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Design And Assembly Of Microcapsules And Hollow Fibers For In-Vitro Culture Systems And Organ-On-Chip Systems

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CHAPTER 1
INTRODUCTION

1.1. Statement of the Problem

Organ and tissue transplantation are currently the most widely and prosperous therapy for severe, end stage disease including articular cartilage damage and acute liver injuries. Despite the success of these therapies, there are hindered by lack of organ and tissue donor, the necessity of a lifelong immunosuppression and its potential for serious complications. Tissue engineering has emerged as a successful approach to address these issues by building 3D tissue structures \textit{in-vitro} which can be later be implanted \textit{in-vivo}.

Liver is a complex organ which is responsible for a myriad of task in our body including albumin and urea synthesis, bile production, drug metabolism and detoxification among many others. Acute and chronic liver injuries including hepatitis, alcoholic liver, drug toxicity and infections are lethal and input a lot of economic burden on health sector. Currently, liver transplantation is the only treatment for end stage liver disease and suffers from the fact that there is always lack of organ donors compared to organ recipients. According to U.S Organ Procurement and Transplantation Network (OPTN), as of June 19th, 2020, there were 12,418 patients on waiting list for liver transplantation. As can also be seen from fig. 1.1, after kidney, liver is the highest demand organ for transplantation. Liver transplantation is also a costly procedure with an approximate cost of $600,000 according to the United Network for Organ Sharing (UNOS) in addition to the lifetime need for immunosuppressive therapies. Apart from success of temporary liver support systems such as artificial liver, which can accomplish liver functions for short period of time (days to weeks), there is still a long-road to fabricate an \textit{in-vitro} liver which can function like a native liver. Building a complete liver which can replace and function like a native liver, is the goal of liver tissue engineering.

Therefore, liver studies have gained lot of attention among tissue engineering and regenerative medicine community. Currently, most of the research on tissue related disease and culture systems, are
focused on building 3D \textit{in-vitro} models which can resemble the \textit{in-vivo} conditions. These systems are more complex, require different fabrication, assembly and analysis techniques compared to the traditional 2D culture systems \cite{4}. Tissue generation is one of the key parameters of \textit{in-vitro} systems which should be optimized to gain maximum rate of tissue generation\cite{5}. 3D cell culture systems can accelerate the fabrication and generation of new tissue due to their ability to simulate the \textit{in-vivo} conditions \cite{6}. Due to diversity of liver function from drug metabolism to hormone synthesis, multiple groups have designed \textit{in-vitro} liver models which capture a single function of the liver \cite{7,8}. So far, it is totally clear that a single \textit{in-vitro} liver model, due to high complexity of this organ, cannot handle all functions and duties of the liver. Hence, any model design should be able to mimic a specific portion of the liver. Despite all the advances in the design and fabrication of \textit{in-vitro} liver models or “liver-on-chip” systems, there are still challenges regarding their application including loss of hepatocyte function and lack of bile duct presence in these systems. These challenges were the main motivations behind this research to come up with a novel, modular approach to build a liver on chip based on microcapsules and hollow fibers.

![Fig. 1.1. Organ demand in U.S. as of June 2020 (UNOS)](image)

\begin{itemize}
  \item Kidney-83.7% 
  \item Liver-11.6% 
  \item Heart-3.3% 
  \item Lung-1.2% 
  \item Other-1.5%
\end{itemize}
1.2. Approach to the Problem

Generating a dense tissue containing cells and extracellular matrix (ECM) proteins, outside of the body is one of the prime goals of tissue engineering. These dense tissues can be later used for organ transplantation or organ studies outside of the body. For many years, seeding and growing cells inside the scaffolds (top-down approach) was the gold standard in tissue engineering (fig. 1.2). Despite some advantages of these models including their high mechanical properties, they suffered from many drawbacks including inability of nutrient and growth factor diffusion into the scaffold, lack of cell migration inside the scaffold resulting in non-uniform cell distribution and inability to provide a uniform cell distribution inside the scaffold.

![Fig. 1.2. Illustration of the traditional top-down approach and novel modular approach to create an engineered tissue.](image)

The modular (Bottom-up) approach addresses all these issues by building a smaller modular microtissue which can later be assembled to fabricate a larger, macroscale tissue. Uniform cell distribution and control of cell aggregation by adjusting the dimensions of the microstructure, is one of advantages of
modular approach. There are a variety of microtissue geometries which can be used in the modular approach including micro molds, microbeads, and microfibers. In all these designs, cells are surrounded in an ECM like microstructure resembling the native ECM structure \(^{9-11}\). Assembly of the repeating microtissues can also be done by a variety of methods including photo crosslinking, 3D bioprinting and, layer by layer stacking of cell sheets\(^ {12}\). The fabricated 3D tissue can later be used for implantation to replace the damaged organ and tissue or, can be used inside organ-on-chip bioreactor for disease studies and drug toxicity tests \(^ {13}\).

This project is focused on building a bile duct on-chip by assembling microcapsules and hollow fibers in a perfusion bioreactor. Hepatocytes alongside MSCs and VECs were encapsulated inside a glycosaminoglycans (GAG) and Chitosan microcapsules and liver epithelial cells (cholangiocytes) were seeded inside the hollow fibers, presenting the bile duct. In the first section of the project, a comprehensive study was done on optimizing the cell encapsulation using electrospraying (ES) technique. For this mean, mesenchymal stem cells (MSCs) were encapsulated at different formula and voltages to find the optimum conditions regarding cell viability and function inside the microcapsules. Successful differentiation of encapsulated MSCs to three lineages of adipocytes, osteocytes, and chondrocytes, further verified the safety and potency of this method for regenerative medicine applications. Co-culture of hepatocytes with other cell types including MSCs have shown to preserve their metabolic activity inside \textit{in-vitro} systems\(^ {14}\). ES method was later used to encapsulate hepatocyte-MSCs- Vascular endothelial cells (VECs) as our modular compartment. MSCs will help maintaining the hepatocytes functionality while VECs participate building a vascular network inside microcapsule facilitating the transfer of nutrient inside the microcapsule. An \textit{in-vitro} bile duct was also made by fabrication of hollow fibers seeded internally with cholangiocytes. Eventually, cholangiocytes hollow fibers and hepatocytes microcapsules were assemble inside a perfusion bioreactor, representing our bile duct-on-chip system.
2.1. An Intro to Tissue Engineering

The conventional approach of tissue engineering (top-down) involves combining living cells with a natural or synthetic scaffold that is biocompatible and biodegradable to build a 3D construct with the same or better functionally and mechanical stability of the original tissue\textsuperscript{15}. This approach has four basic components to construct the desired 3D structure: 1) Cells. 2) Growth factors. 3) Extracellular matrix. 4) Scaffold.

1. **Scaffold**: scaffolds are the building block of the top-down approach. They are biocompatible, degradable and have a porous structures which can be fabricated from either natural material such as chitosan, collagen and fibrin or synthetic polymers such as polyglycolide, polylactide and polylactide coglycolide\textsuperscript{16}. They might have a simple structure such as sponge like sheets or highly complex structures with intricate pores and channels fabricated using new 3D printing techniques. In general, all scaffolds designed and used in TE should degrade slowly after implantation. Therefore, the choice of the biomaterial used for fabricating the scaffold is of great importance. There are three individual groups of biomaterials, ceramics, synthetic polymers and natural polymers which are used in the fabrication of scaffolds for tissue engineering\textsuperscript{17}. Each of these materials have their own advantages and disadvantages, therefore, current studies are greatly focused on mixed biomaterial to maximize the performance of the scaffold. For instance, recently ceramic scaffolds such hydroxyapatite and tricalcium phosphate have become popular in bone tissue engineering\textsuperscript{18}. These scaffolds are known by their high mechanical stiffness (Young module), low elasticity and hard brittle surface. From a bone perspective, they exhibit excellent biocompatibility due to their chemical and structural similarity to the mineral phase of native bone. The interactions of osteogenic cells with ceramics are important for bone regeneration as ceramics
are known to enhance osteoblast differentiation and proliferation. In other researches different ceramics have been used in dental and orthopedic surgery to fill bone defects and to coat metallic implant surfaces to improve implant integration with the host bone\cite{19}.

2. **Cells:** source of the cells used in seeding the scaffold is the most important parameter in provoking the immune response after implantation. In general, there are three types of cell source used in TE: autologous which are the cells from the patient and have the lowest immune response stimulation, allogenic which are the cells from a healthy donor who is not immunologically identical to the patient and xenogeneic which are the cells from different species\cite{20}. There has been a lot of research on using each of these cell types in TE. The autologous and allogenic cell sources are the best for TE applications but due to lack of these cells and shortage at the time of demand, xenogeneic cells studies have attracted a great attention\cite{21}. Although, the future of using xenogeneic cells for tissue repair remains controversial due the potential for transmitting animal pathogens to humans\cite{22}. In the best scenario, xenogeneic cells can temporarily support a defected tissue until either a human donor organ becomes available for transplant or the tissue repairs itself. In one research pig liver cells have been cultured in a bioreactor to be tested on a patient suffering from a liver injury until a liver transplant can be performed\cite{23}. Allogeneic cells have also been used successfully to treat skin ulcers, diabetes, and liver disease\cite{24,25}. Patients with diabetic or venous skin ulcers have been treated with two FDA approved living skin products engineered in the lab\cite{26}.

3. **Growth Factors:** use of growth factors (GF) is one of the important aspects that must be considered to have a successful cell growth inside the scaffold. For example, during the natural healing of the skin after the wound, a complex set of GFs including FGF and VEGF are released to trigger cell proliferation, macrophage activation and angiogenesis. The timing of growth factor release is exactly controlled and is triggered by the progress of repair process. Therefore, use of the GFs in TE is vital to have higher cell proliferation and angiogenesis\cite{27}.

4. **ECM proteins:** ECM proteins are a mixture of cellular and non-cellular components with a super well-organized network, present in all tissues and organs. They provide a physical scaffold for cells
and regulate many cellular processes including growth, migration, differentiation, survival, homeostasis, and morphogenesis. The ECM is mostly made of collagen, glycosaminoglycans (GAGs), fibronectin and elastin. Cells inside ECMs interact with this macromolecular network through their surface receptors, such as integrins, discoidin domain receptors (DDRs), cell surface PGs, and the hyaluronan (HA) receptor CD44. This aim can be achieved by using scaffold which are made of ECM or they deposit the ECM. Ligands are the pathways which cells interact with scaffold throw them. Natural scaffold such as collagen possess these ligands whereas synthetic scaffolds require the deposition of these ligands onto their surface. On the other hand, the mechanical properties of synthetic scaffolds are better compared to natural scaffold.

2.2. Drawbacks of the Top-Down Approach

The goal in the top-down approach is for cells to proliferate and populate the scaffold and make an appropriate extracellular matrix and a microstructure with the help of growth factors, perfusion cultures and mechanical stimulation. This dense microstructure can later be transplanted into the damaged organ or tissue. However, there are several issues that must be addressed to achieve efficient, long-lasting repair of damaged tissues; these include: 1) There must be enough number of cells to fulfill the damaged tissue, in other words cell proliferation must be at maximum level. 2) Scaffold should be able to be vascularized to accelerate the nutrient transport into the cells in the center of the scaffold. 3) Scaffold should be mechanically stable. 4) Cells should not provoke any immune response. Although the top-down approach has a couple of advantages e.g. high mechanical stability of the scaffold, it lacks most of the aforementioned characteristics, especially low rate of cell proliferation and also lack of vascularization which are two key parameters in a successful tissue design. Therefore, to overcome these obstacles, the bottom-up approach was proposed. The bottom-up approach will start with building microstructures such as microcapsules and microfibers, containing cells, to assemble and obtain larger, macroscale tissue.
2.3. Cell Encapsulation Technologies

Cell encapsulation in a biocompatible, biodegradable material using natural polymers and hydrogels has been a focus of research in regenerative medicine for decades. These structures are popular as they provide a genuine microenvironment for cell growth. Cells without protection are at high risk of attack by immune system after implantation which will diminish cell function, eventually leading to cell death. One great approach to overcome this problem is to encapsulate cells in a biocompatible, biodegradable material. The microcapsule environment, if filled with ECM protein can also accelerate cell growth and function by mimicking the in-vivo cell microenvironment. Apart from the in-vivo applications of cell encapsulation, encapsulated cells will be more protected inside the in-vitro organ-on-chip models too, as the capsule membrane will protect the cells from the side effects of perfusion cultures. Biomaterials used for cell encapsulation should possess certain level of porosity, facilitating diffusion of nutrients and oxygen into the cells and removal of toxins. Therefore, specific considerations should be considered when choosing the biomaterials. Rather than the choice of biomaterial, technique used for cell encapsulation will also affect cell function. Fig 2.1. Summarizes the four major type of microcapsules widely used in TE; bulk and hollow representing the cells entrapped in hollow fibers or entrapped in a bulk gel, micromolding which uses molds with specific designs to fabricate different shapes of microcapsules, microbead generation methods which generate cells entrapped in a spherical shape capsule and, microfibers which encapsulate cells inside a filled fiber structure. Each of these types, requires a certain fabrication technique which will be discussed in next section.
2.3.1. Fiber Based Encapsulation

Hollow fibers (HFs) are widely used in TE for encapsulation of neurons, hepatocytes, and muscle cells. Most of these hollow fibers are formed by the gelation process which yields uniform structure but with low mechanical properties\textsuperscript{33}. Normally, cells such as endothelial cells and hepatocytes are seeded inside the lumen of the HFs in which the medium passes through the HF becoming in contact with the cells. Therefore, the choice of biomaterial used for fabrication of these HFs is crucial as it will affect cell function and strength of the HF. Synthetic HFs have high mechanical strength but at the same time are less biocompatible\textsuperscript{34}. Therefore, use of biomaterials is preferred to synthetic polymers. There are multiple methods to fabricate HFs including gelation, extrusion and microfluidic techniques. In the gelation method, HF is formed by neutralization of chitosan with ammonium hydroxide\textsuperscript{35}. The HF made can be optimized by addition of other ECM proteins on the surface of the chitosan HF. HFs can also be made by extruding a polymer solution which is pumped through an outer tube of a nozzle and an aqueous solution flew through a central tube\textsuperscript{36}. These fibers are later collected in an aqueous water bath followed by washing the remaining solvent\textsuperscript{38}. HFs fabrication using microfluidic techniques is another area of research which have attracted a great attention\textsuperscript{37}. These methods are rapid and versatile but at the same time, are mostly focused on use of
synthetic polymers. Apart from HFs, fibers also play a key role in tissue engineering and regenerative medicine. These fibers can be used to make 3D structures by assembling multiple layers of repeating units in which cells can be either be seeded inside the fiber or on the surface. They are multiple techniques for generation of fibers including electrospinning, wet spinning, and microfluidic spinning. Fabrication of fibers in general is less complex than HFs as there is only one material used in generation of these structures.

