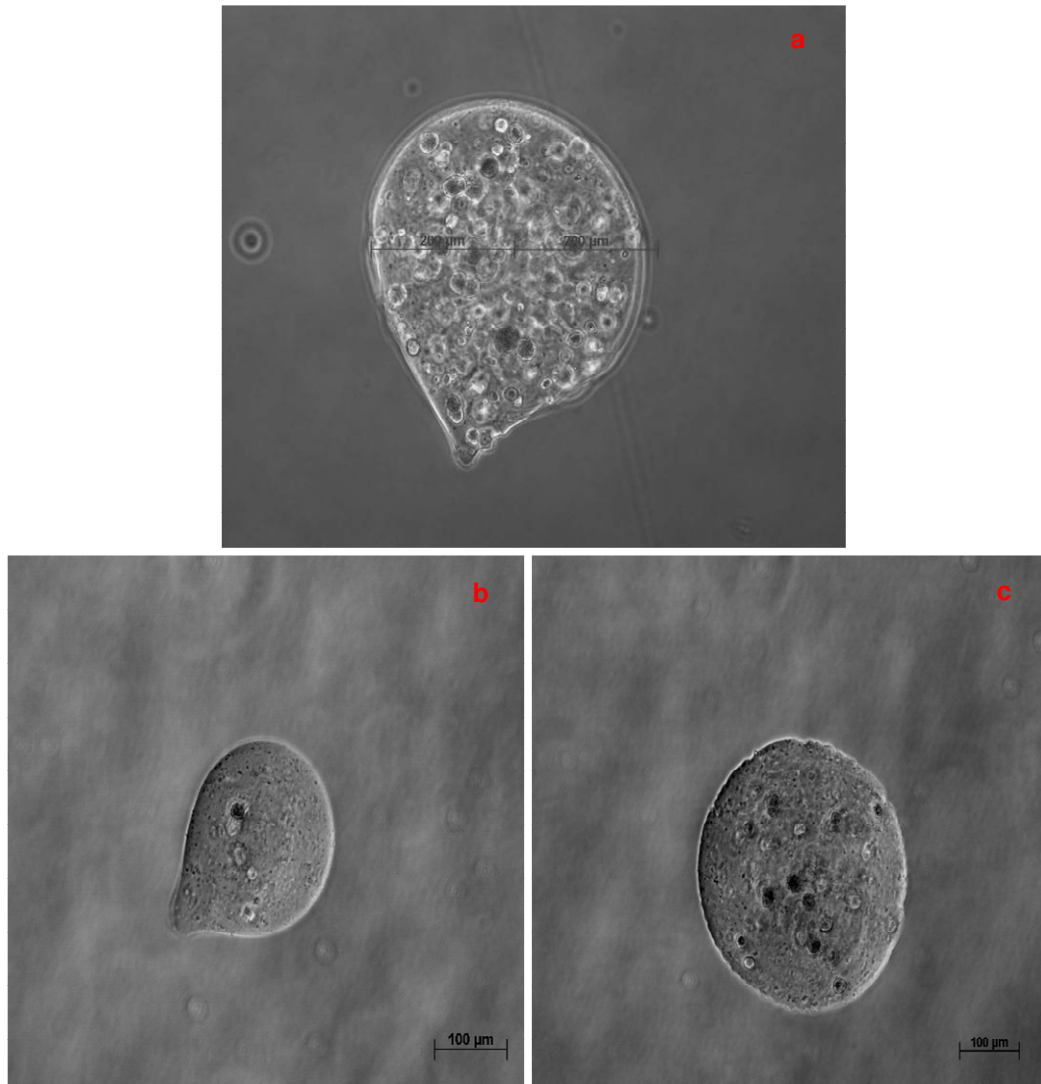
## 4.3 Results

### 4.3.1 Hepatic Progenitor Cell Encapsulation

Figure 4-3 shows transmission microscopy images of hepatic progenitor cell-containing alginate beads with differing diameters (HPCs:  $1 \times 10^6$  cells/ml initial concentration) produced using the Air-Jet encapsulation method (4-3a) and the Electrostatic encapsulation method (4-3b, c). These images demonstrate the overall spherical nature of the bead, range of bead diameter production and the ability to generate beads containing the progenitor cell population. The cells are distributed throughout each alginate bead. The beads in Figure 4-3a and 4-3c have a diameter of approximately 400  $\mu\text{m}$  and the bead in Figure 4-3b has a diameter of approximately 200  $\mu\text{m}$ . This is proof that with both these methods we were able to generate beads with controllable diameters. A noticeable irregularity in the bead's shape is a small tail generated on one end of the bead possibly due to the shear at the needle tip.

The Air-Jet system however suffered from operator inaccuracies in concentrating the air stream over the ejection port. This sometimes resulted in non-uniformity in bead size and shape. There was tremendous variability between encapsulations depending on the operator's hand position and movement. Another major concern was the shear stresses that the cells were exposed to with this method. Upon experimentation very little metabolic activity was demonstrated in cells encapsulated using this method. This finding necessitated research into other possible encapsulation techniques.

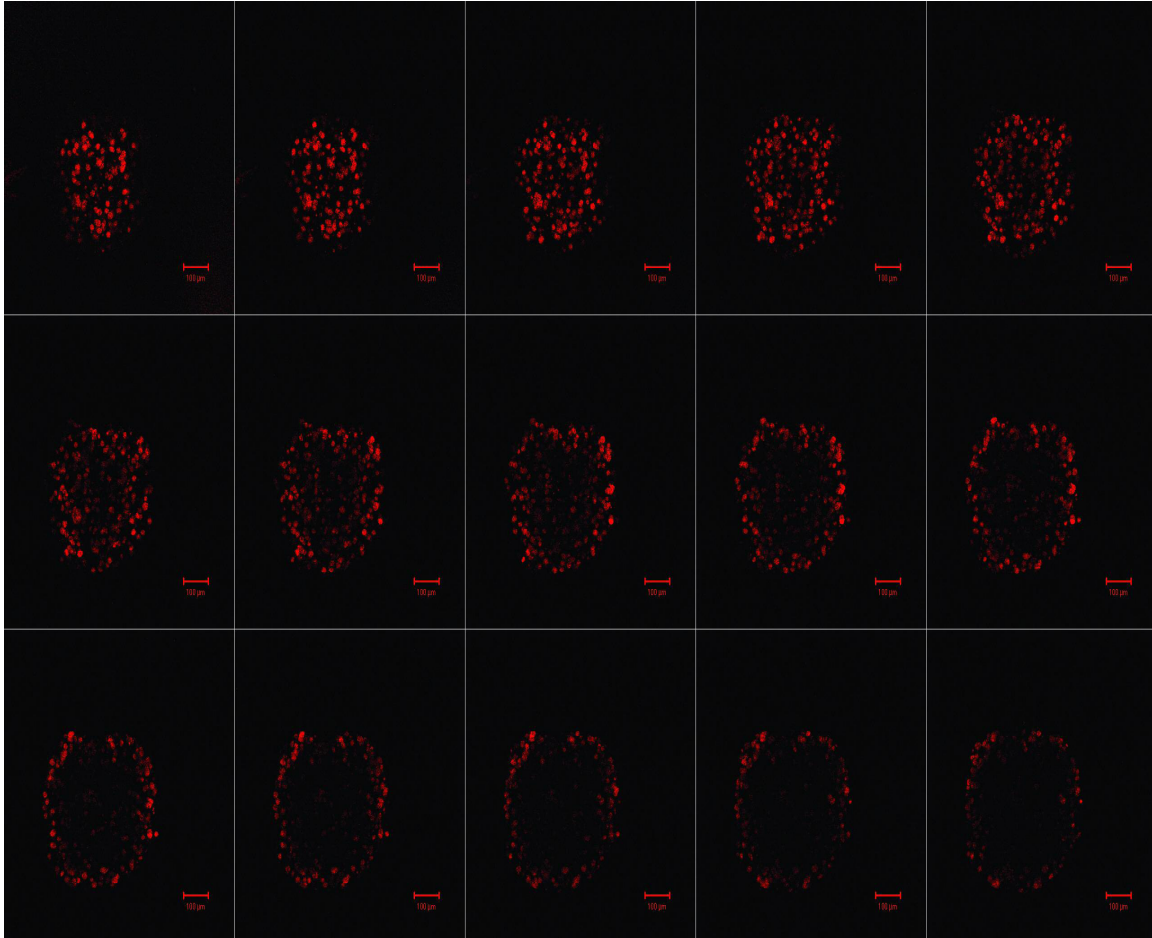
The electrostatic encapsulation method had more stability and resulted in improvements with respect to uniformity and reproducibility. In long-term culture (beyond 3 weeks) the alginate beads maintained their physical characteristics (e.g. shape).



**Figure 4-3: Hepatic Progenitor cells encapsulated in sodium alginate beads**  
(a) Using the Air-jet system and (b, c) The Electrostatic system. Mag: 10X

### 4.3.2 Assay for Cellular Viability using Confocal Microscopy

Viability of encapsulated hepatic progenitor cells generated using the Electrostatic method was determined by confocal microscopy using the Mitotracker® dye which is incorporated into the mitochondria of living cells.



**Figure 4-4: A series of confocal images of HPCs within an alginate bead. Mag: 10X**

The encapsulated cells remained viable for the duration of the experiment (day 0-21 in culture). Figure 4-4 shows confocal images of the encapsulated cells stained with Mitotracker®. The images were in consecutive focal planes 7.9 µm apart. There is no evidence of cell death within the core of the bead.

### 4.3.3 SEM to Analyze Bead Morphology

Figure 4-5 shows a SEM micrograph of an alginate bead produced with the electrostatic encapsulation method. The image shows a spherical bead of approximately 150-200  $\mu\text{m}$  with many surface imperfections. These are present because alginate is a hydrogel and undergoes shrinkage during the ethanol dehydration procedure. An uneven surface is partly due to the encapsulated cells that are protruding out of the surface (possibly due to expansion of the cell mass inside).