2.3.2. Microcapsules

Spherical shaped capsules or microcapsules, are another encapsulation structures which have been used for many years. These microcapsules can be made both from natural or synthetic polymers with synthetic polymers possessing higher mechanical strength but lower cell interaction and cell adhesion properties. The microcapsule membrane should be permeable for diffusion of nutrients and toxin removal from the interior space. Spherical shape of the microcapsule is another advantage of these structures regarding mass transport properties which they offer the highest surface to volume ratio compared to other geometrical shapes. Moreover, capsule size in the range of 100-500µm allows their implantation close to the blood vessels without oxygen depletion. These structures are also mechanically more stable compared to other modular structures. Microcapsules can be made by different techniques including microfluidics, electrospayin, air extrusion, lithography, and emulsion. All these methods begin with formation of a uniform droplet which is later stabilized by addition to a second solution to form a solid membrane around the droplet. The bonding between the first and second solution can be either chemical or physical, depending on the nature of the two polymers used. Microcapsules can also be seeded externally with cells or proteins, acting as both microcarriers and microcapsules for drug delivery applications (fig. 2.2). Based on this, different biomaterials have been used in TE with the most widely used one being alginates. Alginates are widely available, easily gel but at the same time are not completely biocompatible. Hence, other biomaterials were introduced to overcome this limitation including, chitosan, collagen, and different type of GAGs. These biomaterials have shown to promote cell function and proliferation in addition to their high
biocompatibility and low immunogenicity\textsuperscript{42}. Beside the type of the biomaterial used in fabrication of microcapsules, size and shape of the microcapsules is another critical factor in determining the cell function inside the capsule. Researchers have found that immune response was lower in smaller microcapsules compared to the larger ones\textsuperscript{43}. Moreover, it has been seen that the cell viability is higher in smaller (<100\textmu m) microcapsules due to higher O\textsubscript{2} and nutrient diffusion into the microcapsule\textsuperscript{44}. Roughness of the microcapsule surface is another factor which should be avoided due to stimulating the immune response\textsuperscript{45}. To address all these requirements, there is a need for an encapsulation technique being able to generate small, uniform, smooth microcapsules with high level of reproducibility. Among the available microcapsule generation techniques, microfluidics and electrospraying method have shown a great advantage over the other methods\textsuperscript{46,47}. These two methods can generate large number of microcapsules in short period of time with high level of accuracy and reproducibility which are crucial for regenerative medicine applications. In the next part these two techniques will be discussed in detail.

\textbf{Fig. 2.2.} Major properties of microcapsules. By addition of proteins or cells to the capsule membrane, they can be optimized to microcarrier structures.
2.3.2. Microfluidic Based Encapsulation Techniques

The obstacles and limitations of the microencapsulation methods is overthrown by using microfluidic based encapsulation techniques. These techniques give better control over size, shape, and production rate of the microcapsules in addition to cost effectiveness and time saving. The most important advantage is the high reproducibility of the encapsulation results compared to other encapsulation methods. Cells are generally encapsulated by gelation in a hydrogel solution to form a solidified complex\(^{48}\). The materials used for this method are of great importance since they should be both biocompatible and biodegradable. Natural polymers such as chitosan and sodium alginate and synthetic polymers such as polyethylene glycol (PEG) are poly-lactic-glycolic acid (PLGA) are amongst the most widely used materials for this mean. Microfluidic based techniques can be divided to two groups based on the design used: T-junction design and flow focusing. Tan et al explained the fabrication of monodisperse alginate microbeads using a droplet forming T-junction microfluidic channel\(^{10}\). This instrument let them to precisely control the bead size and uniformity with adjusting the two flowrates. They also utilize calcium carbonate nanoparticles inside the sodium alginate solution which induces the internal gelation. Some examples of the microencapsulation using microfluidic techniques is illustrated in fig. 2.3.

Fig. 2.3. Encapsulation and 3D liver tissue construction methods using microtechnology: (A) 3D encapsulation of hepatocytes and transplantation increased the survival rate of a liver failure-induced mouse model, (B) In situ encapsulation of liver spheroids using concave micromolds, (C) Formation of heterotypic
hepatic micro-organoids with a controlled cellular organization developed from hydrogel microfibers and (D) optical images of the developed 3D hepatic cord structure⁴⁹.

2.3.3. Electrospaying Technique

One of the great challenges in the encapsulation techniques mentioned above is the difficulty in adjusting the microcapsule size and uniformity. In the bulk and hollow method we cannot fabricate capsules in the range of micro and although in microfluidic systems, capsules will be in the micro range, since the capsules are formed at the interface of two liquid, the capsule properties will be greatly dependent on the interfacial tension between two liquids and in other words to the intrinsic characteristic of the two liquids used for encapsulation. That’s why most of the research in the field of microfluidic is based on water in oil(w/o) or oil in water systems(o/w) or sodium alginate calcium chloride since the gelation process in these systems are so fast and there is no instability in the formation of capsules or fibers. Electrospraying is a liquid atomization method using high electrical forces to dominate the surface tension of the liquid. Droplets formed in this method are highly charged and can be in the range of nanometer⁵⁰. The size and the charge of the droplets can be controlled by the voltage applied to the system and the flowrate of the liquids mainly. Electrospraying technique operates on the principle of an applied potential difference between two electrodes. One electrode in this case is a conducting stainless-steel needle accommodating the flow of media, while the second is a grounded electrode, which could geometrically be varied from a ring, or point, to a plate or beaker. The applied voltage to the liquid containing electrode will result to voltage difference between tip of the electrode and the ground. It has been shown that variation in ground electrode can control a majority of the generated droplet trajectories⁵¹. Electrospraying could be a useful technique to encapsulate cells in 3D microstructures in a rapid and convenient fashion. Although the effect of electrospraying on cell viability, proliferation and differentiation still needs to be studied more in detail. Electrospinning, similar method with the same principles as electrospraying, has been explored for over a century with applications in both the physical and biological sciences for the fabrication of nano to micro sized fibers and scaffolds. Although, the principle of the two methods are the same in which both of them are driven by electric fields, electrosprying method is used for generation of droplets while electrospinning is used for generation of...
micro to nano sized fibers. The difference between the electrospinning and electrospraying techniques lies in the density of the polymer solution and the voltage that is applied to it. Studies have shown that a critical polymer concentration called $c_{cv}$ can determine the transition between electrospinning and electrospraying. This critical concentration can be found for each type of polymer solution and represents the critical number where disintegration of the fiber structure will begin. In order to produce fibers, the polymer concentration, $c$, must be chosen such that a threshold ratio $c/c_{cv}$ is overcome. Hence, at viscosities lower than a certain value, there will be formation of droplets rather than fibers. The ratio of $c/c_{cv}$ can be found experimentally for each polymer solution with known values of $c_{cv}$. The electrospraying is basically an easy and versatile process: first the polymer solution is loaded into a syringe pump and the tip of the metal needle is attached to a high voltage power source. The power can be from 0-30 kV and the needle size can vary from 16-24 G based on the desired capsule size. The droplets are then formed at the tip of the needle and are then collected into an either a plate or can also be collected into a beaker containing another solution for further improvement of the microcapsule. In the electrospraying technique, there are several parameters which all have an inter-dependent influence on droplet size, distribution, encapsulation efficiencies, loading capacities and in vitro release profiles. These parameters include voltage, needle size, distance to collector, and polymer flowrate. Therefore, although the electrospraying techniques seems a simple method, due to number of parameters affecting the whole process, its optimization is highly complex.

2.4. Cell Encapsulation Biomaterials

2.4.1. Chitosan

Chitosan derived from chitin is a linear polysaccharide composed of (1-4)-2-acetamido-2-deoxy-β-D-glucan (N-acetyl D-glucosamine) and (1-4)-2-amino-2-deoxyβ- D-glucan (D-glucosamine). The structure of the chitosan is shown in Fig. 2.4. The deacetylation degree (DD) of chitosan, which illustrates the number of amino groups along the chains, is calculated as the ratio of D-glucosamine to the sum of D-
glucosamine and N-acetyl D-glucosamine. The criteria is that the deacetylation degree should be at least 60% to name the compound chitosan. Chitosan is the second most abundant polysaccharide after cellulose which is usually found in the vertebrates as crustacean shells or insect cuticles. The most prominent advantage of chitosan over chitin is the presence of the amino groups. Chitosan with protonated amino groups becomes a polycation that can subsequently form ionic complexes with a wide variety of natural or synthetic anionic species, such as lipids, proteins, DNA and some negatively charged synthetic polymers as poly (acrylic acid). On the other hand, chitosan is the only naturally occurring polysaccharide which is positively charged. The amino and alcohol functions along chitosan chains enable this polysaccharide to form stable covalent bonding with other species. Other specific characteristics of the chitosan include antibacterial, antifungal, mucoadhesive, analgesic and hemostatic properties. Moreover, chitosan can be degraded to non-toxic residues which the rate of degradation depends on the molecular weight and deacetylation degree of the chitosan. All these special features have made chitosan a useful and promising biopolymer in tissue engineering.

![Chemical structure of chitosan](image)

**Fig. 2.4.** Chemical structure of chitosan.

### 2.4.2. Collagen

Collagen is the most widely protein found in the body and is one of the major constituents of extracellular matrix which is well known for the excellent biocompatibility and biodegradability and low antigenicity. In the development or healing of tissues and organs, collagen is involved in controlling the cell behaviors such as adhesion, proliferation and differentiation and plays an important role in the formation of extracellular matrix. Collagen is the primary component of skin and bone which constitutes around 25% of body’s total dry weigh. So far, 29 distinct types of collagen have been found in which all of
them display a typical triple helix structure. Collagen fibers are mostly composed of types I, II, III, V and XI. Collagen molecules are made of three α chains that arrange together according to their molecular structure. Each α chain is made up of more than a thousand amino acids based on the sequence -Gly-X-Y-. Glycine is the major component which should be present at every third amino acid position to promote a tight assembly of the three α chains of the triple collagen molecule. The other empty X and Y positions are packed with 4-hydroxyproline and proline. Although many types of collagen have been described, only a few types are used to produce collagen-based biomaterials. Type I collagen is currently the gold standard in the field of tissue-engineering.

![Fig. 2.5. Chemical structure of collagen.](image)

### 2.4.3. Glycosaminoglycans (GAGs)

GAGs are a group of highly sulfated, complex polysaccharides that play multiple major biological roles in the body. They can be arranged into four main groups based on the different repeating disaccharide groups: hyaluronan (HA), chondroitin sulfate (CS), dermatan sulfate (DS), heparin (HP), heparan sulfate (HS), keratan sulfate (KS). Rather than hyaluronic acid, all other GAGs are sulfated in which heparin is the most sulfated GAG among all others. GAGs are categorized based on three characteristics; first monomer type present in the GAG, second the position of glycosidic linkages and third is the amount of sulfation. Due to the presence of acidic sulphate and/or acid groups (COO-), all GAGs are negatively charged. GAGs play a primary role in the integrity of the tissue and play an important role in embryo development and organogenesis. Most of the macromolecules including proteins interact with GAGs. They also play a major role in...
role as a lubricating agent in articular cartilage due to their low compressibility and high viscosity. Facilitating cell migration and cell integrity inside the tissue is two other roles of GAGs. Variation in the GAG synthesis inside body including their down regulation and upregulation also plays an important role in development of disease\(^{30}\). GAGs are triggered when interacting with different proteins. Multiple researches have illustrated that specific sequences within heparin and HS act as protein binding sites\(^{56}\). This suggests that polysaccharides can act as informational molecules and indicated the importance of developing analytical methods to sequence GAG molecules\(^{56}\). Chemical structure of different types of GAGs is illustrated in fig. 2.6.

![Chemical structure of different GAGs](image)

**Fig. 2.6.** Chemical structure of different GAGs\(^{56}\).

### 2.5. Liver Structure

Liver which weighs around 1.2-1.6 kg in adult humans is the largest solid organ in the body, responsible for complex set of functions including hormone production, synthesis of all plasma proteins, bile and urea in addition to generation of immune proteins for both adaptive and innate immune system. One other function of liver is bile production which is vital for food digestion\(^2\). Liver tissue is made of small
lobules. The liver lobule is the well-defined structural unit of the liver (fig.2.7A). Each lobule has a hexagonal design with a diameter of approximately 1 mm and the thickness of about 2 mm. In adults, the lobule consists of hepatocyte plates, which diverge from a central vein in the center of the hexagon. All the adjacent hepatocytes in each lobule are joined by tight junctions. The tight junctions are separated by the bile canaliculi with a diameter of about 1 μm. The human liver contains about one million lobules. At each vertex of a lobule there is a portal triad which is consisted of portal vein for delivering most of the oxygen to liver, hepatic artery for most of the nutrients required and bile duct for collecting bile accumulated in bile canaliculus. Portal triads are grouped together by connective tissue. Portal vein supplies about 80% (in volume) of the blood required by the liver and it contains the nutrients absorbed from the digestive system. Cells inside each lobule can also be divided by their distance from the central vein (Fig.2.7B). In this manner cells closer to triads absorb most of the nutrients and oxygen. It has been seen that the oxygen concentration drops from 13% v/v to 4% v/v by going from region 1 to 3. This oxygen concentration regulates the expression of different genes including pyruvate carboxykinase-1 which makes the carbohydrate metabolizing enzymes. This phenomenon is called the metabolic zonation of liver. Liver cells can be divided in to two general group, first is the parenchymal cells which includes the hepatocytes, second the non-parenchymal cells which include cholangiocytes, liver sinusoidal endothelial cells and Kupffer cells (macrophages). In the next section each of these cells will be explained in detail.

Fig. 2.7. (A) liver lobule, (B) organization of the liver lobule and acinus (C) Lobular zonation of different metabolic pathways.
2.5.1. Hepatocytes

Hepatocytes are the liver parenchymal cells which do most of the metabolic functions and account for most of the total liver cell population by weight and volume (80%)\textsuperscript{57}. Most circulating plasma proteins such as albumin, transporters, protease inhibitors, blood coagulation factors and modulators of immune complexes and inflammation are expressed by hepatocytes. Homeostasis of molecules such as cholesterol, triglyceride, bile acid, vitamin A and D and metabolism of the compounds such as heme and bilirubin are also done by hepatocytes. In addition, liver is responsible for detoxification of ammonia and regulation of pH which both these processes need urea, therefore urea synthesis in liver is used as key marker for hepatocyte activity. Hepatocytes are surrounded by sinusoid from the sides and which provides the blood into them (fig. 2.8). Liver sinusoids are covered with sinusoidal endothelial cells which play a great role in regulation of hepatocyte activity\textsuperscript{58}. Bile canaliculus are also spread around the hepatocytes, responsible for collection of bile.

![Fig. 2.8. Location of hepatocytes surrounded by sinusoids inside the liver. Portal triads in composed of bile duct, hepatic artery, and portal vein. It should be noted that the flow direction of bile and blood are opposite inside liver. The arrows show the direction of the flow in sinusoids and bile canaliculi\textsuperscript{57}.](image-url)
2.5.2. Biliary Epithelial Cells (Cholangiocytes)

Cholangiocytes are the epithelial cells of the bile duct. Their impairment and lack of function causes biliary disorders such as ischemic cholangiopathy and biliary atresia which are among the highly morbid liver disease \(^{59,60}\). They are responsible for bile modification through a series of adsorptive and secretory processes which are regulated by calcium signaling \(^{61}\). Multiple gastrointestinal hormones such as secretin, somatostatin and bombesin are responsible for modification of bile by cholangiocytes. Biliary tree is a super complex network of tubular structures inside the liver which varies in size from the extrahepatic region of around 800µm to interlobular ducts of around 15µm. Same as the variation in bile duct diameter, the cholangiocytes which line the bile ducts also vary in size from 6 to 15µm (fig 2.9A&B)\(^{62}\). Also, their morphology varies with the flattened, cuboidal shape in small ducts and columnar structure in large ducts. Cholangiocytes are polarized cells having an apical and basolateral membrane and adjacent cells are attached with tight junction proteins which are located on the apical side. Bile, which is mainly composed of bile salts and water, is transported into the canaliculi resulting in a “bile-salt-dependent” transport\(^{63}\). This bile then enters the lumen and canals of herring followed by transporting into the biliary tree. It is in this section which the bile primarily modified by cholangiocytes (mostly by large cholangiocytes). Bile modification in this area is done by secretion of Cl\(^-\), HCO\(_3\)- and water from cholangiocytes and reabsorption of amino acids, bile acids and glucose into the cholangiocytes (fig 2.7C) \(^{64}\). Therefore, these cells play a critical role in liver metabolic activity especially in bile modification.
2.5.3. Liver Sinusoidal Endothelial Cells

Liver sinusoidal endothelial cells (LSECs) are the cells which form the wall of the liver sinusoid and make up to 15-20% of the liver cells but only 3% of the total liver volume. LSECs are specialized endothelial cells of the liver sinusoid which are recognized by the lack of a basement membrane. Apart from protecting the endothelium as a barrier, LSECs also participate in the following inflammatory reactions: (1) They can detect the patterns associated with pathogens such as lipoteichoic acid, Lipopolysaccharide, N-acetyl muramyl peptide (2) They secrete cytokines and chemokines to activate leukocytes. (3) They express different adhesion proteins that help attachment of leukocytes to the injury site. One of the main characteristic of LSECs is the lack of diaphragm and a basal lamina leading to the presence of fenestrae. This makes them as a porous barrier between blood components and hepatocytes. These cells are also responsible for transportation of blood from branches of portal vein and hepatic artery into the central vein of the liver lobule. The other key role of the LSECs is that they promote vascularization and angiogenesis.

2.5.4. Kupffer Cells

Kupffer (KC) cells are the quiescent macrophages of the liver which in case of liver damage they synthesize and secrete the pro-inflammatory cytokines tumor necrosis factor a (TNFa) and IL-1b. They represent about 35% of the non-parenchymal liver cells in normal adult human. They are in the liver sinusoids and are attached to the LVECs. They are the first line of defense against bacteria, bacterial endotoxins and microbial debris derived from gastrointestinal tract and transported to the liver via portal vein. They represent 80-90% of the tissue macrophages in the body in which out of this amount, 80% of the KC in the body reside in the liver. This shows the importance of the KC as a critical barrier against foreign body particles and bacteria. KC are present throughout the liver, but there are differences in the population density, cytologic characteristics and physiologic functions of KC in different zones of the
hepatic acinus. Large KC are mostly located in the periportal region of the liver acinus which is the first point of contact for the pathogens and bacteria. These KCs have higher lysosomal enzyme activity compared to the other KCs\textsuperscript{66}.

2.5.5. \textit{Mesenchymal Stem Cells (MSCs)}

MSCs were introduced more than 40 years ago by Friedenstein et.al as a group of adherent cells isolated from the bone marrow which were non phagocytic, possess a fibroblast like structure and have the potential to be differentiated to adipocyte, chondrocytes, muscle, tendon and bone tissue. MSCs can also be isolated from other tissue such as adipose tissue, umbilical cord blood and muscle connective tissue\textsuperscript{67}. One other major characteristic of them is is their potent immunomodulatory properties which interact with both adaptive and innate immune system. Another great feature of MSCs is their tissue regeneration capability. Tissue regeneration after transplantation of MSCs at the sight of injury is believed to be due to secretion of growth factors which act in a paracrine model. In general, MSCs effect on tissue after transplantation can be categorized in four section of cytoprotective effect, provasculogenic effect, anti-inflammatory effect and antifibrotic effect. Due to these promising characteristics of MSCs, they have widely been used in co-culture systems with other cell types such as endothelial, muscle, cardiac, neurons and liver cells to preserve and retain the cell function inside \textit{in-vitro} culture models\textsuperscript{68–71}. MSCs helps growth and survival of the native cell at the site of implantation by secretion of growth factors and ECM proteins. It is believed that MSCs antifibrotic effect on cardiomyocytes is due to altering the heart ECM structure through paracrine signaling, resulting into faster healing\textsuperscript{72}. It was also observed that direct injection of MSCs into rat heart decreased the fibrosis and accelerated the cardiac healing process\textsuperscript{73}. These vast evidence of the beneficial effect of MSCs in healing and regeneration of tissue has transformed them as a great tool in the co-culture systems resulting to cell survival and preventing tissue and cell fibrosis.

2.6. \textit{Organ-on-Chip Systems}

The first practical 2D cell culture systems were developed around 100 years ago. In spite of their great impact on advancing the cell related studies, they lack the ability to support tissue specific and
environment required for many cell types; in other words they cannot mimic the *in-vivo* microenvironment which is essential for proper growth and activity of many cells. These defects were the motivation behind fabrication of complex 3D systems which could come up with all these obstacles. Therefore, 3D cell cultures systems were proposed and developed around 50 years ago to utilize ECM proteins, hydrogels, and different natural polymers alongside the cells to fabricate a complex 3D cell culture system to better mimic the *in-vivo* conditions. These systems could mimic *in-vivo* conditions much better than traditional 2D systems with more accurate results with respect to protein synthesis, drug toxicity tests and cell metabolism. However, these systems also have some limitations. For instance, cell organoids which are a widely used 3D cell culture systems, are highly variable in size and shape and it is also challenging to maintain cells in a fixed and stabilized position in 3D culture systems. Another drawback of the 3D cell culture systems is the inconvenient sampling procedures for cell metabolic activity and related procedures; for instance protein synthesis analysis in 3D culture systems requires special imaging techniques and the conventional protein quantification assays might not work due to the thickness of the 3D culture, preventing the diffusion of synthesized proteins out of the system. Moreover, these systems still lack the precise design and architecture of the *in-vivo* systems such as the interfaces between vascular endothelium and connective tissue which is essential for the development of the nearby cells and organs. At last, since most of these systems are designed and implemented under static culture conditions, some physical parameters like shear stress, transport phenomena, compression and extension which are required for cell and organ growth cannot be applied inside these 3D culture systems. To overcome the aforementioned limitations, researchers came up with an idea of developing a system which can mimic the 3D structure of the desired organ or tissue under perfusion conditions, while at the same has the advantage of easy sampling, easy operation, high image quality and high reproducibility of the results. Organ on a chip, or miniaturized organs inside a bioreactor, can address all the limitations of the current 3D culture system, in addition to providing a small-scale organ model for disease studies and drug toxicity tests. Organs-on-chips are devices for cell assembly and proliferation under perfused and organ alike environment to model physiological functions of tissues and organs. One great advantage of these systems is that there is no requirement to build the whole organ inside
one chip as the organs are super complex and rather, each organ can be subcategorized into smaller sections and each section can be designed in a separate bioreactor. Later by assembling these sections, the whole organ structure can be recapitulated. The simplest design of these systems is a perfusion bioreactor which contains cells and medium. Addition of more cell types, ECM proteins and vascular network in these systems make them more complex and relevant to \textit{in-vivo} tissue and organ microenvironment. Moreover, physical forces such as fluid shear stress and mechanical forces can also be applied to these systems to allow analysis of organ-specific responses including analysis of bone and cartilage structure. One example of these systems is illustrated in Fig. 2.10 in which multiple liver cells are aligned in a perfusion bioreactor mimicking the liver sinusoid structure.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2_10.png}
\caption{Liver-on-chip system mimicking the liver sinusoid using parenchymal and non-parenchymal cells, (A) microfluidic design, (B) fluid flow inside chip, (C) image of the chip.}
\end{figure}

One great advantage of organ-on-chip systems is the capability of the system to control a wide array of parameters including medium flow, number and type of cells, structural design of the system and ease of sampling from the system. This characteristic makes them a superior model compared to traditional organoid and 3D culture systems which are not able to control the physical parameters of the process. One of these parameters which affect cell growth and proliferation is fluid flow. For instance, since viscous forces dominate over inertial ones at small length scales, the flow will be laminar if the diameter of the microfluidic channel is less than 1mm. Moreover, shear stress can be controlled independent of chemical or physical properties of these systems just by changing the flowrates or channel dimensions. In these
systems, fluid mechanic software such as COMSOL® can be used for prediction of flow rates and channel dimensions. The ability to use a porous membrane to separate two parallel channels has enabled analysis of tissue barrier function and absorption and secretion in these systems. By culturing two cell types on each side of the membrane, interactions between the vascular endothelium and parenchymal cells can be mimicked in a very precise manner unattainable by traditional 3D culture systems. These chips also allow the replication of complex mechanical microenvironment of living tissues in-vitro. For instance, the effect of shear stress on cell migration and proliferation can be analyzed using these systems. Some examples of these systems are illustrated in fig. 2.11.

![Image](image.png)

Fig. 2.11. (a) a microfluidic system for encapsulation hepatocyte along fibroblast, (b) kidney on a chip, (c) gut on a chip.

2.7. Liver-on-chip Systems

Liver on a chip system have three main applications(fig 2.12): 1) Simulating the liver function under an in-vivo-like microenvironment to understand liver physiology in detail and study liver disease in-vitro; 2) Studying the effect of different ECM proteins and parenchymal-non parenchymal cells interactions to find an optimal Bio ink and cell structure to be used for 3D bioprinting of liver tissue; and 3) Use in drug toxicity tests and drug induced liver injuries in the drug development phase. Liver on a chip models are of great significant as they are a tool to mimic the liver in-vivo conditions that can be used for drug toxicity tests to reduce the number of animals used in phase two of drug development. If the proposed design can mimic both the structural and physiological functions of the liver in detail, the cultured tissue inside the liver-on-chip model also has the potential to be used as a building block for the bottom–up assembly of a liver organoid for transplantation, such as (bio-artificial liver) and as an in-vitro liver model.
for drug toxicity tests. To achieve this goal, various techniques including cell aggregation, cell patterning and assembly techniques have been used to build 3D liver tissue inside these devices.

Fig. 2.12. 3 main application of liver-on-chip models which are drug toxicity tests, fabrication of an artificial liver structure and studying liver disease. The current models lack a main characteristic; they do not include the biliary tree structure which is an important part of liver structure.

2.7.1. Fabrication of Liver Microtissue Inside Liver-on-chip Systems

One primary application of a liver on a chip model is to build a tissue model which can generate liver specific functions including albumin, urea, and bile synthesis inside the system. This structure should include liver major parts including liver sinusoids, bile ducts, and vascular structure to provide sufficient nutrients and oxygen to the parenchymal cells while at the same time being able to remove the toxins accumulated inside the system. These two characteristics are vital for long-term survival and function of liver parenchymal cells. These fabricated tissues inside the liver-on-chip models can also be implanted and replace the damaged liver tissue if they possess all the functional and vascular structure of the native liver tissue. Micropatterned liver models are one of the first 3D liver models which were developed to analyze the growth and activity of parenchymal cells on different ECM proteins and culture conditions. Despite the success of these models to mimic the parenchymal-ECM interaction, they lack the vascular structure of
native liver which is crucial for long-term culture of hepatocytes (fig. 2.13)\textsuperscript{82}. Du et al recently built a bile duct-on-chip model which replicates the bile duct physiology in detail but lacks the hepatocytes as the main liver parenchymal cells\textsuperscript{8}. This and other similar researches including the work of Sampaziotis et al and Chen et al on building an \textit{in-vitro} bile duct hollow fibers seeded with cholangiocytes, are great progresses toward building a comprehensive liver-on-chip model but they still lack hepatocytes inside the system\textsuperscript{83,84}. Another approach to build a complex tissue model containing all liver sections is liver decellularization techniques and use the obtained structure as a scaffold to build liver tissue and vascular network\textsuperscript{85}. Although these methods were successful in many aspects including the improvement of vascularization compared to previous models, they still possess drawbacks including lack of a proper cell source for recellularization and xenogeneic immune problems has hampered their broader application. Hence, there is a great demand to fabricate a liver-on-chip model which can mimic the native structure of the liver as much accurate as possible regarding the hepatocyte function and metabolic activity.