**Figure 4-5: Scanning electron micrograph of an alginate bead.**

Two interesting features that can be observed in this picture are the depression at the front end of the bead and the tail at the far end. The depression represents the region where the droplet struck the  $\text{CaCl}_2$  surface and the alginate



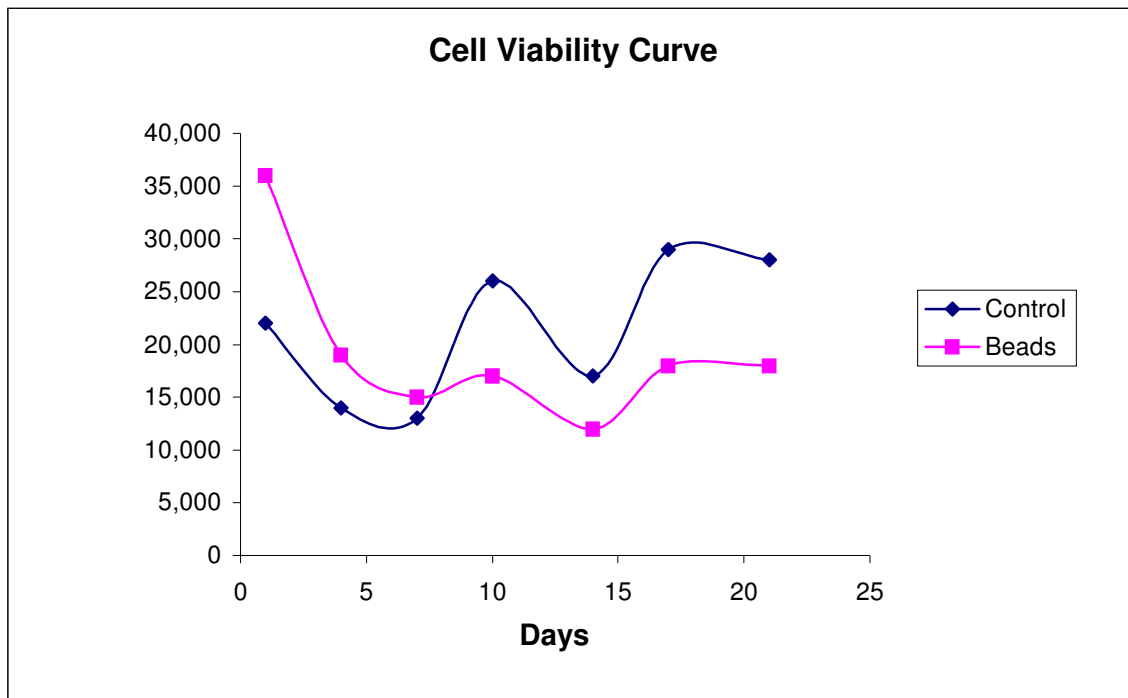
thread that breaks away from the ejection port forms the tail. These morphological imperfections are of concern because of issues related to adequate nutrition distribution. A few iterations of the fixation protocol for SEM were performed. The standard SEM fixative solution had a chelating agent which dissolved the beads instead of fixing them. After a few iterations, the problematic agent was removed to fix the problem.

Transmission electron microscopy was also performed (Data not shown) on the beads. The TEM micrographs of beads showed cells with poorly defined intracellular organelles. In subsequent experiments, this was found to be as a result of the TEM processing technique and not because of the encapsulation method. Cells that were obtained by breaking open the beads after encapsulation showed intracellular structure. It was decided not to pursue TEM to image encapsulations because of these technical challenges.

#### **4.3.4 MTT Assay to Analyze Cellular Proliferation**

It was necessary to analyze the proliferation characteristics of the cells to make functional comparisons between encapsulated HPCs and those cultured on two dimensional collagen coated dishes. A colorimetric assay based on mitochondrial breakdown of MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] was used for this purpose. The assay results showed that there was considerable cell loss in the first few days after the cells were isolated. This can be attributed to the culture conditions that are more attuned to proliferation and differentiation of HPCs and not the freshly isolated heterogeneous cell population. The other factor contributing to this cell loss was the stress undergone by the cells during the initial

procedures (isolation, encapsulation etc.). After about day 6 in culture, the cells recovered from the initial insults and proliferation could be demonstrated by the increase in cell number. By about day 7 mature hepatocytes whose proliferation had been stopped secondary to the addition of DMSO (day 4), start dying. This decrease in cell number was compensated by the proliferating HPCs and there is only a slight depression in the cell growth curve. After about three weeks in culture, cells entered a stationary phase of their cell cycle.



**Figure 4-6: Growth characteristics of encapsulated and 2-D cultured HPCs. (MTT Assay)**

These characteristics were true for both encapsulated and non-encapsulated HPCs which proved that the encapsulation procedure did not have a long term impact on the growth of these cells. It also proved that the encapsulated cells were adequately nourished and there was no significant death due to necrosis. The

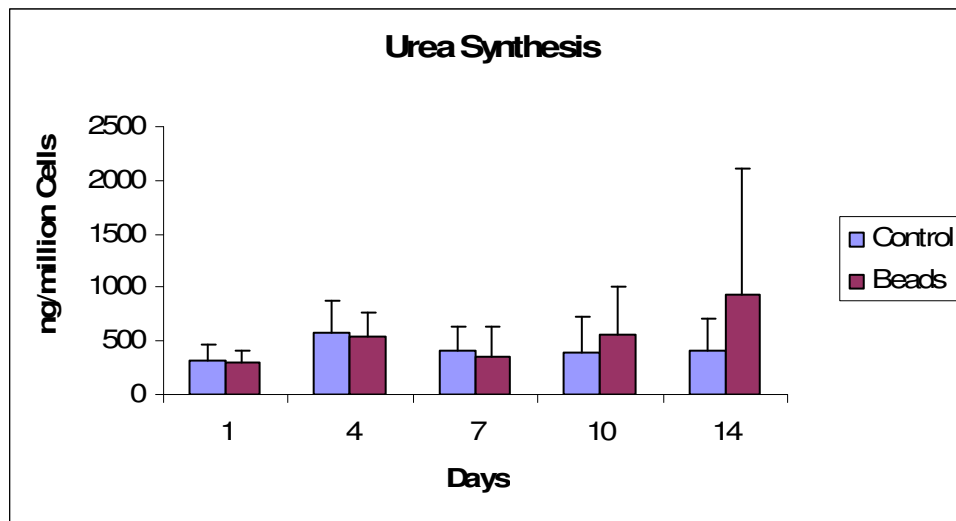
numbers from the viability experiments shown in Figure 4-6 were used to normalize the metabolic assays described below.

### **4.3.5 Analysis of Hepatocellular Function of Encapsulated HPCs**

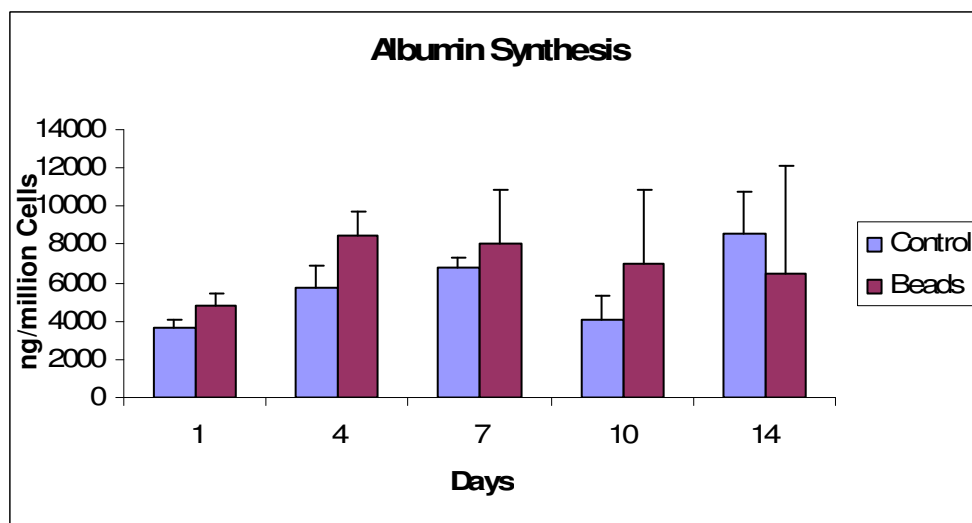
#### **4.3.5.1 Urea Nitrogen Metabolism and Albumin Production**

The cells' ability to undergo urea-nitrogen synthesis and their ability to produce albumin was studied to analyze hepatocellular function of the encapsulated HPCs. Figure 4-7 compares urea metabolism in the encapsulated hepatic progenitor cells versus a control (hepatic progenitor cells in routine tissue culture). This assay was evaluated at select time points during 2 weeks of culture and demonstrates persistence of urea metabolism during that time.

Figure 4-8 represents a graph for albumin synthesis by encapsulated HPC over a period of two weeks. Albumin synthesis by the encapsulated progenitors occurred at a rate comparable to cells grown on control dishes. The data from both the urea nitrogen and albumin synthesis assays were normalized for cell number obtained using an MTT assay.



**Figure 4-7: Comparison of Urea Metabolism by encapsulated and control HPCs.**



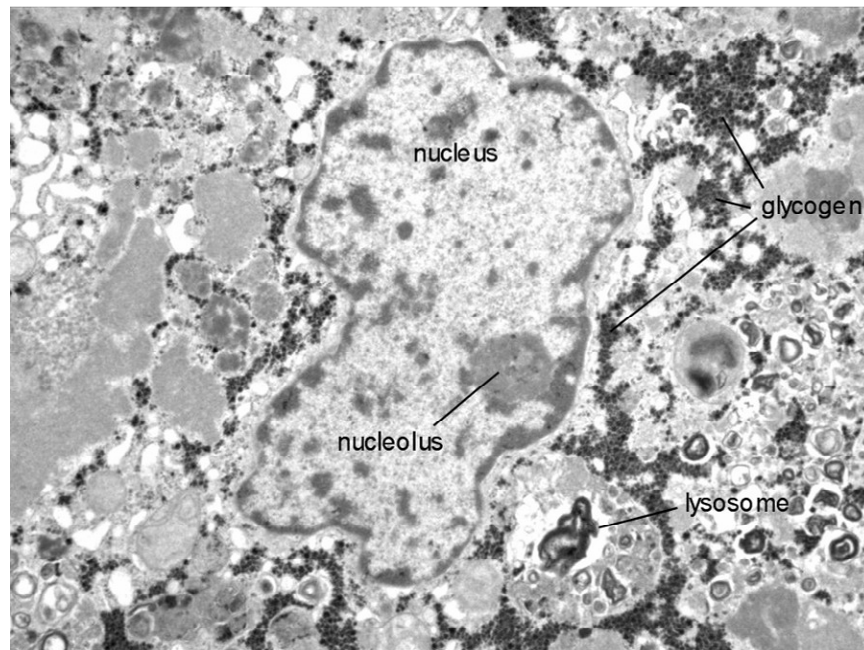
**Figure 4-8: Comparison of Albumin synthesis by encapsulated and monolayer HPCs.**