![Liver models fabricated using micropattern technology](image)

**Fig. 2.13.** Liver models fabricated using micropattern technology; (A) A microarray with different ECM proteins coated to analyze hepatocytes function on each surface \textsuperscript{86}. (B) A microarray for investigated the effect of different GFs on hepatocyte function \textsuperscript{87}. (C) a microarray pattern mimicking the liver lobule physiology using hepatocytes and endothelial cells \textsuperscript{88}. (D) A piezo electric printing technique for fabrication of collagen and poly-L-lysine micropatterns for cell based assays \textsuperscript{82}. 

2.7.2. Liver-on-Chip for Drug Development

Currently, the cost for bringing a single drug into market from the initial phase is around $400 million. This cost can be greatly reduced by performing pre-clinical drug testing using *in-vitro* models to remove the false lead candidates. Liver *in-vitro* models which are used for drug development tests are based on high throughput well plates which do not mimic the exact liver physiology. This results into false predictions and results from these systems which in turn results to more cost for the whole process of drug development. To overcome these challenges, microfluidic liver-on-chip models were developed using an array of parenchymal and non-parenchymal cells in an ECM like microstructure. Moreover, as the ADME/tox reactions also occurs in other organs, models have been proposed to mimic this whole process by designing a human-on-chip model (fig. 2.14).

![Diagram of human-on-a-chip model](image)

*Fig. 2.14.* The human-on-a-chip model for modeling a complex, dynamic process of ADME/Tox.
CHAPTER 3

CENTRAL HYPOTHESIS and SPECIFIC AIMS

In this research, we investigated the application of microcapsules and hollow fibers in tissue regeneration and assembly in addition to building organ-on-chip devices. Microcapsules provide an enclosed microenvironment which can control cell aggregation and due to their hydrogel nature, they facilitate nutrient and growth factor diffusion. Hollow fibers as another part of our modular approach, can mimic the vein, artery, or bile duct function, in collection and transporting the fluids, inside the organ-on-chip system.

The central hypothesis to test the feasibility of using microcapsules and hollow fibers for cell encapsulation, differentiation and building organ-on-chip models. We will show that by using ECM proteins inside the microcapsule structure, providing the required growth factors and specific culture conditions such as perfusion and hypoxic, one can accelerate cell growth and facilitate the differentiation of MSCs inside microcapsules to multiple lineages of adipocytes, osteocytes and chondrocytes. Differentiation of MSCs to chondrocytes was a primary focus in this project compared to the other lineages as differentiated MSCs have great potential in treatment of osteoarthritis. Later, a comprehensive method will be proposed for fabrication of mechanically stable and elastic hollow fibers. These hollow fibers can be use internally or externally for cell seeding in multiple TE applications. The specific usage of the fabricated hollow fibers in this project is to build an in-vitro bile duct. This bile duct would be optimized prior to use in the liver-on-chip model. Eventually, microcapsules will be used for co-encapsulation of three different cell types, hepatocytes, MSCs and vascular endothelial cells. These microcapsules will be later combined with the hollow fibers seeded with cholangiocytes to test the feasibility of building a liver-on-chip model capable of generation and collection of bile.
**Fig. 3.1.** Central hypothesis. Fabrication of uniform microcapsules and hollow fibers with a specific method and using them for multiple application including cell and tissue regeneration, stem cell differentiation and use in organ-on-chip models which in this project the focus of the study has been on a liver-on-chip model. It should be noted that transfer of the microcapsules alongside the hollow fiber to be tested inside the chip is the very last step of the project and prior to that these two tissue structures have to be optimized both qualitatively and quantitatively.

**Fig. 3.2.** Another hypothesis tested in this research was to test the differentiation capability of MSCs to chondrocytes inside the microcapsules. These microcapsules can be assembled and implanted into the affected area of the cartilage. The current project has been focused just on the first part.

The *specific aims* of this project are to:
1. Investigating the feasibility of ES in formation of uniform, small microcapsules alongside optimization and characterization of this technique.

2. Studying the differentiation capability of MSCs inside microcapsules to three lineages of adipocytes, osteocytes, and chondrocytes.

3. Generation of co-encapsulated hepatocytes, MSCs and VECs microcapsules using the ES technique and assessing the hepatocytes function under perfusion culture.

4. Fabrication of hollow fibers seeded internally with cholangiocytes and their functional optimization.

5. Assembly of hollow fiber and hepatocytes microcapsules inside the liver on chip model to investigate the feasibility of bile synthesis inside the system.

3.1. Overall Research Design

In first part a comprehensive study on stem cell microencapsulation using the ES techniques was done. MSCs were used in this section and their viability and differentiation to the three lineages was assessed. A detailed study was accomplished on chondrogenic differentiation due to the great research focus on this area in the past few years. In second part, hepatocyte, MSCs and VECs were co-encapsulated using the ES and the metabolic function of hepatocytes in these microcapsules were studied in detail. Additionally, hollow fibers seeded internally with cholangiocytes were fabricated in the next section mimicking the hepatic bile duct. These hollow fibers were also characterized both regarding their cellular and material aspects. Ultimately, hepatocytes microcapsules and hollow fiber seeded with cholangiocytes was assembled in the liver bioreactor to assess the feasibility of bile collection using this system.

3.2. Significance and Rationale

Generation of dense, multicellular, and organ specific tissue is the goal of tissue engineering field. Microcapsules alongside with hollow fibers are two great shapes which can provide a desirable microenvironment for cell growth and reorganization. These two structures are the building blocks of the
modular tissue engineering in which by their 3D assembly using techniques such as 3D bioprinting, one can obtain larger tissue structures in short period of time with a high resolution and dimensions. Speed, convenience, and high throughput capability are three main factors in generation of microcapsules. This study is significant as we introduced a novel, rapid and convenient technique for the cell encapsulation. Initially, MSCs were used in the electrospraying technique for cell encapsulation and its cell viability and growth was analyzed. One great capability of this method is to generate large number of capsules in short period which is crucial for preserving cell function and viability during the encapsulation process. We further demonstrated that the high voltage does not affect the cell viability and function. Another significance of this study is the demonstration of the potency of the encapsulated MSCs differentiation to chondrogenic lineage. Most of the studies done on the cartilage tissue engineering has been focused on application of scaffolds but this research for the first time has investigated the employment of microcapsules to generate the articular cartilage tissue. Another great potency of this method is the ease of assembling the microcapsules to larger 3D structures which is currently under investigation by this group. The electrospraying was later used to encapsulate three cell type together at the same time including hepatocytes, MSCs, and VECs. This represents another power of this modular structure in forming multicellular tissue structure.

Hollow fibers are another 3D structure which can simulate tissue structures such as veins, arteries, and bile ducts. Another significance of this project is the design and fabrication of bioengineered hollow fibers using a variety of biomaterials with high mechanical properties and cell attachment properties. For this first time this study has proposed a GAG based hollow fiber seeded with cholangiocytes capable of bile modification through secretory and adsorption process. Research done by others in the field of bile duct tissue engineering has been focused on hollow fiber with low mechanical properties which are not applicable for implantation surgeries. Eventually, the microcapsules and hollow fiber designed were transferred into the liver bioreactor to test the bile formation in this system. This study is also of high significance as it is the first research done using both hepatocytes and cholangiocytes to mimic the biliary
structure of liver. We believe the methods and techniques proposed in this study will provide researchers with an efficient, high-throughput assay for generation of dense, highly vascular and functional 3D tissue structures in-vitro.
CHAPTER 4
CHARACTERIZATION OF MICROCAPSULES

4.1. Introduction

Cell encapsulation in a proper microenvironment using natural polymers and hydrogels is important, particularly, for the rehabilitation of functional tissues capable of repairing or replacing the damaged organs. Non-encapsulated cells are prone to damage and attack by host immune system after implantation. This will diminish the cell viability and metabolic activity and shortens the efficiency of the implanted scaffold. One great way to overcome this obstacle is to encapsulate cells inside biopolymers which degrade inside the body after implantation. Consequently, the most important objective of encapsulation is for the cells to retain their function and secrete hormones and cytokines in the capsule while at the same time be protected from the host immune system. Additionally, stem cells microencapsulation regulates stem cell phenotype by providing the appropriate microenvironment for them to grow. For this mean, there are different encapsulation techniques available, the most common ones including microfluidics, coaxial compressed air flow, electrospraying (ES) and micro molding methods. Microencapsulation generally refers to fabrication of spherical capsules in the range of 100-1500µm in diameters. In this chapter, the feasibility of two methods including microfluidics and electrospraying to generate uniform, small (100-500µm) microcapsules was investigated. Furthermore, cell function including viability and growth inside the generated microcapsules was also analyzed.

4.2. Aim and Rationale

The main specific aim of this chapter is to yest the feasibility of the microfluidic technique and electrospraying method in making uniform and small (<500µm) microcapsules. These microcapsules should be mechanically stable and keep their structure throughout the culture period. The rationale behind the aim is that the current encapsulation methods including compressed air flow method have low throughput efficiency which can be improved. Furthermore, the high throughput characteristic of these
methods is vital for regenerative applications. Also, we will investigate how changing the encapsulation material will affect the cell viability. Next, among these two methods the most feasible and efficient method for MSCs encapsulation will be chosen for the next set of experiments.

4.3. Experimental Approach

**Study 1:** Analyzing the Feasibility of the microfluidics encapsulation method to generate small (100-500µm) and uniform microcapsules.

The effect of the operating parameters in the microfluidic method including the flow regime, size of the channels and the design of the microfluidic on the size and uniformity of the microcapsules were investigated.

**Study 2:** Analyzing the feasibility of the ES method on generating uniform and small (100-500µm) microcapsules.

The effect of operating parameters in the ES method including voltage and needle size on microcapsule size and uniformity was investigated. Additionally, the effect of microcapsule formulation on size and uniformity of the microcapsules was studied. This study allowed us to approve ES method as a successful encapsulation technique.

**Study 3:** Characterization of the microcapsules generated using the microfluidics and ES method.

Cell viability, growth and organization inside the microcapsules was analyzed to see the effect of encapsulation method and microcapsule formula on these parameters. This study allowed us to acquire a comprehensive knowledge to compare the two encapsulation methods already tested and choose the best one for the rest of the project.

4.4. Materials and Methods

Concisely, for each the two encapsulation methods, the efficiency and yield of the method was compared based on the capsule size, uniformity, and encapsulation speed. Encapsulation speed is of crucial
importance as for fragile cell lines such as hepatocyte or lung epithelial cells the more time the cells our out
of their culture environment, the more loss of their viability and their functionality. After choosing the
best encapsulation method, the microcapsules were cultured for 30 days and the cell function including
viability, growth and organization in microcapsules was analyzed using both quantitative and qualitative
assays.

4.4.1. Cell Culture Conditions

All chemical and reagents were purchased from Sigma-Aldrich unless otherwise noted. MSCs were
isolated from the femurs and tibiae of a 6 month old Sprague-Dawley (SD) weighting between 200-250g
according to the established protocols. Concisely, after the rat has been euthanized with CO₂, femur and
tibiae were removed and were cleaned thoroughly using sterile gauze and PBS. Afterward, the epiphysis of
the bones were removed and the contents of the bone marrow were removed by flushing with an 18G needle
containing 20ml of LDMEM+10%FBS+gentamicyn at 37°C and the contents were passed through a 70µm
cell strainer to remove any fats and any other contaminating cells, followed by centrifugation at 300g for
5min. MSCs were then seeded onto 100mm culture plates at a density of 10,000cell/cm², cultured up to
80% confluency and subcultured afterwards. MSCs at passage 4 were used in all experiments.

4.4.2. Fabrication of Microfluidic Chips

To investigate the effect of microfluidic design on formation of microcapsules, multiples designs
were made using PDMS as the base material. Microfluidic chips were fabricated using an established
protocol. The whole illustration of the fabrication process is depicted in Figure 4.1. Briefly, the template
was first designed using the CAD software which was then 3D printed using 3D printer (Ultimaker3,
Netherland) with ABS polymer as the material. Meanwhile, Sylgard silicone elastomer (184) kit (Dow
Corning, Midland, MI) was made by mixing the polymer solution and the curing reagent in a 10:1 ratio
according to manufacturer protocol. The polymer solution was then degassed to remove all the air bubble
formed during the mixing process and the ABS template was then submerged carefully inside the uncured
polymer. The curing and hardening of the PDMS was done in oven at 60°C for 1hr. The whole mold was
then suspended inside acetone solution to dissolve the ABS template. This will leave us with a microfluidic chip with hollow channels inside the PDMS as the acetone will only dissolve the ABS and not the PDMS. This method is convenient, fast, and versatile which does not require the sophisticated instruments used in soft-lithography methods which makes it applicable in any lab. In this study, multiple designs including flow-focusing and T-junction microfluidics were tested and their efficiency in generating microcapsule was investigated.

**Fig. 4.1.** Multistep fabrication of the microfluidic chip; (A) Making the ABS template using a 3D printer. (B) Addition of PDMS solution to the 35 mm plate. (C) Submerging the ABS template inside the uncured PDMS solution followed by curing at 60°C for 1hr. (D) Submerging the whole structure inside acetone to remove the ABS and leaving the PDMS with empty channels. Same procedure was used for making the T-junction microfluidic i.e. by changing the design of the ABS template. The angle of the flow-focusing can be changed based on the design from 90° to 45° depending on the application.
4.4.3. Fabrication of Microcapsules Using Microfluidics

Fabrication of droplets using aqueous two-phase system (ATPS) in microfluidics requires the separation of two phases (continuous and the dispersed) at the junction point 97. In this study, the disperse solution contains dextran (MW=229,000) +CMC+CSA and the continuous phase contains PEG (MW=8000) at different concentration. CMC was added to the dispersed phase to increase stability of the generated droplets and CSA was added as a highly biocompatible material. Table 4.1 illustrates a detailed concentration of each component used for generation of microcapsules. The concentration range of DEX and PEG were chosen based on the previous studies done by other researchers98,99. After preparation of the continuous and dispersed phases, the two phases were delivered into the microfluidic channels at desired flowrates using a syringe pump. Following the formation of the droplets at the end of the microfluidic device, they were added to a stirring chitosan solution and due to polyelectrolyte complexation between CSA and chitosan, a higher mechanically stable microcapsules were formed. The capsule formation under the four conditions of table. 1 were then analyzed. The two microfluidic devices used in the experiments are depicted in figure 4.2.

Table 4.1. Concentrations of the continuous and disperse phase used in the study

<table>
<thead>
<tr>
<th>Run #</th>
<th>DEX (%w/v)</th>
<th>CMC (wt%)</th>
<th>CSA conc (wt%)</th>
<th>PEG 8000 (wt%)</th>
<th>Interfacial tension (mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (control)</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>2 (control)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>1.5</td>
<td>4</td>
<td>10</td>
<td>0.014</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>1.5</td>
<td>4</td>
<td>20</td>
<td>0.042</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>1.5</td>
<td>4</td>
<td>10</td>
<td>0.051</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>1.5</td>
<td>4</td>
<td>20</td>
<td>0.158</td>
</tr>
</tbody>
</table>
Fig. 4.2. Microfluidic devises used for encapsulation. ABS template is still not removed in these pictures for better visualization of the channels; (A): flow-focusing device. (B): T-junction device. (C) Illustration of the microcapsule generation using flow-focusing microfluid. First, the droplets will be formed due to phase separation of PEG-DEX, the formed droplets will then be directed to stirring chitosan solution to form more mechanically stable and biocompatible microcapsules. (D) Illustration of the microcapsule generation using T-junction microfluid. It should be noted that the DEX solution is the continuous phase which also contains GAG solution, and the PEG solution is the dispersed phase. D1=200μm and D2=100μm.

4.4.4. Fabrication of Microcapsules Using ES Method

The ES system consisted of a high voltage (HV) power supply (Gamma High Voltage research, Ormond Beach, FL), magnetic stirrer and a syringe pump for precise control of the solution flow (Braintree Scientific, MA) which is illustrated schematically in Fig.4.3. To generate microcapsules using the ES method, MSCs were first trypsinized, resuspended in the GAG (HA+CSA at different concentrations) as the polyanion solution (10×10^6 MSC/ml GAG), and transferred to a 1 ml syringe. The suspension was then delivered by the syringe pump to the tip of an 18 or 22 G (OD) stainless steel needle (Fig. 4.3). The needle was connected to the negative electrode of the HV system with the polycation chitosan solution (50 ml)
serving as the positive electrode. The potential difference was adjusted to the desired value, and droplets generated at the tip of the needle were collected into 50 ml of stirred chitosan solution. In all experiment the distance from the tip of the needle to the chitosan surface was kept at 12mm. It was observed that for distances over 12 mm the droplets would go to the sides of the chitosan container rather than inside the solution and for lower distances the spark was formed between the tip of the needle and chitosan solution. Polyelectrolyte membrane formation around each droplet was instantaneous and the cells were encapsulated completely. The microcapsules formed were washed twice with normal saline to remove excess chitosan solution, followed by a brief wash with 0.1 wt.% polygalacturonic acid (PGA) solution to achieve surface stabilization and to prevent inter-capsule adhesion. The microcapsules were then equilibrated with culture medium for 60 min at 4°C and were then transferred to the incubator for culture or used for other analysis.

**Fig. 4.3.** Electrospraying (ES) setup used for generation of microcapsules. The GAG+Cells are first transferred into a syringe pump and the tip of the needle is then attached to the high voltage system.

### 4.4.5. Microcapsule Size and Uniformity Analysis

In the first set of experiments, the microcapsules without cells were generated in the microfluidic and ES method and were then analyzed based on their size and uniformity. Afterward, the best fabrication method and formula based on the two criteria of microcapsule uniformity and smaller size were chosen.
Initially microcapsules made using the microfluidics were analyzed. In all experiments a sample size of 100 microcapsules were used to analyze the data and the size distribution. As the efficiency of the microfluidic method was low and the resulted microcapsules were not in the same size range (discussed in the results section) the cells were not used in this method. In addition to size comparisons, the shape of the microcapsules was analyzed in the ES method as an indicator of the capsule uniformity. Here, the circularity (Eq. 1) was used as a tool to measure the uniformity of microcapsules formed in each voltage with the corresponding needle size and GAG formulation. “A” and “P” represent area and perimeter of the microcapsules, respectively. Values equal to 1 represent perfect circle while smaller values suggest deviation from circularity meaning higher non-uniformity of the microcapsules. All results were calculated for 100 capsules in each experiment⁴⁰⁰.

\[ f = \frac{4\pi A}{P^2} \] (1)

4.4.6. MSCs Viability and Growth Inside Microcapsules

After finding the optimized conditions of the ES method, MSCs were encapsulated in three different GAG formulations under the optimized conditions and the cell viability and cell growth over 30 days was analyzed. For this mean, different quantitative and qualitative assays were used including Calcein AM-EthD-1, Hoechst 33258 and H &E staining for cell viability, growth, and cell organization, respectively. Additionally, SEM images were captured to analyze the membrane morphology and location of cells inside the microcapsules. Cell viability was investigated using Calcein-AM/ethidium homodimer (Cytotoxicity kit L3224, Invitrogen) according to the manufacturer recommendations. Microcapsules were then imaged under a fluorescence microscope (Nikon Diaphot 300) and the number of dead and live cells in a sample population of ~100 microcapsules were counted. Cell proliferation inside microcapsules was quantified using the Hoechst 33258 fluorometric method which binds to the double-stranded DNA according to manufacture instructions. Concisely, 30 microcapsules were crushed, cell lysis buffer (0.1% SDS+10 mM Tris-HCL +1mM EDTA) was added to the solution and then cells were lysed for 2hr at room
temperature. Afterward, 200µl of 2µg/ml Hoechst solution was added to the lysed cells solution and fluorescence was read at Ex/Em of 360nm/460 nm. To generate the standard curve, calf thymus DNA at concentration between 0-10µg/ml was used. For H&E staining, briefly, capsules were fixed in 10% neutral buffered formalin overnight at 4°C. Dehydrated in ethanol series, cleared in xylene, embedded in paraffin, and subsequently sectioned to 7µm sizes. Sliced sections were mounted on charged glass slides (MTC bio, NJ, USA) for better adhesion and used for subsequent staining. Routine H&E staining (Leica Biosystems) was used to detect the cell proliferation and morphology inside the microcapsules. Cell organization inside and the microcapsules, membrane structure and morphology of microcapsules were analyzed using scanning electron microscopy (JEOL JSM-7600). To prepare the samples for SEM imaging, microcapsules were first fixed in 2.5% (v/v) glutaraldehyde solution for 24hr at 4°C followed by three washes with PBS, 48hr of freeze drying and sputter coating with gold prior to imaging.

4.5. Results

4.5.1. Characterization of Microcapsules Generated Using Microfluidics

The main challenge in the formation of droplets using microfluidics is that the continuous and disperse phases should have a high interfacial tension\(^{101}\). By use of water in oil (w/o) systems this objective is achievable as there is a high interfacial tension in water/oil systems. In the PEG/DEX aqueous systems studied here, the low interfacial tension of the system (10 1mN/m) makes it a challenge to fabricate uniform droplets compared to w/o systems which is around 30mN/m\(^99\). ATPS systems are different than w/o systems in that both phases are water based. The mixture of PEG and DEX above a certain concentration causes the mixture to phase separate. Additionally, the biocompatibility of these two polymers has broadened their use in biomedical applications compared to w/o systems. The dimensionless capillary number, \(Ca\), is a critical factor in determining the formation of droplets in microfluidics which is defined as \(Ca = \frac{\eta U}{\gamma}\) where \(\eta\) and \(v\) are the viscosity and velocity of the continuous phase and \(\gamma\) is the interfacial tension between the continuous and disperse phase. Another dimensionless number which pinpoints the
formation of droplets is the Weber number (W) defined as \( We = \frac{\rho U^2 d}{\gamma} \) where \( \rho, U, d \) are the density, velocity and flow diameter of the disperse phase respectively and \( \gamma \) is the interfacial tension between the two phases. When \( We < 1 \) and \( 0.01 < Ca < 1 \), surface tension dominates over the drag forces leading to the formation of the droplets in these system\(^{102} \). As can be seen, droplet formation depends on multiple intrinsic characteristics of the two fluids in addition to the geometry and design of the microfluidic device. It should be noted that in these systems the dripping regime is desired, and the jetting regime is undesired. By analyzing variables in the \( We \) and \( Ca \) numbers, it can be observed that increasing the interfacial tension between the continuous and dispersed phase is the only way to shift into the dripping regime. This can be done by using higher MW of PEG or increasing the PEG concentration. This phenomenon can be observed in fig4.4. as in the low PEG concentration (10wt\%) no droplet formation was observed while at higher PEG concentrations (20wt\%) droplet formation could be clearly observed. It should be noted that the interfacial tension also depends on the DEX concentration as it can be seen from table.1.

![Image](image.png)

**Fig. 4.4.** Formation of the droplets in the flow-focusing microfluidic device. (A) Jetting regime formation when using the low concentration of PEG as the continuous phase (10wt\%) and DEX(5wt\%). (B) Formation of droplets when using highest concentration of PEG (20\%) and DEX (10wt\%). Higher concentration results in higher interfacial tension between the two phases and results in lower Capillary number which leads to droplet formation.

Coalescence and merging of the droplets together are another issue in generation of droplets in the microfluidic system as it can be seen from fig. 4.5. Small droplets formed in these microfluidic systems will eventually coalesce due to their unstable nature (low interfacial tension). One way to prevent the droplet coalesce is to add a surfactant to the disperse solution which acts as a stabilizer. In general, there are two
methods to prevent droplet coalesce in microfluidics. The first is the passive merging which is can be done appropriate design of the microfluidic channel and the second is the active merging which can be done by the of using electric-controlled methods such as to inputting high voltage into the system\textsuperscript{103,104}. Utilizing these two methods have shown to prevent the droplet coalesce in these systems. As can be seen in from fig. 4.5, in all conditions of the study the droplets tend to merge and the size of the formed droplets increase by increasing the interfacial tension (fig4.6) which we believe is due to formation of more droplet in the high concentration system.

![Fig. 4.5. Droplets formed in flow-focusing microfluidic device with a different formulation.](image)

![Fig. 4.6. Mean diameter of the droplets formed using the flow-focusing device at different continuous and disperse formulations. It should be noted that higher interfacial tension between the two phases results in larger droplets.](image)

At last, by adding these droplets into the stirring chitosan solution, it was observed that microcapsules were formed due to ionic complexation between chitosan and CSA (fig. 4.7). These microcapsules were non-uniform, large (>500µm) and completely unstable which make them unusable for
regenerative applications. The limitations and pitfalls of this system directed our attention to focus on the ES as an alternative method for cell encapsulation.

Figure 4.7. Formation of microcapsule hydrogels after addition of droplets to the chitosan solution. Increase in microcapsule size is also clearly seen here by changing the GAG formulation uses in the disperse phase.

4.5.2. Characterization of Microcapsules Generated Using ES Method.

In the first set of experiments the microcapsule size in each test was measured and the optimized conditions were determined (fig 4.8. & fig. 4.9). It was observed that the capsule diameter was decreased by increasing the voltage from 10 to 14 kV in all three microcapsules formulation with both needle sizes. Although, the reduction in capsule diameter was more obvious with the (0.5% HA + 4% CSA) compared to the two others. This was mostly due to the lower viscosity of this solution when using 0.5% HA concentration. Solutions with higher viscosity require higher voltages for the electrostatic forces to overcome the surface tension of the liquid. Hence, it would be harder for them to detach from the tip of the needle compared to lower viscosity solution in which in yields to larger microcapsules at the same voltage used.
Fig. 4.8. Effect of voltage and formulation on size of the microcapsules made with and 18G blunt needle. (A1-A3): microcapsules made with 0.5%HA+4%CSA at 10kV, 12kV and 14kV. (B1-B3): microcapsules made with 1%HA+4%CSA at 10kV, 12kV and 14kV. (C1-C3): microcapsules made with 2%HA+4%CSA at 10kV, 12kV and 14kV. Scale bar everywhere 200µm.

Fig. 4.9. Effect of voltage and formulation on size of the microcapsules made with 22G blunt needle. (A1-A3): microcapsules made with 0.5%HA+4%CSA at 10kV, 12kV and 14kV. (B1-B3): microcapsules made with 1%HA+4%CSA at 10kV, 12kV and 14kV. (C1-C3): microcapsules made with 2%HA+4%CSA at 10kV, 12kV and 14kV. Scale bar everywhere 200µm.
As can be seen from Fig. 4.8 and 4.9, by decreasing the needle size, the average capsule size would also decrease. This trend was seen in all three GAG formulations. Among the three GAG formulations, the 2% HA+4%CSA formulation had the highest viscosity compared to the other two. The effect of viscosity can be also seen in the lower uniformity of the microcapsules formed compared to the other formulations as these capsules had tails due to higher viscosity and higher surface tension. The higher surface tension results in more difficult detachment of the droplets from the needle tip and results in a droplet elongation that contributes to tail formation. The higher viscosity retards the tendency of free droplets to restore a spherical shape during free fall, and the tear-drop shape is ultimately immobilized upon formation of the capsule membrane.

Fig. 4.10. Effect of GAG formulation, voltage and needle size on microcapsules mean diameter. (A): microcapsules made with 18G blunt needle. (B): microcapsules made with 22G blunt needle. Values are ± standard deviations from measurement of 100 microcapsules.