#### **4.3.5.2 Periodic Acid Schiff's (PAS) Assay for Glycogen Storage**

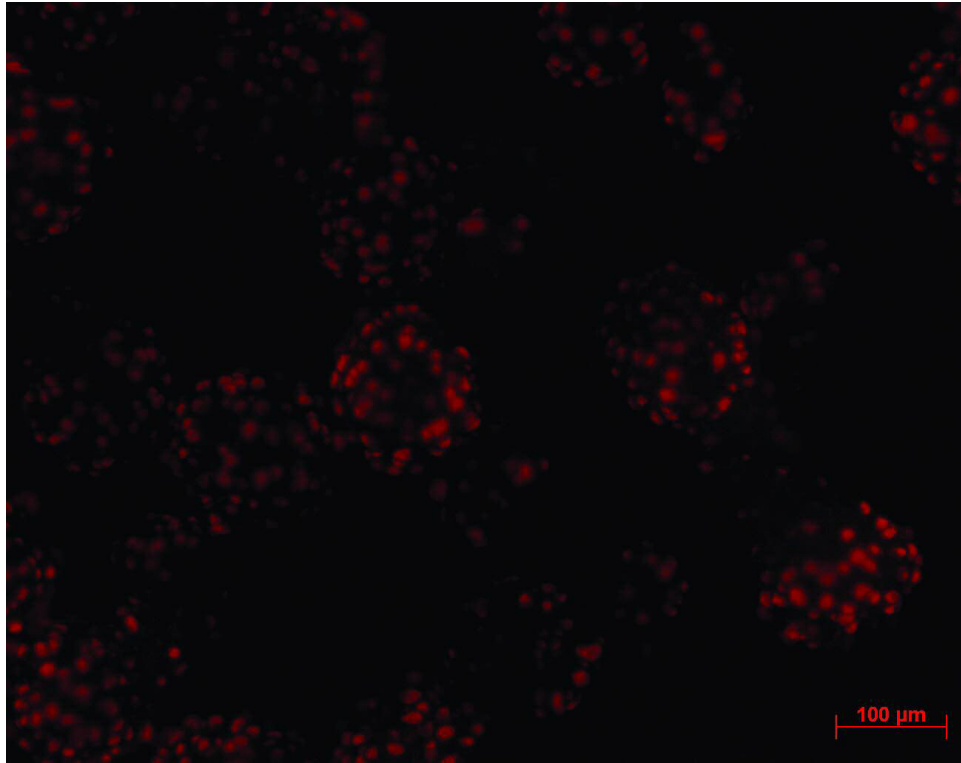
The Periodic Acid Schiff's (PAS) assay demonstrates glycogen storage within a cell; another functional characteristic of hepatocytes. This is a complementary

assay to evaluate the impact of the electrostatic encapsulation process on the hepatic progenitor cell viability and metabolic function. Glycogenesis (conversion of glucose to glycogen) and its subsequent storage is a primary function of hepatocytes. Glycogen particles are found in the cytoplasm of hepatocytes and are a marker for functional maturation. Figure 4-9 shows a histological image of a mature hepatocyte depicting glycogen particles in the cytoplasm.

This experiment helped analyze if the encapsulated progenitors were proceeding along a hepatic-differentiation pathway or undergo stress induced de-differentiation and loss of function.



**Figure 4-9: Glycogen particles in the cytoplasm of a mature hepatocyte.**  
(Image Source: <http://www.humpath.com>)



**Figure 4-10: Fluorescence image of PAS staining of encapsulated HPCs.**

Glycogen stores inside the cell are oxidized into aldehydes by periodic acid during the procedure. The aldehydes react with the Schiff's reagent forming a colored final product. Figure 4-10 demonstrates encapsulated cells stained for glycogen particles using the PAS assay 7 days post-encapsulation. Alginate beads in the image have a diameter of approximately 200μm based on confocal measurements. Encapsulated HPCs were also observed to have glycogen stores that persist beyond 3 weeks in culture.

## **4.4 Conclusion and Future Plans**

This novel demonstration of electrostatic encapsulation of hepatic progenitor cells provides a successful step in creating a cellular niche that supports the ex vivo culture of somatic-derived progenitor cell (or stem cell) populations. The three dimensional character of the encapsulated beads allows us to increase the cell density compared with traditional tissue culture experiments. This process also favors development of an optimal microenvironment for the hepatic progenitor cells. The encapsulation process provides a framework or matrix for the hepatic progenitor cells thus allowing them to play a dynamic role in developing an ideal three dimensional architecture. Potential applications for this method of progenitor cell encapsulation include drug screening and the development of bioreactors.

### **4.4.1 Future Plans**

The results from the ELISA and the PAS assays allow us to raise exciting scientific questions about the phenotypic changes in the encapsulated cells during later time points in culture. Experiments are also currently ongoing to incorporate alginate encapsulated cells in NMR compatible bioreactors. This allows the device to be used as a perfusion bioreactor and also to study cell metabolites during drug screening.

Future experiments will build on these results and try to improve the encapsulated cell viability. The immediate plan is to translate the macroscale encapsulation experiments described herein to the microfluidic platform to

enhance viability by reducing fluid stressors and also to reduce the operating volume and cell number requirements.

The microfluidic encapsulation device will also utilize natural extracellular matrices like collagen as encapsulation substrates to provide cell-matrix interactions that are critical to progenitor cell proliferation and differentiation. This revolutionary “Lab-on-a-chip” device will also greatly enhance analysis of effects of drugs/cytokines etc. on progenitor cells cultured on 3-dimensional matrices by reducing their quantity.



## **5 Microfluidic Encapsulation System**

### **5.1 Microfabrication and BioMEMS**

Recently, there has been huge progress in the fields of microelectronics, microfabrication and micromachining technologies. These are collectively now called MEMS and its applications in biomedical engineering have spurred a new field known as BioMEMS. The technology can trace its origins to computer chips manufacturing processes and companies that were trying to make them more powerful and less expensive. This has already made its impact on decreasing the cost of technology in extensively used devices like computers and hand held devices. [82]

The field of medical diagnostics has been searching for the development of novel devices which offer great control on functionality at a reduced cost. The new field of BioMEMS has enabled researchers to apply these concepts to varied areas in biotechnology and clinical medicine.

### **5.2 Microfluidics**

Microfluidics is a multidisciplinary field combining principles of physics, chemistry, engineering, biotechnology and the new field of BioMEMS which enables creation of devices with micron-sized dimensions. [83]. It can be defined as the science of manipulating nanoliter or microliter volumes of fluid with great precision and control. The field is relatively new, making an emergence in the

1990s and has generated great interest in creation of a myriad of “Lab-on-a-chip” devices. This technology can allow for the creation of micro-chemical reactors, micro/nano sensors and actuators to single cell analysis systems and high throughput bio-assays.

### 5.3 Fluid Behavior at the Microscale

The dominant forces at the microscale are very different from those experienced in everyday macroscale situations [84]. Precise understanding of these physical phenomena is critical for miniaturization. It would be erroneous to assume that a large scale device would exhibit similar characteristics when shrunk down to micron dimensions [85]. The phenomena that play a major role in a microfluidic environment are laminar flow, diffusion, fluid resistance, effect of surface to volume ratio and surface/interfacial tension.

Flow at the microscale is always linear because of the dimensions of the microchannels [86]. This effect is quantitatively explained by the most important dimensionless number in fluid dynamics, the Reynolds number (Re). In fluid mechanics, the Reynolds number is the ratio of inertial fluid forces ( $\rho v_s$ ) to viscous forces ( $\mu/L$ ). This ratio thus quantifies the relative importance of these two forces for given flow conditions. Thus, it is used to identify different flow regimes, such as laminar or turbulent flow [87]. The Reynolds number (Re) is less than 2300 for laminar flow and the flow becomes turbulent as Re approaches 2300. It is mathematically represented as follows; ('L' being the principle dimension of the flow chamber)

$$Re = \frac{\rho v_s L}{\mu} = \frac{v_s L}{\nu} = \frac{\text{Inertial forces}}{\text{Viscous forces}}$$

Due to the small dimensions of microfluidic chambers the Reynolds number is typically much less than 100 [88]. This explains the dominance of laminar flow at the microscale.

The laminar flow profile provides us great control over fluid flow and the ability to precisely manipulate fluid particles inside the microchannel architecture. However this laminar flow regime is problematic in the building of chemical reactors because of insufficient mixing at the microscale [89, 90]. The other possibility is the creation and transportation of discrete droplets of fluid that can function as micro-reactors and sensors. As explained later in this document, these fluid droplets can also be used to hold cells, protein, growth factors etc. for purposes of tissue engineering.

The surface to volume (SAV) ratio also plays a significant role in microchannels. The surface to volume ratio increases by orders of magnitude compared to macroscale situations because of the dimensions at the microscale. This phenomenon can be observed in “Lab-on-a-chip” chemical reactors. High SAV causes reactions to occur faster at the microscale due to faster migration of reactants inside the reaction chamber. The high SAV also provides for improved heat transport characteristics. For example, rapid removal of excess heat in microchannels makes capillary electrophoresis more efficient (102).

## **5.4 Surface/Interfacial Tension**

Surface tension is an effect within the surface layer of a liquid that causes that layer to behave as an elastic sheet. This is a very commonly observed phenomenon in everyday life. A layer of oil on a puddle of water, the water strider

walking on water and dew drops on leaves are all due to the effects of surface tension. The effects of interfacial tension are greatly pronounced at the microscale because of the small dimensions involved that increase the surface free energy of the fluid. This leads to effects like capillarity where fluids can be moved through a microchannel for long distances without any active pumping mechanism. The smaller the channel dimensions the longer the fluid travels. In nature this is observed in plants that use channels a few nanometers wide to pump water and nutrients from their roots to the leaves. The increased effects of interfacial tension can also be harnessed to cause flow focusing in a microchannel as explained in detail later in this document.

## **5.5 Biological Applications of Microfluidics**

Advances in microfabrication techniques and the invention of the “soft lithography” process [91] has led to this technique being increasingly applied in biology and has created a new field called *Biomicrofluidics*. The applications of biomicrofluidics are endless; from microscale perfusion cell culture systems to DNA purification systems to high throughput bio-assays. The advantages are ease of manufacture, very small operating volumes usually in microliters and nanoliters and faster thermal and chemical transfers due to the high surface to volume ratios.

The small operating volume is highly favorable in the drug testing industry where it could result in huge financial savings by reducing the amount of drug/compound needed for a test. One of the biggest applications has involved

biomicrofluidics where it is possible to mimic microscale tissue structures like capillaries and cell-cell junctions. [92]

Such systems can also provide ideal environments for cell culture and fluidic behavior similar to in-vivo environments. Biomicrofluidics has numerous applications in the field of tissue engineering including precise cell patterning, control over topography and in microscale bioreactors and biohybrids.