Quantitative results for the capsule size assessment are shown in Fig. 4.10. As shown, the decrease in the capsule size in formulation 1 is higher compared to the two others, likely due to the lower viscosity and surface tension of the solution. Beside the capsule size, the uniformity of the microcapsules is another factor that affects diffusive transport in implantation procedure. The results for the microcapsules circularity formed by 18G and 22G needle are shown in Fig. 4.11. These results suggest that increasing the voltage from 12 kV to 14 kV results in a reduction in uniformity across the whole range of microcapsules. These results suggest that using solutions with higher viscosities in addition to yielding larger capsules,
also generates fewer uniform microcapsules across the whole range of voltages and needle sizes. Moreover, by comparing the results for two needle sizes, it can be clearly seen that the effect of needle size on circularity of microcapsules is negligible compared to effect of voltage and GAG formulation. Similar results have been reported in other ES studies where deviations from circularity have been reported as being related to higher voltages and higher viscosities of the electrosprayed solution.\textsuperscript{105} On the contrary, use of higher voltages in the ES method results in smaller microcapsules but on the other hand it has been reported to affect the cell viability and metabolic activity to a significant degree, as higher voltages can potentially cause cell membrane damage leading to cell death.\textsuperscript{106–108} Hence, voltage of 12 kV and 22G needle size were determined as the optimized conditions to achieve both smaller and more uniform microcapsules. The capsules formed under these optimized conditions were later used for encapsulation of MSCs and analyzing the cell function.

**Fig. 4.11.** Effects of GAG formulation and voltage on microcapsule circularity. (A) Capsules formed with an 18G needle. (B) Capsules formed with a 22G needle. Values close to 1 represent perfect circles, any deviation from this value represents noncircular capsule. Values are means standard deviations from measurements on >100 capsules.
4.5.3. Effect of GAG Composition on Cell Viability and Growth in the ES Method.

Effect of GAG formulation on cell viability was investigated in detail. Quantitative results were obtained by counting the number of live and dead cells in a sample population of ~100 microcapsules. Cell viability was done at two time points: one day after encapsulation and 30 days after culture inside the medium. All cell viability analysis was conducted on microcapsules made at 12 kV using the 22G needle. Fig. 4.12 illustrates the cell viability analysis using Calcein-AM and EthD-1 staining at the two time points. Fig 4.13 shows the corresponding quantitative cell viability results. As can be seen, the cell viability decreases in all three formulations from day 1 to day 30. It was observed that MSCs preserved their viability after one day of culture (>90% in all conditions) which represents the safety of the ES. After 30 days of culture, the low HA concentration presented the lowest cell viability which can be due to lower presence of HA inside the microcapsules. As in the higher HA concentrations there is a chance of higher HA presence inside the capsule which provides an ECM microenvironment for cells. Although, the high HA concentration might also lead to thicker membranes which results to lower nutrient diffusion. More histological experiments are required to verify how HA concentration would affect cell viability. DNA quantification results also presented a same trend as higher cell growth was observed in 1%HA+4%CSA formulation. So far, these results suggested that the 1%HA+4%CSA was the best GAG formulation as was seen from both cell viability (fig. 4.13) and DNA quantification results (fig. 4.14).
**Fig. 4.12.** Cell viability analysis for different microcapsule formulation using Calcein-AM EthD-1 dye 1- and 30-days post encapsulation. Top row shows capsules made using 0.5% HA+4% CSA. Middle row shows capsules made with 1% HA+45% CSA and bottom row shows capsules made with 2% HA+45% CSA. Scale bar everywhere 200µm.

![Viability Graph](image1)

**Fig. 4.13.** MSCs viability one day and 30 days after encapsulation. Data are ±SD from three independent culture runs. (n=3)

![MSC Viability Graph](image2)

**Fig. 4.14.** DNA quantification results using Hoechst 33258 fluorometric method. Cell growth in all three types of microcapsules can be observed representing the advantageous effect of microencapsulation on cell growth. Microcapsules made using 1% HA+4% CSA revealed highest cell growth after 30 days of culture which is mainly due to appropriate membrane thickness compared to other two conditions. Data are shown as ±SD. (n=3)
4.5.4. Cell Organization Inside Microcapsules

H&E staining of the microcapsules was done to investigate the cell morphology inside the microcapsules with different GAG formulations (Fig. 4.15). A can be seen, cells tend to attach to the capsule membrane due to presence of GAGs inside membrane. Moreover, higher cell density can also be seen here in the 1%HA+4%CSA formulation which verifies our previous observations. To further investigate the capsule and membrane morphology, SEM images were taken (fig. 4.16). As can be seen, the capsules have a hollow structure with cells mostly attached to the membrane. Moreover, the porous structure of the membrane can also be seen which facilitates diffusion of nutrients into the microcapsule.

Fig. 4.15. Cell organization and growth analysis inside microcapsules using H&E staining after 30 days of culture. (A) Cells mostly attached to membrane of capsules made with 0.5%HA+4%CSA. (B) Highest cell growth was observed in 1%HA+4%CSA formulation. (C) Cells also show average growth in 2%HA+4%CSA but due to higher membrane thickness, they will be lower diffusion of nutrients into the microcapsule. Scale bar everywhere is 100µm.

Fig. 4.16. SEM images of the microcapsules. (A): whole structure. (B): microcapsule membrane showing the high porosity of the membrane. (C): cells encapsulated inside the capsules which are attached to capsule wall.
4.5. Summary and Discussion

Cell microencapsulation has been used in regenerative medicine field for decades to protect cells from external environment and providing a 3D structure for cell growth. There are multiple cell encapsulation techniques including microfluidics, micro molding, electrospraying and air-nozzle methods. Primary parameters affecting the type of encapsulation method to be used include; type of biomaterial used for encapsulation, encapsulation efficiency with regard to cell viability and functionality, encapsulation speed and convenience. Cell encapsulation using microfluidics is a versatile and cost-effective method which can generate large number of capsules in short period of time. In this chapter we first analyzed the feasibility of microfluidic methods to generate microcapsules using GAG-Chitosan as our encapsulation biomaterial. Multiple microfluidic devices were generated using 3D printing technology which were able to generate microcapsules with diameter ranging from 120-300µm. It was observed that the microfluidic technique could generate small, uniform microcapsules using different concentrations of dextran and PEG, yet, after generation, most of the microcapsules were attached to each other making large, non-uniform microcapsules which is undesirable. As the cell encapsulation using microfluidic technique is highly dependent on interfacial tension of the two phases in contact with each other, we believe testing higher MW of PEG and dextran might result in more stable microcapsules which are less likely to stick to each other. In general, we found that microfluidic method is not a suitable technique for cell encapsulation when using GAG-chitosan as the encapsulation materials since the interfacial tension of GAG-Chitosan is 300 times lower than the conventional water/oil capsule generation using microfluidics.

In the next part, we investigated the feasibility of ES method for MSCs encapsulation. Other groups have shown successful result in electrospraying of stem cells without encapsulation. Here, for the first time we have shown that the ES is able to encapsulate MSCs in a GAG-Chitosan microcapsule with high cell viability after encapsulation. In this study we analyzed the effect of voltage, GAG formulation and needle size on microcapsules size generated using the ES technique. We found that by increasing the voltage and decreasing the needle size, the size of the microcapsules would decrease which is desirable as smaller
microcapsules lead to higher nutrition and O\textsubscript{2} transport to the cells which results in higher cell growth and viability in long-term cultures. We also noted that the increase in voltage should be limited up to 12 kV as in higher voltage (14kV) the uniformity of the generated capsules was disrupted substantially in addition to the damage of the cell membrane. We found that using 12kV, 22G size needle and GAG formulation of 1%HA+4%CSA resulted in the most uniform capsules with the highest cell viability after 30 days of culture. DNA quantification using Hoechst also showed that 1%HA+4%CSA resulted in the highest cell growth after 30 days of culture which is mainly due to the proper membrane thickness of the microcapsules. The results obtained in this chapter were significant as they made the foundation of the remaining chapters. The microcapsules generated under these optimal conditions were used for the rest of the experiments.
CHAPTER 5
MULTILINEAGE DIFFERENTIATION OF MSCs INSIDE MICROCAPSULES

5.1. Introduction

Multilineage differentiation capacity of MSCs is one of their primary traits which has been investigated by many groups for regenerative medicine applications\textsuperscript{67,113}. MSCs can differentiate to different lineages including adipocytes, chondrocytes, and osteocyte. MSCs have been isolated originally from bone marrow but they can also be found in other tissue such as lungs, skeletal muscle cells, synovium, cord blood and adipose tissue. Multiple factors affect the efficiency and degree of differentiation of MSCs in \textit{in-vitro} cultures including the presence of ECM proteins, presence of growth factors and mechanical stimulus such as agitation\textsuperscript{114,115}. In this chapter, the differentiation capability of encapsulated MSCs in different type of microcapsules and under different culture conditions was analyzed both quantitatively and qualitatively using multiple assays. Adipose tissue is vital for energy maintenance through storage of lipids which is accomplished by adipocytes. One of the main applications of adipocytes differentiated from MSCs is their use in soft-tissue regeneration and reconstruction including skin and oral mucosa\textsuperscript{116,117}. Moreover, as the cartilage damage repair using MSCs has attracted a great attention in the past years due to its promising results, a specific section is dedicated to detailed analysis of MSCs differentiation to chondrocytes\textsuperscript{118}.

5.2. Aim and Rationale

The primary specific aim of this chapter is to assess the differentiation capability of encapsulated MSCs in microcapsule. MSCs multilineage capacity is their principal characteristic which must be investigated prior to their use in any regenerative medicine application. There are multiple parameters which determines the differentiation direction of MSCs (fig. 5.1). Amongst these factors, cell-ECM interaction and Cell-Cell interaction, both play pivotal role by determining the cell faith\textsuperscript{119}. The ECM composition and structure including presence of collagen and proteoglycans is a key factor in differentiation
Another factor is the oxygen level which has been shown to affect the MSCs differentiation greatly\textsuperscript{121,122}. The next aim of this chapter was to analyze the effect of these parameters namely ECM composition and hypoxic conditions on differentiation of MSCs, especially their differentiation to chondrocytes. Therefore, a thorough investigation was done on chondrogenic differentiation of MSCs inside microcapsules by both quantitative and qualitative assays. These microcapsules can be further used for addressing the articular cartilage damage.

![Diagram](image)

**Fig. 5.1.** MSCs biochemical and physical differentiation stimulants. The most dominant parameters include how the MSC-ECM interact and the MSCs culture conditions.

### 5.3. Experimental Approach

**Study 1:** Evaluation of adipogenic and osteogenic differentiation of MSCs.

Microcapsules containing MSCs were generated using the ES method using the optimized formulation achieved in the previous chapter. These microcapsules were then cultured under adipogenic and osteogenic conditions separately for 30 days and their differentiation was evaluated after one month of culture under these conditions using both qualitative and quantitative assays.

**Study 2:** Effect of ECM composition on chondrogenic differentiation of MSCs.
In this study the effect of presence of collagen inside microcapsule on chondrogenic differentiation of MSCs was investigated. Two type of capsule was made, one with collagen and without collagen which were then cultured for 28 days inside chondrogenic culture. Chondrogenic differentiation of MSCs inside microcapsules was then investigated using both quantitative and qualitative assays.

**Study 3:** Investigating the effect of perfusion and hypoxia on chondrogenic differentiation of MSCs.

Effect of perfusion to accelerate the nutrient diffusion to the cells inside the microcapsule was investigated. Furthermore, as native cartilage resides in a low oxygen (2-5%) microenvironment inside the body, it makes common sense to provide very similar conditions *in-vitro* to further improve the chondrogenic differentiation of MSCs. In this study, encapsulated MSCs were also cultured under low oxygen level (2-5%) and their differentiation to chondrocytes was investigated using both quantitative and qualitative assays.

**5.4. Material and Method**

**5.4.1. Fabrication of the Perfusion Culture Bioreactor**

The bioreactor for the culture of microcapsules was fabricated as explained previously using the 3Dprinting method (Fig. 5.2). The working volume of the bioreactor is 150mm$^3$ and dimensions of the working section are 40mm×5mm×2mm.
Fig. 5.2. Step by step procedure of making the perfusion chip. (a) 3D printed template was made using 3D printer (Ultimaker 3, Utrecht, Netherland). (b) template was attached to glass slide (25mm×75mm). (c) glass slide containing the template was transferred to the box and the SYLGARD 184 Silicone Elastomer Kit (DOW Chemical, MI, USA) was added followed be degassing and curing in the oven. (d) cured PDMS template was removed from the box and after plasma treatment, glass slides were attached to the top and bottom part of the PDMS template made. The size of the working section of the chip is 40mm×5mm×2mm with a working volume of 150mm$^3$.

5.4.2 Adipogenic Differentiation of MSCs Inside Microcapsules and Analysis

All chemicals used were purchased from Sigma-Aldrich unless otherwise noted. MSCs were isolated as explained before and were encapsulated at concentration of (10×10$^6$ cells/ml GAG) using the ES method as describes before under the optimized GAG formulation of 1%HA+4%CSA. Microcapsules were then cultured for four weeks inside adipogenic culture medium containing LDMEM+ 15%FBS+ 0.5mM 1-methyl-3-isobutylxanthine+1µM dexamethasone+10µg/insulin+0.2mM indomethacine+1%gentamycin, according to the established protocols$^{112,115}$. The cultured capsules were then analyzed with different assays to confirm the differentiation to adipocytes.
To confirm the adipogenic differentiation of MSCs, they were stained with BODIPY 493/503 (Molecular Probes, OR) which is a tracer of lipid trafficking. Additionally, this stain can be used for neutral lipids and as a tracer for oil and other nonpolar lipids. In addition to fluorescence staining of the lipids, expression of adipogenic markers including FABP4 (fatty acid binding protein 4) and PPARG (peroxisome proliferator activated receptor gamma) which is a regulator of adipocyte differentiation was analyzed using the qRT-PCR machine. Briefly, around 50 microcapsules were taken at the end of the culture, crushed and the total RNA was isolated using the TRIsure RNA isolation reagent (Bioline, UK) using isopropyl alcohol as precipitant according to the manufacturer protocols. To analyze the purity of the isolated RNA the absorbance ratio A260/A280 equal to 2 was considered as pure RNA sample without any contaminating DNA. Two step cDNA synthesis method was used for running the qRT-PCR experiments. RNA was first reverse transcribed to cDNA using the SensiFAST cDNA synthesis kit (Bioline, UK). The acquired cDNA, they were then mixed with forward and reverse primers (Table 5.1) and the SensiFast SYBR Lo-ROX kit (Bioline, UK). This mixture was then put into the QuantStudio RT-PCR machine (Applied Biosystems, MA, USA) to run then qRT-PCR. GAPDH was used as the house keeping gene and the expression levels of each marker was quantified using the $2^{-\Delta\Delta CT}$ method.

**Table 5.1.** Primer sequence used for qRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
</tr>
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| FABP4 | F: AATTGCTTGCTTATTAGTGAA  
R: TGGATGTTATGAAAGGC |
| PPARG | F: AATATAGCCAAGTCACTGTCA  
R: CCTGATGAATAAGATGGAGTC |
| OC | F: GGTCTGATAGTCTGTCACAA  
R: CTGACCTCACAGATGCCA |
| ALP | F: GCCCTTGAGGTTTGGTC  
R: AACCCAGACAAAGCATC |
| SOX9 | F: CGAAGAGCCACCGAACAG  
R: CTGAGATTGCCCAGTG |
| COL2A1 | F: GGCCAGGATGCCAATTAG  
R: CCTGTACCAGGTACCT |
| ACAN | F: CCCCAAGGAATCCCTAGCTGC  
R: TGAGGGATGCTACGTC |
| GAPDH | F: GGGGAAGGTGAGGTCGGAG  
R: ACGGTGCATGGAATTTGC |
5.4.3. Osteogenic Differentiation of MSCs Inside Microcapsules and Analysis

MSCs were encapsulated using the ES as described before at a concentration of \((1 \times 10^6\text{cells/ml GAG})\) using the optimized GAG formulation of 1%HA+4%CSA. Microcapsules were then cultured for four weeks inside the osteogenic medium containing LDMEM, 10mM \(\beta\)-glycerophosphate, 0.1 \(\mu\)M dexamethasone, 50\(\mu\)g/ml L-ascorbic acid 2 phosphate and 10\(\mu\)g/ml insulin with a medium change of every 3 days. Expression of osteogenic markers including osteocalcin (OC) and alkaline phosphate (ALP) was used to further confirm the differentiation (primer sequence used in from table 5.1). GAPDH was used as the house keeping gene and the procedure was done as explained previously.

5.4.4. Chondrogenic Differentiation of MSCs Inside Microcapsules Under Static and Normoxia

As described earlier, chondrogenic differentiation of MSCs was investigated more in detail compared to the two other lineages. Since the cartilage cannot heal itself after trauma and damage, there are multiple challenges in repairing the damaged tissue. Tissue engineering and regenerative medicine can help overcome these challenges by building modular construct containing high cell densities. In this section, the effect of GAG formulation, perfusion culture condition and hypoxic culture on chondrogenic differentiation of MSCs has been investigated. The overall experimental procedure for this section is illustrated in fig. 5.3. Two formulations were used for making the microcapsules, the first including 1%HA+4%CSA and the second containing 1%HA+4%CSA+1mg/ml Collagen I (to analyze the effect of collagen on cell differentiation). After encapsulation of MSCs at concentration of \(10 \times 10^6\text{cells/ml GAG}\) using the two formulas mentioned earlier using ES method, microcapsules were transferred to two separate 100mm culture plates inside incubator and cultured inside the chondrogenic medium containing HDMEM supplemented with 100nM dexamethasone; 10ng/ml TGF-\(\beta\)1(Peprotech, NJ, USA); 1.25mg/ml bovine serum albumin; 0.2 mM L-ascorbic-2-phosphate; 1% insulin-transferrin-selenium and 1% Penicillin streptomycin with a medium change of every three days for 28 days. Samples of medium and microcapsules were collected from the plates weekly for differentiation analysis. As the microcapsules with the collagen
exhibited higher potential toward chondrogenic differentiation, in the next set of experiments microcapsules with this formula were used.

Fig. 5.3. Schematic of the experimental setup and timeline of the experiments. Following MSCs isolation from rat bone marrow, cells were cultures up to 4th passage, trypsinized and were encapsulated using the ES method in two different microcapsule formulations at a concentration of (10^7 cells/ml of GAG). Both types of microcapsules were then cultured under static conditions for 4 weeks and the chondrogenic differentiation was monitored. The capsules containing collagen were used as the base model due to higher chondrogenic differentiation yield compared to capsules lacking collagen. Accordingly, fresh capsules were made with collagen and transferred into two perfusion culture systems, one under normoxia and the other under hypoxia to analyze the effect of perfusion and hypoxia on differentiation yield. The results were compared with static conditions and normoxia.

5.4.5. Chondrogenic Differentiation of MSCs Inside Microcapsules Inside Perfusion Culture

To analyze the effect of oxygen tension on chondrogenic differentiation of MSCs, 200 microcapsules made with the formulation containing collagen were transferred to the perfusion chip and the chip was transferred to a normoxic incubator containing 21%O₂+5%CO₂+78%N₂ and for the hypoxic conditions same number of microcapsules were transferred to the second perfusion chip and put inside the hypoxic incubator containing 2%O₂+5%CO₂+93%N₂ which were then cultured for four weeks with a medium change of every three days. The flow inside the perfusion chip was adjusted to constant flow of 1ml/min. The results of the normoxic and hypoxic conditions were compared with each other to analyze the effect of oxygen tension on chondrogenic differentiation of MSCs.
5.4.6 Analysis of Chondrogenic Differentiation of MSCs Inside Microcapsules

Multiple quantitative and qualitative assays were used to investigate the chondrogenic differentiation of MSCs inside microcapsules. Cell staining using the fluorescence Calcein AM stain was done to analyze the cell aggregation and formation inside the microcapsules. In brief, after two and four weeks of culture inside chondrogenic medium capsules were washed twice with PBS and were incubated in 2μM Calcein AM working solution for 1hr followed by washing with PBS and imaging under the fluorescence microscope. Size of the aggregates were then quantified in a population of 100 microcapsules for each condition. H&E staining of the microcapsules were done to further investigate the cell growth inside microcapsules in each condition. To visualize the sulfated GAG (sGAG) deposited by the differentiated MSCs, toluidine blue & fast green staining (Sigma Aldrich) was done according to manufacturer instructions. Collagen type II (COL2A1) which is the dominant collagen inside the articular cartilage (~90%) was stained using COL2A1 antibody (Invitrogen, MA, USA). In brief, samples were fixed in 10% neutral buffer formalin for 20min followed by dehydration, clearing, paraffin embedding, sectioned to 7μm ribbons, and were loaded onto charged slides. Slides were then gone through dewaxing, dehydration in ethanol series followed by antigen retrieval using 0.05% trypsin solution for 10min at 37°C. Samples were then blocked for 2hr at room temperature inside 1% BSA followed by incubation overnight at 4°C with COL2A1 monoclonal antibody. HRP conjugated secondary antibody was then used and DAB substrate kit (abcam, MA, USA) was utilized for color development. In addition to qualitative analysis, quantitative analysis including the DNA content, sGAG content, total collagen content and chondrogenic gene expression analysis was performed to verify the chondrogenic differentiation. DNA content was quantified using Hoechst 33258 fluorometric method by crushing 30 microcapsules followed by washing the crushed microcapsules inside the cell lysis buffer (0.1%SDS=10mM Tris-HCL+1mM EDTA) for 2hr at room temperature. 200μL of 2mg/ml of Hoechst solution was then added to the lysed cells followed by reading the fluorescence at Ex/Em of 360nm/460nm. Calf thymus DNA (0-10 µg/ml) was used to generate the standard curve. sGAG content was quantified using the colorimetric dimethyl methylene blue (DMMB)
assay and using chondroitin sulfate A as standard as explained elsewhere\textsuperscript{125}. Briefly, 30 microcapsules from each condition at each time point was collected and crushed in a 2ml microcentrifuge tube and 1ml of the papain digestion solution (300µg/ml) was added to the tube followed by incubation for 1hr at 60°C to digest all the interfering proteins inside the solution. 100µL of digested sample was then mixed gently with 200µL of DMMB and absorbance was read at 525nm using spectrophotometer. Total collagen content was measured using the hydroxyproline assay as discussed before\textsuperscript{126}. In brief, 30 microcapsules were crushed and then were hydrolyzed in 6M HCL solution for 3hr at 115°C in water bath. Hydrolyzed samples were then mixed with buffered chloramine T solution in a ratio of 2:1 for 20min at 25°C. After addition of DMAB solution and incubation at 60°C for 20min followed by reading the absorbance was read at 550nm using spectrophotometer. These results were then converted to the total collagen content following the 1:10 ratio of hydroxyproline to collagen. Expression of chondrogenic markers including SOX9, ACAN and COL2A1 was analyzed as described before using qRT-PCR (Primer sequence in table 5.1)

5.4.7. Analysis of Capsule Wall Permeability

Capsule wall permeability is of great importance as it will determine the diffusion of nutrients and waste into and out of the microcapsule, respectively. In this section the permeability of the two type of microcapsules with two different GAG formulation was analyzed. Permeability of the capsule wall was assessed by measuring the rate of diffusion of labelled BSA from the microcapsule as discussed elsewhere\textsuperscript{127}. This rate was later employed for calculating the overall mass transfer coefficient. Briefly, 60 capsules from each formulation was let to equilibrate with Hanks balanced salt solution (HBSS) containing 2mg/ml BSA and 1mg/ml FITC-BSA for 48hr at 4°C. This process saturates the internal section of the microcapsules with BSA in addition to saturating the BSA binding sites on capsule wall. Microcapsules were then washed three times with HBSS followed by resuspension in quartz vial containing HBSS and were transferred to shaker at 120rpm. The rate of outward diffusion of FITC-BSA was then measured by
reading the fluorescence of the solution every 15 min at Ex/Em of 495/520 nm. The following formulas were used to measure the mass transfer coefficient (K) and permeability (P):

\[ V \frac{dc}{dt} = KA(C_c - C) \]  

(1)

\[ NC_c V_c + V C = NC_\infty V_c + V C_0 = M \]  

(2)

\( M, V, V_c, \) and \( N \) represent the total mass of solute present in the cuvette, volume of external solution, volume of capsules and number of capsules, respectively. \( C, C_0, C_c \) and \( C_\infty \) represent the concentration of solute in external solution, initial extracapsular concentration, concentration of solute inside capsules and initial intracapsular concentration, respectively. Solving equations (1) and (2) it will yield:

\[ \ln(Q) = \left( \frac{KA(V + NV_c)}{NV_c} \right) t \]  

(3)

\( Q \) is a dimensionless concentration number defined as:

\[ Q = \frac{M - (V + NV_c)C_0}{M - (V + NV_c)C} \]  

(4)

By plotting \( Q \) vs time we can find the value of K. The permeability of each type of microcapsule can also be found using the following formula by knowing the average capsule wall thickness which can be found from the images captured by microscope:

\[ K = \frac{P}{\delta} \]  

(5)

5.5. Results:

5.5.1. MSCs Present Differentiation to Adipocytes Inside Microcapsules

MSCs displayed the first sign of adipogenic differentiation after 14 days of induction inside the adipogenic medium as seen from fig. 5.4. The number of lipid droplets increase more after 28 days as can also be seen. In addition to the qualitative imaging analysis done, expression of primary adipogenic markers
including FABP4 and PPARγ were upregulated compared to microcapsules in a non-induction medium which was used as the negative control. MSCs cultured on a normal culture plate and induced under adipogenic conditions for four weeks were also used as the positive control to investigate the effect of electrospraying on gene expression. Higher expression of PPARγ in microcapsules compared to the MSCs on culture plate was observed (fig. 5.5A) which represents higher cell activity inside microcapsules compared to 2D culture that leads to higher expression of the PPARγ.

5.5.2. MSCs Present Differentiation to Osteocytes Inside Microcapsules

Calcium deposits were visible inside the microcapsules after 2 weeks of induction inside the osteogenic medium. The size and density of these calcium deposits increased by 60% after 28 days of culture inside the osteogenic medium which confirms the capability of MSCs to differentiate into osteocytes inside the microcapsules (fig. 5.4). Gene expression analysis of two main osteogenic markers osteocalcin (OC) and alkaline phosphatase (ALP) showed a significant upregulation of both markers compared to non-induced MSCs inside microcapsules which represents the efficient differentiation of MSCs inside microcapsules. The expression of OC in microcapsules containing the MSCs is even higher than the MSCs cultured on the plate inside the induction medium (fig. 5.5B.) which shows the advantage of 3D culture systems compared to the traditional 2D cultures on the plate. On the other hand, expression of ALP was seen to be lower inside the microcapsules compared to the culture plate. This might be due to large size of the ALP protein (86 kDa) which prohibits the efficient isolation of the RNA from the microcapsule samples compared to the OC which is 6kDa. The expression of OC and ALP in the non-induced microcapsule culture was seen to be negligible.
Fig. 5.4. (A1-A2): Staining of the MSCs differentiated to adipocytes at day 14 and day 28 using BODIPY 493/503 fluorescent dye, as can be seen the intensity of the fluoresce oil droplets increase over time which represents the formation of more adipocytes after 28 days. (B1-B2): formation of calcium deposits inside microcapsule cultured inside the osteogenic medium. As can be seen more calcium is deposited at day 28 compared to day 14. Scale bar everywhere is 200µm.

Fig. 5.5. (A) Quantitative gene expression results for the two main adipogenic markers FABP4 and PPARG. Results were compared with a positive control and negative control including the MSCs cultured on plate inside the induction medium and MSCs microcapsules cultured using the non-induced medium, respectively. (B) Expression of osteogenic markers including osteocalcin (OC) and Alkalinephosphate(ALP). Data are shown as ±SD and represented as a fold ratio using GAPDH as housekeeping gene (n=3). (*P<0.05, **P<0.001).
5.5.3. Cells Aggregation Inside Microcapsules Cultured in Chondrogenic Medium

All microcapsules in different conditions cultured inside the chondrogenic medium presented cell aggregate formation after four weeks of culture (fig. 5.6A-D). Though, the size of the aggregate was higher in microcapsules containing the collagen. In both type of microcapsules with and without collagen, it was observed that that aggregate size increases 51% and 49% from day 7 to day 14, respectively, which represents the prompt effect of cell entrapment on cell aggregate formation and growth (fig.5.6E). In perfusion culture, cells start to make aggregates after 2 weeks of culture while under static conditions, they are still spread around the microcapsule. Furthermore, it was observed that most of the cell aggregates form close the capsule membrane due to the presence of GAG binding sites on the capsule membrane. At last, it was observed that culturing the microcapsules containing collagen under hypoxia and perfusion resulted in the largest cell aggregates after 4 weeks of culture inside the induction medium.
Fig. 5.6. Cell aggregation in microcapsules. The first and third column are the bright field and the second and fourth columns are the fluorescence images of the viable cells stained by Calcein AM. MSCs in capsules cultured under static and normoxia conditions, (A),(B), are yet spread around the microcapsule after the second week of culture while those under perfusion culture make aggregates at the second week (C),(D). The size of the aggregates in all four conditions of the study reach maximum after 4 weeks of culture and most of the aggregates are formed around the capsule membrane. In addition, the capsule membrane kept the integrity and uniformity over the 28 days of culture under static conditions. Creating hypoxic conditions further increased the aggregate size. Capsules cultured under perfusion showed deformation at the fourth week of study which can be due to the shear stress input from the perfusion culture. (E): shows size comparison in capsules cultured under static conditions. The average size of cell aggregate in capsule containing collagen is higher compared to capsules formed without the collagen. Sharpest increase in aggregate size was observed from day 7 to 14 (51%) and from day 14 to 21 (42%) for capsule lacking collagen and with collagen, respectively. Data are shown as ±SD. All scale bars are 200µm. (S= static, N=normoxia, P=perfusion, H=hypoxia Col=collagen).