## **5.6 Microfluidic Devices- Design and Fabrication**

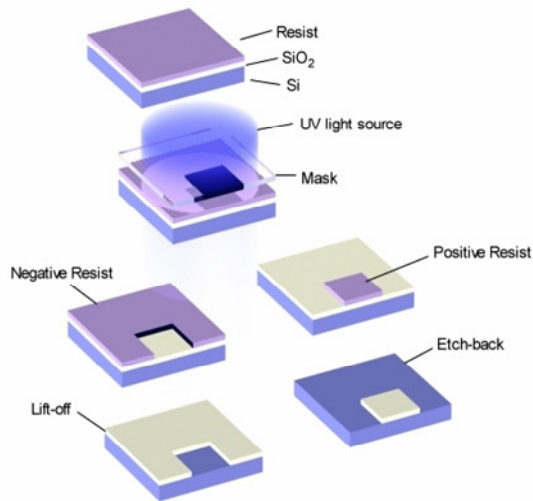
Microfabrication methodologies originally designed in the field of integrated circuit (IC) design and microelectronics have revolutionized the field of microfluidics. The most popular technique for the manufacture of biomicrofluidic devices involves photolithography to manufacture of the silicon masters followed by soft lithography to create a mold of the design using an elastomeric material.

## **5.7 Photolithography**

Photolithography (also optical lithography) is a process used in microfabrication to selectively remove parts of a thin film. It uses light to transfer a pattern from a photomask to a light-sensitive chemical (photoresist) on the substrate. A series of chemical treatments then engraves the exposure pattern into the material underneath the photoresist.

There are two kinds of Photoresists: Positive tone and Negative tone photoresists depending on their light interaction property. Positive tone resists become soluble in developer solution upon exposure thereby leaving behind the unexposed regions. Negative tone resists harden upon exposure to light and the unexposed regions wash away when exposed to developer solution. The only

limiting factor in the fabrication process is the resolution obtainable in the photomask printing process. The schematic of the lithography process is shown in Fig 5-1.

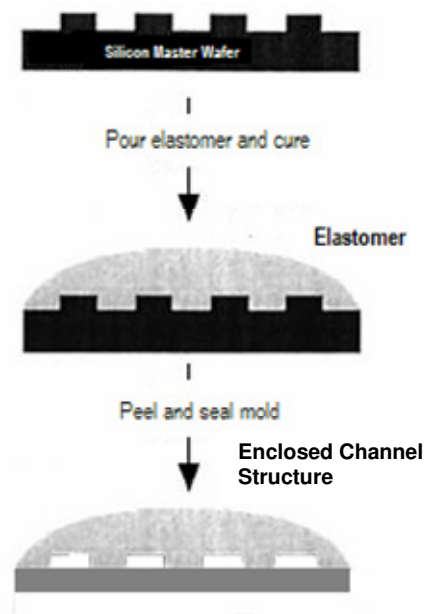


**Figure 5-1: Photolithography Process**  
(Ref: <http://britneyspears.ac/physics/fabrication/Image46.jpg>)

## 5.8 Soft Lithography

Soft lithography refers to the technique of replicating the structures on a patterned substrate using shape conforming, curable elastomeric materials. The process involves pouring a two-part polymer (mix of elastomer and curing agent) over the micropatterned substrate (usually silicon) and allowing it to cure.

The cured elastomer will retain the pattern on the substrate and can be peeled off. If the substrate is manufactured by photolithographic processes, a negative tone photoresist is used for forming a depressed structures and a positive tone for raised features in the elastomer. The schematic for a typical softlithographic process is shown in Fig 5-2.



**Figure 5-2: Soft Lithography Process**

Currently, the most commonly used material for biomicrofluidics is Polydimethylsiloxane (PDMS). The popularity of PDMS is due to its great material properties. Cured PDMS is flexible but sturdy enough to manipulate. It is biocompatible as its preparation does not involve any toxic solvents subsequently allowing it to be used in cell culture. It is also highly inert and can thus be used to build devices that are used in bioassays and for testing of compounds.

The other important property of PDMS that is beneficial when used in conjunction with cells etc. is its gas permeability. PDMS is highly gas permeable and this prevents the occurrence of hypoxia which is commonly observed with enclosed cell culture designs. It is also optically transparent and is usable in even highly sensitive microscopy techniques like DIC imaging and fluorescence.

## **5.9 PDMS - Wetting Characteristics**

Cured PDMS is hydrophobic in nature with a contact angle of about  $119^\circ$  [93]. This property of PDMS has its advantages and disadvantages when used in biomicrofluidic applications. When used as a flow chamber, its wetting property is advantageous because it prevents the fluid constituents from sticking to the PDMS walls and clogging the channels. Channel obstruction is an enormous problem due to the small features of microfluidic devices.

With PDMS devices being increasingly used as cell culture vessels and scaffolds, the hydrophobicity of native PDMS requires to be altered. The wetting properties of the PDMS can be modified by either exposing it to oxygen plasma [94] or by coating the surface of the PDMS with a material that can present a binding domain [95]. It is now possible to precisely pattern such binding sites on the PDMS thus allowing the investigator to perform diverse analysis on a single microfluidic chip.

## **5.10 Droplet Generation at the Microscale**

Uniformly shaped droplets can be formed at the interface of two immiscible fluids within a confined microfluidic channel. This phenomenon is due to the basic energy conservation principles and is dependent on the geometry of the interface and the surface characteristics [96] of the microchannel walls. Two fluid forces are active at a fluid junction in a microfluidic channel of two immiscible fluids. One is the viscous force that tries to maintain a laminar flow to the outlet because of the low Reynolds number environment. The other is the capillary force trying to minimize total surface of interaction between the two fluid



phases by splitting one into spherical shaped droplets which represents the least surface area of all shapes.

### 5.11 Capillary Number (Ca)

Microfluidic droplet generation can be quantitatively explained by a number called the Capillary number (Ca). In fluid dynamics, the capillary number expresses the relative effect of viscous forces and interfacial tension at the interface between two immiscible fluids. It can be defined as:

$$Ca = \frac{\mu G r_s}{\sigma} \quad ; \quad G \approx \frac{Q_o}{w^2 h} \quad \Rightarrow r_s \propto \frac{1}{Q_o}$$

$\mu$  - Viscosity of the liquid

$G$  - Fluid shear rate

$r_s$  - Radius of spherical droplet

$\sigma$  - Surface or Interfacial tension between the two fluid phases

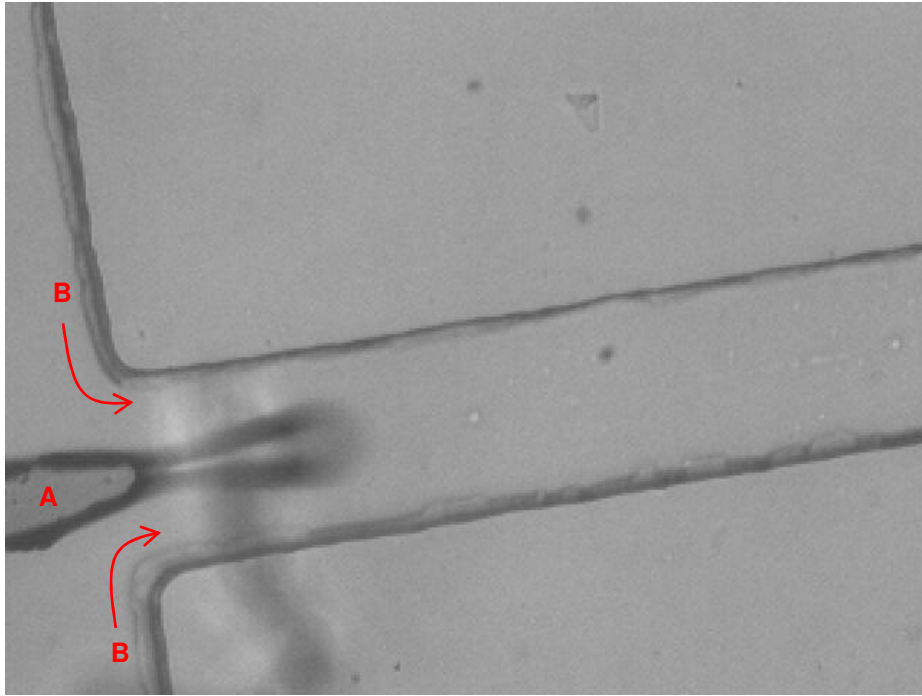
$Q_o$  - Flow rate of Oil

The size of the generated droplets can be controlled by the capillary number and from this equation it is obvious that this can be simply achieved by varying the applied fluid velocities.

### 5.12 Mechanism of Droplet Generation

Fig 5-3 shows a simple “T junction” interface between two immiscible fluids A and B. As explained above, two forces act on the fluid stream A. The viscous drag of the fluid tends to push it towards the outlet in a linear stream. This however impedes the laminar flow pattern of Fluid-B. This competition of the viscous forces leads to a slow “pinching” or “shear focusing” effect that causes elongation of Fluid-A until it reaches its stability limit and pinches off into droplets.

Surface interactions of Fluid-A with Fluid-B and the channel walls, the capillary forces shape the fluid packet into a spherical shape to conserve surface energy.



**Figure 5-3: “T-junction” depicting droplet generation at the interface of two immiscible fluids**

### **5.13 Droplet Generation – Literature Review**

The study of interface driven motions of fluid and fluid breakup into cylinders and other shapes (97) began with the work of Plateau and Lord Raleigh (98-100). This has historically involved break up of a fluid stream in another bulk fluid where the two fluids are immiscible with one another. The resultant droplets had a huge drop size distribution. The production of mono-disperse droplets has generated much interest. This is partly due to the need for such a tight size distribution in biological applications. Most of the work has focused on two

modalities for droplet generation at the microscale: “T-junction” geometry [101] or a multi-tiered flow focusing device (FFD) using micro-capillary architectures [102]. As explained above, the T-junction employs a shear-rupturing mechanism brought about by the competition between viscous forces at the junction. Many different modifications of the T-junction have been developed to achieve maximal focusing of fluid forces [101]. The possibility of accurate spatial prediction of these micro-reactors as well as the capability to form dynamic patterns [103] has also spurred interest in this field. The microencapsulation of biological materials (proteins, growth factors etc.) in liquid vesicles has also gained interest [104]. The dynamics of the microfluidic environment also allow for production of polymer particles from a pre-polymer solution [105, 106].

Although the possibility of cellular encapsulation in polymer particles inside a microfluidic shear focusing device has been discussed earlier [118, 119], not much has been accomplished in the scientific community. This is in part due to the unavailability of biocompatible materials that are capable of being manipulated in this environment to produce successful encapsulation.

Much of the need for cellular encapsulation is in the fields of tissue engineering and drug testing. The creation of spherical polymer particles with embedded cells would allow for 3-dimensional cell culture, cell transplants, drug metabolite analysis. Advantages of the microfluidic platform for cellular encapsulation include the necessity for lower operating fluid volumes, lower cell stressors and the possibility of downstream analysis, all on a single chip.

## 5.14 Project Rationale

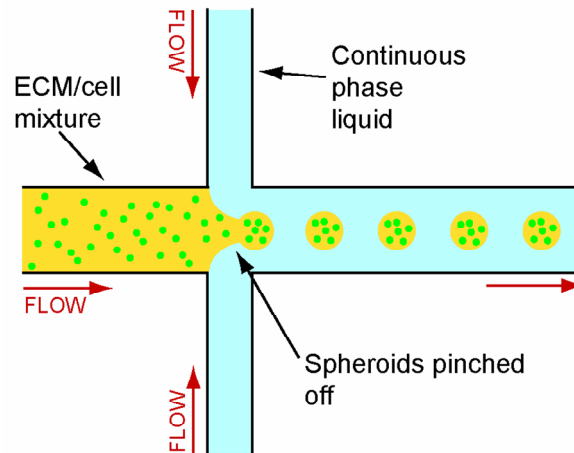
As mentioned earlier, a new system was required for encapsulation of sca-1<sup>+</sup> hepatic progenitor cells (sca-HPCs) for purposes of cell culture and delivery. Microfluidic encapsulation systems that require low operating volumes on the order of milliliters and exerting lower mechanical stresses on cells was the perfect alternative.

Conventional cell encapsulation approaches (macro or micro scale) utilize natural matrices like alginate and synthetic materials like Poly-L-lactic acid (PLLA) etc. that have been approved by the FDA for use as suture materials. Since encapsulation of hepatic progenitor cells was the major rationale behind this project, it was hypothesized that collagen would be the ideal encapsulation matrix. Encapsulation of cells in a collagen droplet requires environmental conditions that are favorable to collagen gelation within the microchannel framework to eliminate the need for further batch processing [106].

## 5.15 Materials and Methods

### 5.15.1 Mask Design

The channel design was drawn using Adobe Illustrator software. A single continuous phase channel was split to ensure that the oil arrives at the junction at the same time from the top and the bottom. The output channel was made long and tortuous to keep the generated beads inside the microfluidic network to allow for complete gellation. The device concept is shown in Fig 5-4.



**Figure 5-4: Microfluidic junction showing bead generation**

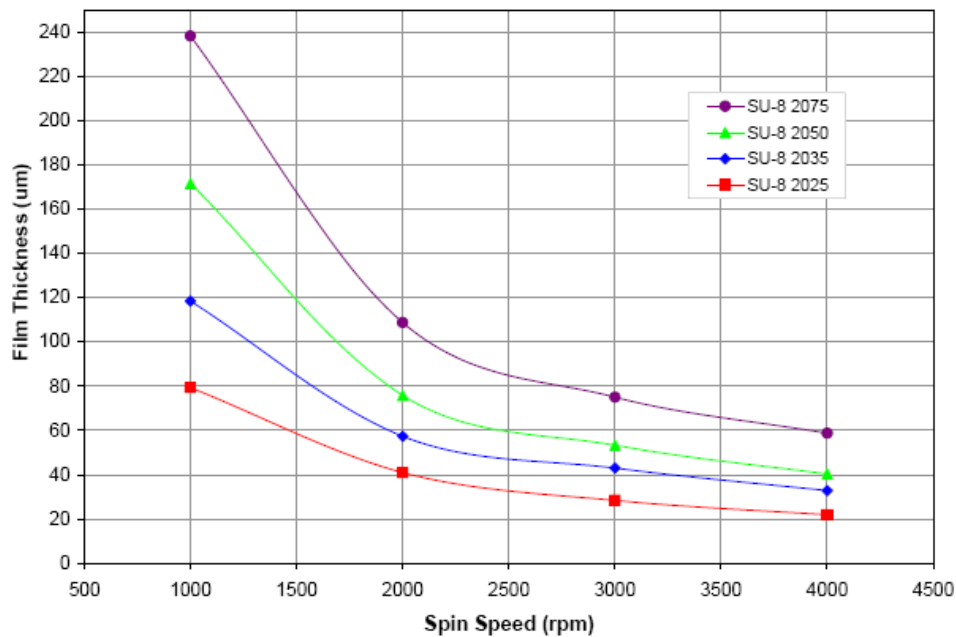
### 5.15.2 Fabrication of Silicon Master

The channel design was printed using a high resolution emulsion printer on transparencies. SU-8 being a negative tone photoresist, most of the UV light needs to be blocked by the mask during photolithography. The channels were

thus drawn clear on a black background to allow light to only pass through the design features.

A 3" silicon wafer is cleaned with acetone and isopropyl alcohol (IPA) twice and dried on a hotplate. A thin coat of SU-8 2050 photoresist is applied using a spin coater. The spin speeds are determined using the graph shown in Fig 5-5 based on the desired height of design features. After an initial bake, the photoresist is exposed to near UV light through the photomask.

Since SU-8 is a negative photoresist, this causes the exposed regions to become less acidic. Upon development using an alkali solution, the unexposed regions of the photoresist get washed away leaving behind the microscale design features.



**Figure 5-5: SU-8 spin speed versus thickness**  
Ref: [http://microchem.com/products/su\\_eight.htm](http://microchem.com/products/su_eight.htm)

### **5.15.3 Soft Lithography**

Soft lithography refers to the technique of using elastomeric materials like Poly-di-methyl siloxane (PDMS) to conform them to structures on a master template. PDMS has been the material of choice for building microfluidic devices due to its excellent conformance to patterned surfaces. It is also optically transparent, inert and gas permeable leading to its usage in the field of biomicrofluidics.

To build the encapsulation device, PDMS elastomer mixed with curing agent (ratio of 10:1) was degassed in a vacuum chamber and cast on the silicon wafer. It was placed on a hotplate and allowed to cure at 80°C for three hours. The PDMS mold was peeled off the wafer after curing and the inlets and outlets were cored out for channel access.

### **5.15.4 Plasma Treatment**

Requirement of a closed channel architecture necessitated sealing of the bottom of the channel network in order to withstand the fluid pressure. This was achieved by bonding the PDMS mold to a glass slide. Bonding was achieved by briefly exposing the PDMS and the glass slide to oxygen plasma. This formed an irreversible bond between the PDMS and the glass slide. In later experiments, the glass slide was replaced by a flat PDMS sheet to provide a uniform hydrophobic environment inside the microchannel.

### **5.15.5 Fluid Phases**

In our previous experiments with encapsulation, calcium alginate was used as the encapsulation matrix. It was postulated that since cell-ECM interactions are critical to maintenance of cellular function, collagen would serve as a better substrate for encapsulation. Vitrogen™ (Cohesion Technologies, Palo Alto, CA) was chosen as the distributed phase because of its gellation properties. Vitrogen™ is sterilized, 97% pure, bovine Collagen-I with the rest comprised of Collagen-III. It forms a stable hydrogel at a pH 7.0 and at about 37°C. Olive oil was chosen as the continuous phase in the bead generation process. It was chosen because it does not swell the PDMS substrate.

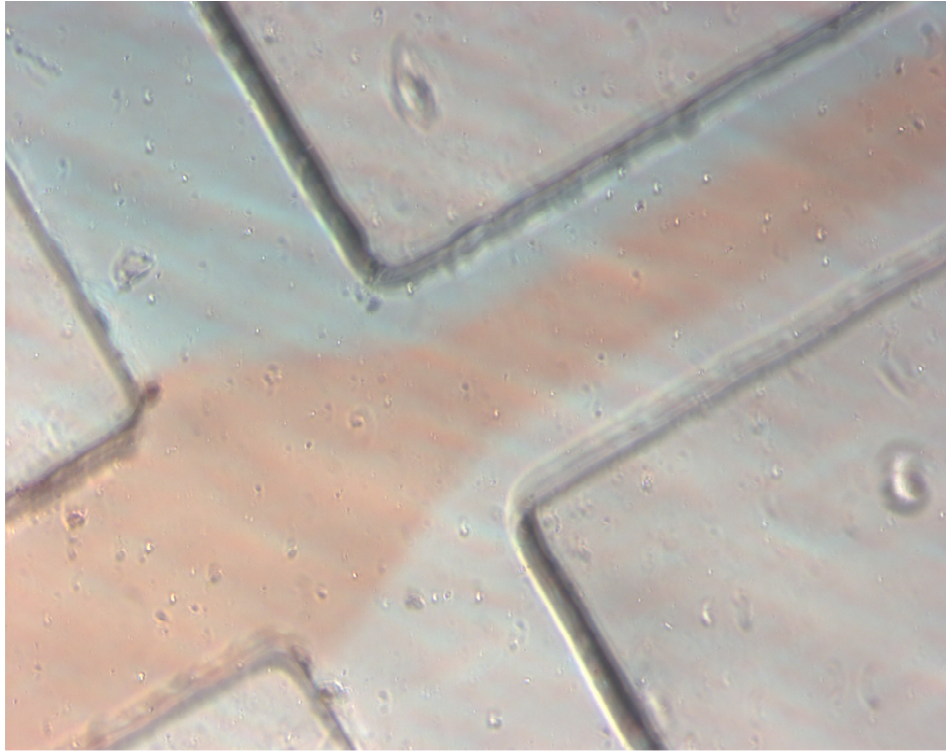
The Vitrogen™ solution is prepared according to the manufacturer's protocol. The cells to be encapsulated are suspended in the collagen solution at a pH of about 7.0. Care is taken to avoid air bubbles. The ECM/cells solution is drawn into 1cc syringe. Olive oil is drawn into another 1cc syringe and both syringes are fixed on micro-flow syringe pumps.

### **5.15.6 Microfluidic Device Construction and Testing**

The pump is connected to the microfluidic device through tubing inserted into the cores at the inlet ports. The device was demonstrated to be free of leaks and could bear the operating fluid pressure without failure. This was verified by flowing colored solutions through the channels and observing the PDMS edges under a microscope. The PDMS device was cleaned using ethanol followed by sterile water. It was allowed to dry under UV light in a laminar flow hood. The working concept was first verified by flowing two colored (blue and red) miscible



solutions. This is shown in Fig 5-6. Since there is no interfacial tension between the two phases and due to the poor mixing characteristics of the microfluidic flow, the two solutions flow parallel to each other.



**Figure 5-6: Flow of miscible fluids in a device with 200  $\mu\text{m}$  wide channels.**

#### **5.15.7 Device Operation**

At the junction point of the three fluid streams, the interfacial tension (between collagen and oil) and the hydrophobic environment of the PDMS chamber preferentially pinches off the collagen/cells stream into spherical droplets. The tortuous outlet network will be gently heated to 37°C. This causes the collagen droplets to gel as they travel through the outlet channel and the gelled beads are tapped out of the cored outlet port.

The bead size is a function of channel dimensions, fluid viscosity and the flow rate. Thus for a given device and fluid phases the bead size can be easily regulated by adjusting the flow rates of the pump. This also offers us control over bead production rate via adjustment of the flow rate of the collagen solution while simultaneous changes can be made in the oil flow to maintain a constant bead size.

The generated beads have a tendency to coalesce as soon as they leave the microfluidic environment. To prevent this, it was necessary to saturate the bead outer surface area with surfactant molecules. This reduced the interfacial tension between the collagen solution and the olive oil and prevented bead coalescence. Tween-20 mixed with the olive oil at a concentration of 2-3% w/w was used as the surfactant. It was chosen because of its widespread usage in biological processes and its ease of availability.

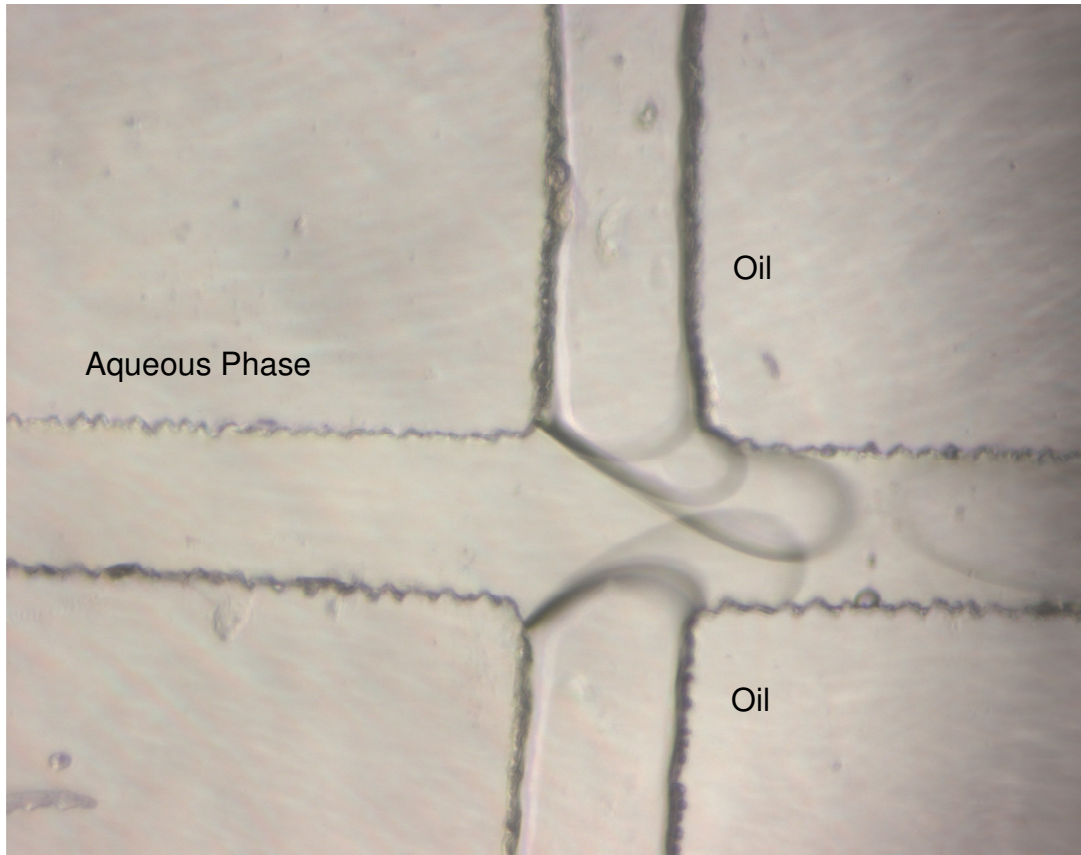
#### **5.15.8 Effect of PDMS Plasma Treatment on Immiscible Fluid Flow**

When two immiscible fluids intersect at a microfluidic junction, one of the fluids is expected to “pinch off” into droplets. This is determined by the interaction of the two fluids along with the wetting properties of the fluid chamber.

Hydrophilic channels form oil droplets and hydrophobic channels are better suited for pinching off the aqueous phase. Upon plasma treatment for purposes of bonding, the natively hydrophobic PDMS becomes hydrophilic. This caused the oil phase to pinch off into droplets. This is shown in Fig 5-7.

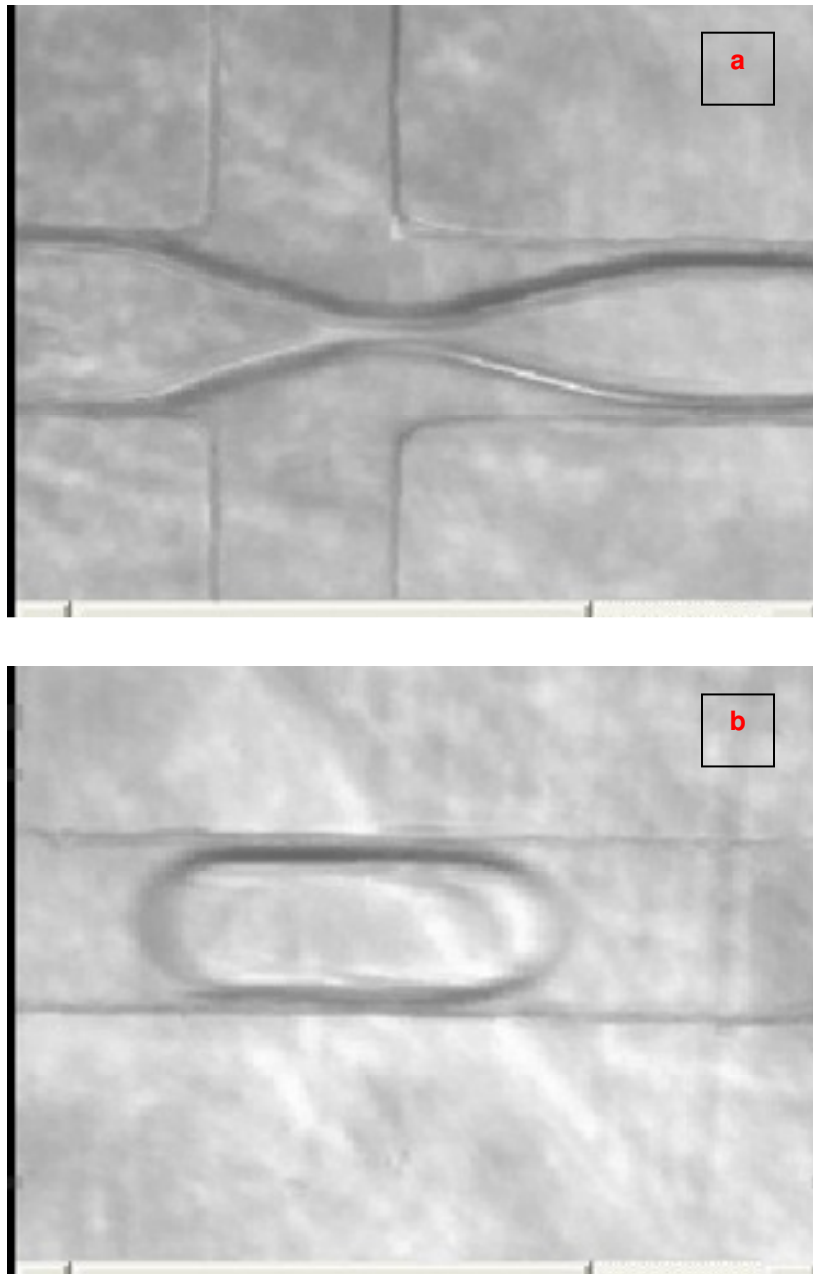
The PDMS mold was allowed to recover its hydrophobicity at room temperature for 3 days to prevent this occurrence. After recovery, it was

observed that the aqueous phase was being “pinched off”. This is shown in Fig 5-8.



**Figure 5-7: Oil droplets being “pinched off” in a hydrophilic environment.**

Channel Width: 100  $\mu\text{m}$ . Mag: 10X



**Figure 5-8: Droplet generation in a hydrophobic environment.**  
Channel Width: 100  $\mu\text{m}$ . Mag: 10X

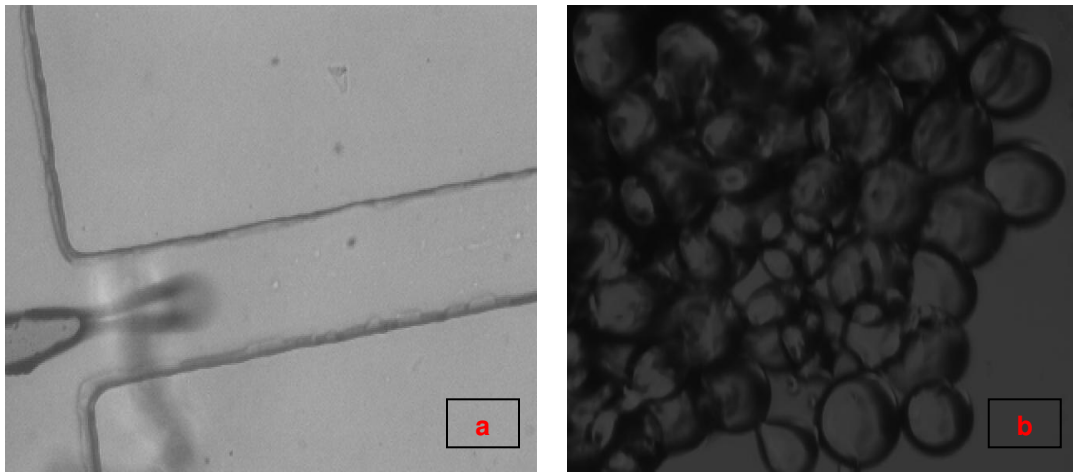
- a. Aqueous phase being “pinched off” in a hydrophobic environment.
- b. Generated droplet moving through the outlet channel.

Another phenomenon that can be observed in Fig 5-8 is the “plug like” nature of the generated droplet. This was because of the hydrophilic nature of the bottom glass slide. This exerts a drag on the produced droplet thus causing elongation. It is thus evident that a uniform hydrophobic environment is needed to generate spherical beads. This was accomplished by using a flat sheet of PDMS made on a non patterned silicon wafer instead of the glass slide as the bottom of the microfluidic device.

## 5.16 Results

### 5.16.1 Collagen Bead Generation

Vitrogen™ beads were produced using the same procedure as described above. Fig 5-9 (a) is a high speed camera image showing Vitrogen™ beads being generated using olive oil as the dispersed phase. Fig 5-9(b) shows the beads that were collected from the outlet port after allowing for gellation.



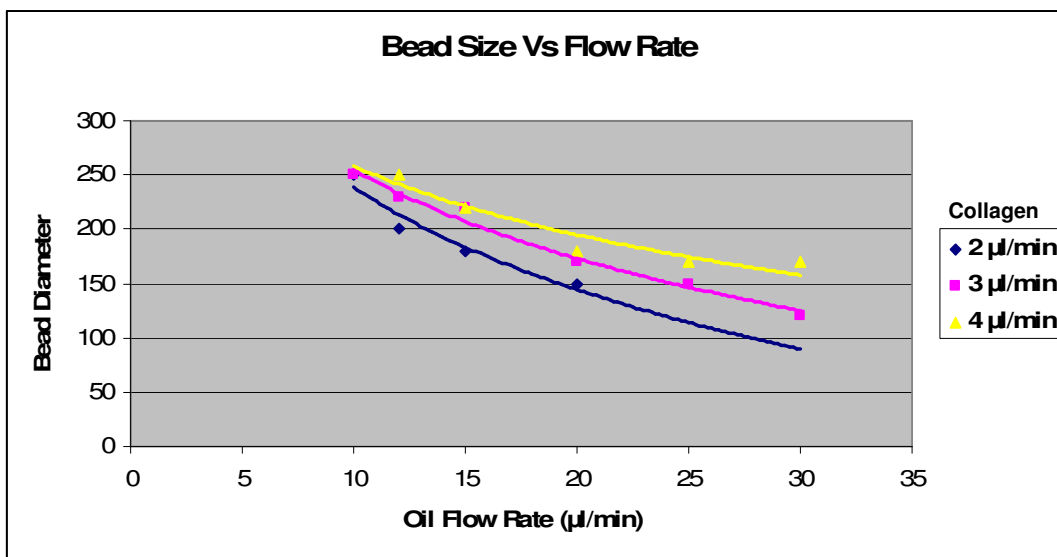
**Figure 5-9: Generation of Vitrogen™ beads at a “T-junction”**  
Channel Width: 100  $\mu\text{m}$ . Mag: 10X

- a. Image of Vitrogen™ droplet being generated.
- b. Collagen beads collected at the outlet port

### 5.16.2 Control over Bead Size

Figure 5-10 shows a graph of bead size versus flow rate of the continuous and discrete phases. The bead sizes increase with increase in the flow rate of the collagen solution and decreases with increased flow rate of the oil phase.

This is predictable because the viscosity of the oil phase is a dominant force at the junction. Increasing the flow rate of the oil phase increases the droplet generation rate thus resulting in smaller droplets. Increasing the flow rate of the collagen solution ensures increased availability of the dispersed phase at the junction. This results in increased droplet size for the same flow rate of the oil but at a faster droplet generation rate.



**Figure 5-10: Effect of fluid flow rates on collagen bead size**

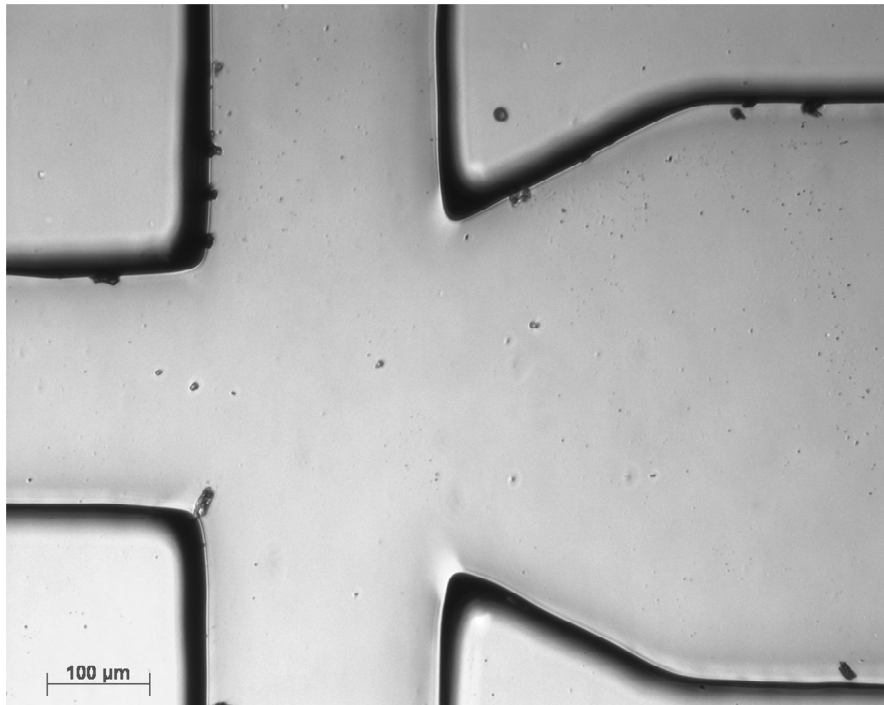
It is possible to create beads with sizes ranging from 50–250 µm. The bead diameter for encapsulation of cells needs to be 100-150µm to provide for optimal number of cells per bead and to maintain nutrient diffusion distances below the “diffusion barrier”.

### 5.16.3 Design Modifications

Fig 5-11 depicts a modified junction architecture where the junction is expanding into the outlet. The channel height in this design was also increased to

150  $\mu\text{m}$  from 100  $\mu\text{m}$ . These changes were made to optimize bead shape inside the channel architecture and to overcome the fluidic resistance of the viscous oil phase.

As depicted in Figure 5-8(b), larger beads are confined and tend to elongate inside the channels, attaining a spherical shape only after reaching the outlet. The other issue associated with flowing viscous fluids in a microchannel was the increased fluidic resistance. This caused the oil phase to sometimes push into the collagen inlet channel at higher flow rates.



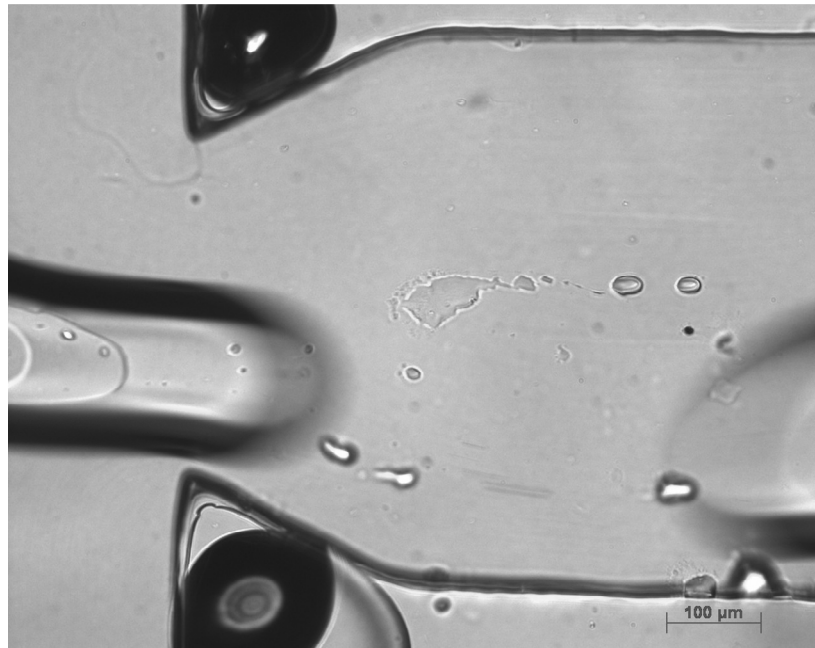
**Figure 5-11: Expanding Outlet with Increased Channel Height**

Since the target bead diameter was 100-150  $\mu\text{m}$  and bead gellation was required to happen inside the channel confines, it was necessary for the beads to attain a spherical shape once they were generated. This was achieved by



increasing the channel height to prevent bead elongation. Vitrogen™ beads were observed to instantaneously attain a spherical shape in the channels with increased height, (Fig 5-12).

Fluidic resistance is directly related to the channel dimensions and decreases with increasing channel width and height. Increasing the channel height also thus decreased the resistance of the oil phase and solved the reverse flow problems.



**Figure 5-12: Bead generation at exit orifice**

The expanding outlet channel also caused the flow focusing effect to be pronounced at the junction of the two fluid phases. In a simple “T-junction” design the viscous collagen phase would sometimes be “pinched off” further downstream instead of at the junction. The expanding geometry caused predictable generation of collagen droplets at the junction. Using simulations it

has been shown (116) that such geometry would cause the maximum fluid velocity to occur at the exit orifice.

#### 5.16.4 Cellular Encapsulation in Vitrogen™ Beads

LA-7 rat mammary tumor cells were successfully encapsulated inside mono-disperse collagen beads. To provide for additional gellation time, beads were confined within the microchannel geometry for 1 hour inside a 5% CO<sub>2</sub>/95% air incubator. A challenge with this system has been that the beads continue to possess a liquid core. Figure 5-13 depicts collagen beads inside the microchannel framework.



**Figure 5-13: Image of cells encapsulated in Vitrogen™ beads.**  
(a) 10X and (b) 20X image of collagen beads showing encapsulated cells (Red arrows).

#### 5.17 Discussion

These experiments are a novel description of microfluidic encapsulation of cells inside collagen microspheres. The system could consistently generate mono-disperse Vitrogen™ beads encapsulating the cells as demonstrated in the

results. With this system it is also possible to accurately control the bead dimensions by adjusting the flow rates of the continuous and dispersed phases.

The generated beads have uniform spherical shape and this symmetry is important to permit predictable and uniform chemical and heat transfer through the beads. This also permits solute transport to and from the cells. The cells are well within the “diffusion barrier” [67] of  $\sim 150\ \mu\text{m}$  and this prevents hypoxia in the encapsulated cells.

Alternative approaches to microfluidic droplet generation involved harsh chemical reagents or UV photo-polymerization to cause gelation of droplets. These steps render the techniques incompatible with cells. Recently Lee et.al (107) have demonstrated successful microfluidic cell encapsulation in alginate beads. Though this is a major step towards microfluidic cell encapsulation, the long term effects of the organic reagents utilized during the encapsulation process on encapsulated cells is yet to be established.

The Vitrogen™ encapsulation protocol is completely benign and none of the system components have a deleterious impact on the encapsulated cells. The gelation is completely biocompatible and does not require any harsh processing steps like UV-exposure.

In conclusion, this demonstration of microfluidic collagen encapsulation creates a three dimensional microenvironment for the encapsulated cells. Potential applications for this method of cell encapsulation include drug screening at the microscale and the micro-bioreactors. This microfluidic technique requires very low operating volumes and can thus be used for

encapsulating rare cell types like hepatic progenitor cells for purposes of transplant. These applications are explained in detail in the next section.

## **6 Conclusions and Future Work**

### **6.1 Conclusions**

Three different tissue engineering approaches for liver tissue engineering are described in this dissertation. The study revolved around developing novel strategies for 3-dimensional culture and in-vivo delivery of bipotent hepatic progenitor cells (HPCs) isolated from naïve adult mice.

The first approach was the creation of cell+scaffold “biohybrids” to provide an optimal 3-dimensional culture environment for the HPCs. As detailed in Chapter 3, this investigation involved culturing HPCs on BD™ collagen composite scaffolds. These scaffolds have a high porosity and an optimal pore size distribution for cellular in-growth.

It was established that both mature hepatocytes and hepatic progenitor cells were able to proliferate on this matrix. The study also investigated the maintenance of hepatic function in HPCs seeded on the collagen scaffolds. This was accomplished by analyzing their capability to produce protein and urea nitrogen. Loss of hepatic function was observed after 14 days in culture. It was felt that this was due to the heterogeneity of the HPC population. This created a need for further purification of this cell population to isolate the true hepatic progenitor cells from the contaminating non-parenchymal cells and other liver constituents like endothelial cells. The degradation properties of the scaffolds

were also found to be lacking. The scaffolds could not withstand the combined effects of the in-vivo environment and cellular contractile forces. They disintegrated in less than a week when loaded with hepatic progenitor cells and transplanted into the intraperitoneal space in mice.

Chapter 4 discusses in detail a cell encapsulation approach using calcium alginate microspheres. The rationale behind this approach was to create an immunoprotective barrier around the HPCs for purposes for cell delivery. It was established that the encapsulation system did not have an adverse impact on cell viability or function. Encapsulated HPCs produced urea and albumin at a rate comparable to cells in monolayer culture. They were also found to maintain differentiated functions like glycogen storage for long periods in culture.

The results from the viability experiments demonstrated a considerable loss in cell number in the first few days in culture. This cell death was attributed to the loss of non-parenchymal cells present in the population. The death was secondary to the culture conditions that are more attuned to the culture of hepatic progenitor cells. These results further emphasized the need for a purified cell population for purposes for tissue engineering and hepatic transplant.

Purification of the heterogenous HPC population using a stem cell marker Sca-1 yielded a bipotent sub-population (sca-HPCs) that is capable of robust proliferation and differentiation into mature hepatocyte and biliary phenotype. The relative rarity and fragility of the sca-HPCs necessitated the development of a tissue engineering technique that had low operating stresses and operating volumes. This led to the development of a microfluidic “Lab-on-a-Chip” platform.

Chapter 5 details this microfluidics/bio-MEMS approach applied to encapsulation of cells in collagen microspheres. This involved manipulating two viscous, immiscible fluid phases in microchannel architectures to create an emulsion of one phase in the other. It was achieved by harnessing viscous and capillary forces that are dominant at the microscale.

Vitrogen™, a mixture of 97% collagen Type-I and 3% collagen Type-III was used as the distributed phase and olive oil (or its derivatives) was used as the continuous (lipid) phase. Handling the viscous collagen solution necessitated extensive modification of existing droplet generation technologies and iterative fine tuning to get repeatable generation of collagen droplets.

Results demonstrate reproducible generation of Vitrogen™ droplets that can be caused to gel in the microchannel framework. The system permitted precise control over droplet size by manipulating flow rates of the two fluid phases. This enabled creation of mono-disperse beads ranging in size from 50-200  $\mu\text{m}$ . Cells were also encapsulated in Vitrogen™ microcapsules thus creating micro-bioreactors for purposes of 3D cell culture, cell transplant and drug analysis.

The issue of incomplete gellation would be addressed by providing a framework wherein the collagen spheres can be provided with an outer shell that is capable of instant gellation. This would enable manipulation of the collagen beads outside the microchannel framework for purposes of cell culture and transplant. This will also permit replacement of the oil phase with culture medium to provide nutrition for the encapsulated cells.

As expounded in this dissertation document, numerous challenges exist in creating a tissue engineering model for hepatic therapy from somatic-derived progenitor cells. Cell encapsulation approaches are a successful step in providing an optimal growth environment for these cells. This document adds to the existing knowledge of encapsulation methodologies and will assist further investigations related to liver tissue engineering and organ regeneration.

## **6.2 Future Work**

### **6.2.1 Alginate Shell for Instant Gellation of Vitrogen™ Bead**

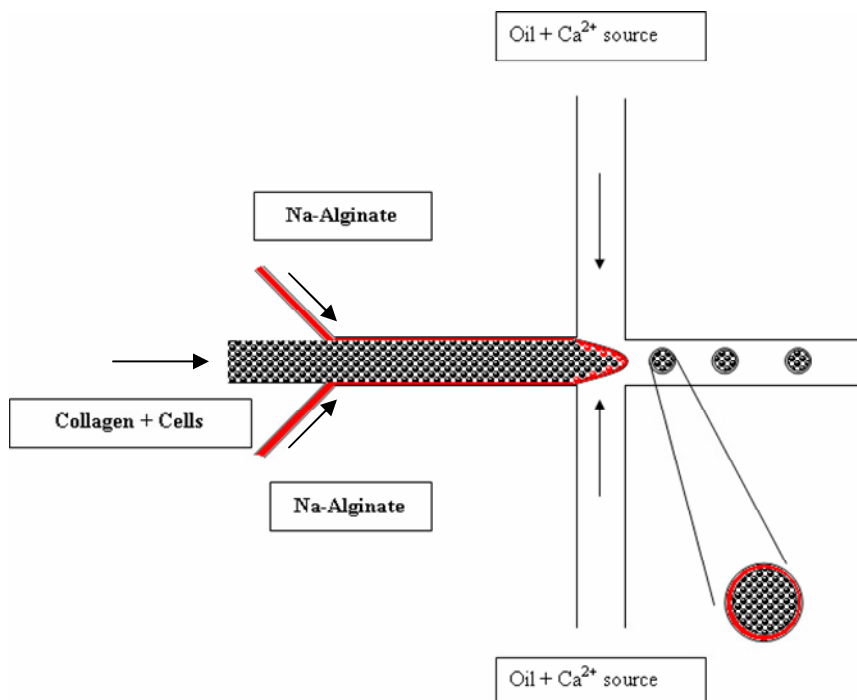
The gellation time of the collagen solution is not instantaneous and is difficult to manipulate. The droplets maintain their spherical morphology and discrete nature until they are confined in the microchannel environment. The laws governing microscale physics cease to exist once the droplets exit the microfluidic environment. This causes fluid mixing and consequently droplet coalescence. This phenomenon can be prevented by the surfactant molecules but once the beads are removed from the oil phase they cease to hold their spherical shape.

Therefore there is a need to provide the beads with a mechanism that would provide for instant gellation. This can be achieved using sodium alginate as an outer shell. Allowing  $\text{Ca}^{2+}$  ions suspended in the oil phase to diffuse into the collagen droplets would cause the outer shell to gel thus encapsulating the liquid core.



The design requires a simple upstream modification in the collagen channel. Two small channels of alginate feed into the collagen channel on either side. The streams of collagen and alginate are miscible and thus will flow parallel to each other as depicted in Figure 6-1 with minimal diffusion because of the short distance in which they are in contact with each other.

After passing through a singular focusing point at the junction with the oil phase, the collagen droplets will possess a shell of alginate around them. The thickness of the shell will be dependent on the ratio of the widths of the individual streams.



**Figure 6-1: Design to provide the collagen-cell solution with an alginate shell.**

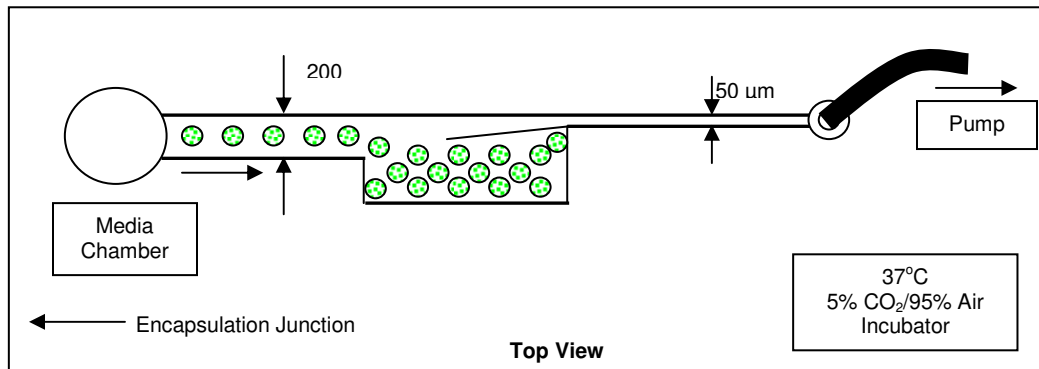
The alginate shell will be discarded after the beads have been in the incubator for 1 day (this incubation period allows for complete collagen gellation) since the host immune responses towards alginate are unknown. Dissolving the

shell will be achieved by treating the beads with a dilute solution of Sodium Citrate that preferentially dissolves the Ca-Alginate.

### **6.2.2 Three Dimensional Microscale Cell Culture**

There is a tremendous amount of recent interest in culturing cells in PDMS based microchannel devices. This interest is due to the advantages offered by microfluidic systems previously detailed in earlier sections. Using a microfluidic chamber allows very few cells to be patterned in a microfluidic chamber and it is subsequently possible to analyze multiple variables on a single chip. These include single cell analyses and analyzing effect of toxins, growth factors, drugs and cytokines on the cultured cells.

Similar to macroscale cell culture systems, cells are still cultured in monolayers on PDMS or glass. A modification in the microchannel outlet design would allow us to entrap a select number of beads within the device and use it as a cell culture chamber. A schematic of such a culture chamber is shown in Figure 6-2.



**Figure 6-2: Device for 3-Dimensional culture at the microscale**

The inlet channel architecture can be simply removed post encapsulation and replaced with a media chamber that will be cored into the PDMS. The beads will be allowed to settle in the micro-culture chamber under gravity and allowed to gel. The oil phase will be flushed out post gellation by operating a pump in the withdraw mode through the outlet port and replaced with media from the reservoir. The small outlet channel and the ledge at the exit combined with the dynamics of the micro-scale flow will prevent the beads from clogging the outlet port.

### 6.2.3 Transplant of Encapsulated Hepatic Progenitor Cells

Hepatic progenitor cell death was associated with previous transplant experiments using alginate microspheres. This drop in viability was felt to be a function of the stresses exerted on the cells during the encapsulation procedure.

The benign nature of the collagen encapsulation system (due to the minimal stressors on the cells) ensures better long term cell viability.

The HPCs inside the collagen microspheres will be transplanted by intrasplenic injection using a 27 gauge needle. GFP<sup>+</sup> donor cells will be transplanted into wild type mice to test for cell survival in-vivo and host immune response to the transplanted beads. Collagen microspheres encapsulating HPCs will be transplanted into transgenic ApoE<sup>-</sup> mice to test for restoration of liver specific function.

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