5.5.4. Collagen Accelerates MSCs Growth and Chondrogenic Differentiation

Histology staining using H&E showed larger cell aggregate formation in microcapsules containing collagen (fig. 5.7). DNA quantification also revealed higher MSCs growth in capsules containing collagen (fig. 5.7E). Additionally, staining the sGAG using toluidine blue also showed higher GAG deposition in microcapsules made with collagen (fig. 5.8). Quantification of sGAG deposition using the DMMB assay also was in agreement with the toluidine blue staining as more sGAG was observed to be found in capsules containing the collagen after four weeks of culture inside the induction medium(fig 8E). IHC staining of collagen type II also showed higher production of this main ECM protein in microcapsules containing
collagen (fig. 5.9). Total collagen content which was also quantified using the hydroxyproline assay verified our IHC staining as can be seen from fig. 9E which shows higher collagen content in microcapsules containing collagen. It should be noted that in these samples the value of initial collagen content present in the microcapsules were subtracted from the final collagen concentration obtained from the hydroxyproline assay. At last, the analysis of chondrogenic markers using the qRT-PCR revealed an increase of the expression of the main chondrogenic markers including COL2A1, ACAN and SOX9 in microcapsule containing collagen compared to microcapsules lacking collagen (fig. 5.10). Ultimately, these results told us that collagen helps MSCs growth, differentiation, and maturation inside microcapsules by providing cell-cell binding and mimicking the native ECM microenvironment by providing a scaffold for cell growth inside microcapsules, therefore, for the rest of the experiments these types of microcapsules were used.
Fig. 5.7. (A-D): H&E staining of the two type of microcapsules cultured under static conditions. As can be seen first signs of cell aggregation can be seen after 14 days of culture. Higher cell growth and aggregation can be observed in capsules containing collagen and cultured under perfusion and hypoxic conditions. (E): DNA quantification results using Hoechst 33258 fluorometric method. Same trend with higher DNA content in capsules cultured under perfusion+ hypoxic condition was observed in the DNA quantification assay. Data are shown as ±SD. All scale bars are 100µm. (*P<0.05, **P<0.001, S= static, N=normoxia, P=perfusion, H=hypoxia, Col=collagen).

5.5.5. Perfusion Accelerates MSCs Growth and Chondrogenic Differentiation

H&E staining of microcapsules cultured under perfusion showed larger cell aggregates compared to static culture of after 4 weeks (fig. 5.7). DNA quantification results verified H&E observations as can be seen in fig. 5.7E. The highest increase in DNA number was observed from day 14 to 28 with a 78% increase which is primarily due to formation of cell aggregates after 2 weeks inside the microcapsule. Staining of the sGAG using toluidine blue stain also showed higher GAG formation under perfusion which was mostly formed around the cell aggregates rather than the individual cells inside the microcapsule (fig.5.8). GAG quantification results using the DMMB assay was in complete agreement with the toluidine blue staining as higher GAG was observed in capsules cultured under perfusion compared to static culture (fig. 5.8E). Moreover, perfusion enhanced the COL2A1 synthesis as can be seen in fig. 5.9. Quantification of total collagen using the hydroxyproline assay also showed higher amount of total collagen synthesized in perfusion culture (fig. 5.9E). It should be noted that the presence of collagen increased the total collagen by 1.5-fold while the perfusion culture increased this value by 3-fold which shows the presiding effect of
perfusion (fig. 5.9E). As can be seen in fig 5.9, most of the collagen type II was also deposited around the cell aggregates. Expression of chondrogenic markers also revealed higher expression of COL2A1, ACAN and SOX9 in prefusion culture compared to the static conditions (fig. 5.10). At last, perfusion culture resulted in higher MSCs proliferation and differentiation compared to the static culture due to higher availability of nutrients and growth factors inside the microcapsule.
Fig. 5.8. (A-D): Toluidine blue and fast green staining of the microcapsules cultured under different conditions. Dark blue spots exhibit the GAG formation over the 28 days of culture in chondrogenic medium. Staining of the capsule membrane also shows the presence of HA inside the membrane. As can be noticed the number of blue spots in the capsules containing the collagen is slightly higher than those without collagen. Data are shown as ±SD. All scale bars are 100µm. (*P<0.05, **P<0.001, S= static, N=normoxia, P=perfusion, H=hypoxia, Col=collagen).

5.5.6. Hypoxia Accelerates MSCs Growth and Chondrogenic Differentiation

Moderately higher cell aggregation was observed in microcapsules cultured under the perfusion and hypoxic (2%) conditions as can be seen from the H&E staining results (fig. 5.7). DNA quantification revealed same trend as can be seen from fig. 5.7E, the highest DNA in sample was observed in combined perfusion and hypoxic condition. DNA content after 28 days in the perfusion culture was 1.2-fold higher than the corresponding static culture. Moreover, higher sGAG was deposited inside the microcapsules cultured under hypoxic conditions compared to normoxic conditions as can be seen from the toluidine blue staining (fig. 5.8D4). GAG quantification using the DMMB assay revealed the same results as higher GAG was produced after 4 weeks of culture inside the hypoxic culture compared to normoxic condition (fig. 5.8E). IHC staining of collagen type II also revealed deposition under hypoxic conditions (fig. 5.9D). Total collagen content quantification using hydroxyproline assay also showed 1.2-fold increase at day 28 of culture compared to normoxic conditions (fig. 5.9E). Expression of chondrogenic markers including COL2A1, ACAN and SOX9 also presented 2.4, 4.5 and 7.1-fold increase, respectively, compared to
normoxic conditions (fig. 5.10). These results indicate the privilege of hypoxic conditions to normoxic conditions in accelerating MSCs growth and differentiation to chondrocytes.

Fig. 5.9. Immunohistology of collagen type II of the differentiated cells after 4 weeks of culture inside chondrogenic medium. (A) 1%HA+4%CSA capsules under static conditions. (B) 1%HA+4%CSA+Col under static conditions. (C) 1%HA+4%CSA+Col under perfusion and normoxia conditions. (D) 1%HA+4%CSA+Col under perfusion and hypoxia conditions. As can be seen, combining perfusion and hypoxia conditions will enhance the collagen type II secreted by cells inside the microcapsules. Hemotoxylin was used as the counter stain (E): total collagen quantification using hydroxyproline assay. In agreement to IHC results, combining perfusion and hypoxic conditions is in favor of more collagen production. Data are shown as ±SD. All scale bars are 100µm. (*P<0.05, **P<0.001, S= static, N=normoxia, P=perfusion, H=hypoxia, Col=collagen).
**Fig. 5.10.** Quantitative gene expression results of the three main chondrocytes markers COL2A1, ACAN and SOX9 for the four conditions of the study. Data are shown as ±SD and presented as a fold ratio using GAPDH as housekeeping gene (n=3). (*P<0.05, **P<0.001, S= static, N=normoxia, P=perfusion, H=hypoxia).

5.5.7. Analysis of Membrane Permeability and Diffusion of GAG into the Medium

Capsule membrane permeability affects both cell viability and growth\(^{128}\). Multiple methods were used to analyze the membrane porosity including SEM images, calculating the membrane permeability and measurement of GAG diffused into the medium. Analysis of SEM images revealed higher porosity in capsules lacking collagen (fig 5.11A-B). Calculating the value of permeability (P) and mass transfer coefficient (K) resulted in 1.2-fold and 4.6-fold higher for P and K, respectively, for the capsules made without collagen compared to those with collagen (fig. 5.11.C). Contraction of collagen inside microcapsules leads to the retention of HA inside the microcapsules which in turn results in lower HA presence inside membrane. Lower presence of HA in membrane causes stiffer chitosan bond in membrane (due to high MW of HA) which results into lower porosity and permeability. In contrast, in capsules made without collagen, higher presence of HA in the membrane leads to weaker bonds between chitosan and HA which in turn results into higher permeability and porosity. GAG measurement inside the membrane is another parameter which presents the membrane permeability. Fig. 5.11D represents the GAG content inside the medium at different time points. GAG accumulation in medium is mainly due to three reasons: initial GAG which was used in encapsulation eventually diffuses out, perfusion causes some of the GAG to come out, GAG synthesized by differentiated cells also might diffuse out. As can be seen from fig. 5.11D, highest GAG content in medium was observed at day 7 which is caused by diffusion of GAG used for encapsulation into the medium and the concentration then has negative slope throughout the 28 days. It can also be seen that the perfusion has in general higher GAG content inside medium which can be considered as the downside of the system. Moreover, as was expected, capsules made without collagen have higher GAG content in the medium which is due to their higher porosity. In conclusion, absence of collagen might be advantageous regarding facilitated nutrient diffusion into capsule but at the same time higher GAG synthesis will also occur which is a downside of capsules lacking collagen.
Fig. 5.11. (A, B): SEM images of the two types of microcapsules cultured under static conditions inside chondrogenic medium (exterior, membrane, and interior, respectively. (C) comparison of mass transfer coefficient (k) and capsule permeability (P) in two type of microcapsules studied. (D): GAG content inside the collected medium in four conditions of the study over 28 days.
5.6. Summary and Discussion

In this chapter we analyzed the multipotency capability of encapsulated MSCs to differentiate into the three lineages including adipocytes, osteocytes, and chondrocyte. MSCs showed differentiation into adipocytes as it was seen from BODIPY 493/503 staining results, in addition to expression of adipocyte markers including FABP4 and PPARγ. Furthermore, MSCs presented differentiation into osteocytes when they were induced for 28 days. Calcium deposits which is the primary indication of osteogenic differentiation, was observed inside the microcapsules after 4 weeks of culture. No major differences were seen in expression of osteogenic markers including OC and ALP for induced MSCs inside microcapsules compared to induced MSCs cultured on plate which implies the multilineage potency of MSCs inside microcapsules. Moreover, MSCs are known as the progenitor cells for chondrocytes in the early embryonic development\(^\text{129}\). Despite MSCs high potency to differentiate into chondrocytes in-vivo, they have limited differentiate capacity in-vitro. To accelerate their in-vitro differentiation, multiple researchers have proposed different strategies including different culture conditions, mechanical stimulus, and chemical stimulus such as presence of GFs\(^\text{130-132}\). It has been seen that ECM structure, perfusion and hypoxia are the most dominant factors among other\(^\text{90}\). In this chapter, we further investigated the co-effect of these three parameters on chondrogenic differentiation of MSCs inside microcapsules. To analyze the effect of ECM, two type of microcapsules were made for cell encapsulation (1% HA + 4% CSA and 1% HA + 4% CSA + 1mg/ml collagen). Hereafter, the effect of collagen on proliferation and differentiation of MSCs inside microcapsules was investigated. Collagen was chosen as it is the primary ECM protein in the articular cartilage which bears multiple traits as: fibrillar properties and self-assembly. It was observed that cell encapsulation would lead to cell aggregation and higher cell growth which is desirable in 3D culture systems. Cell growth quantification by analyzing the DNA content showed that cell number was doubled from 3\(^{rd}\) week to 4\(^{th}\) week of culture for both type of microcapsules. H&E results verified the advantage of collagen in cell proliferation. It is believed that collagen contraction and cell-ECM interaction leads to higher cell aggregation and cell proliferation inside microcapsules\(^\text{42}\). GAG deposition analysis by toluidine
blue staining also revealed higher GAG amounts when using collagen which agreed with GAG quantification results using the DMMB assay. Again, higher amount of GAG was observed around the cell aggregates which is believed to be due to higher paracrine signaling. Paracrine signaling which is known for GAG deposition operates in short cell-cell distances compared to autocrine signaling which operates in large ranges\textsuperscript{133}. Collagen presence also leads to higher collagen type II deposition as was observed both quantitatively and qualitatively. Expression of COL2A1, ACAN and SOX9 showed higher amount in capsules with collagen which is believed to be primarily due to higher SOX9 expression as this gene affects both COL2A1 and ACAN expression. By finding the advantageous effect of collagen on cell growth and differentiation, these types of microcapsules were cultured under perfusion conditions to analyze the effect of mechanical stimulation. In comparison to static culture which the cell aggregates were formed at 3\textsuperscript{rd} week, in perfusion culture aggregates appeared after the first week of culture. Higher nutrient and oxygen are provided to cells inside the perfusion culture which yields to higher cell growth. Specifically, large molecules such as TGF\(\beta\) (25kDa) have higher diffusion rate under perfusion conditions. Larger cell aggregates lead to higher GAG deposition and collagen type II synthesis in the perfusion system compared to static culture. Moreover, higher expression of COL2A1, ACAN and SOX9 further confirm the dominance of perfusion culture. In the last step, to analyze the effect of oxygen tension on MSCs, microcapsules were transferred to a perfusion chip under hypoxic conditions (2\%) and were cultured for 28 days inside the induction medium. It is believed that in human cartilage, hypoxia leads to the upregulation of SOX9 which consequently increases COL2A1 and ACAN expression\textsuperscript{121}. In our results, larger cell aggregates were formed under hypoxic conditions and higher DNA number was also observed in the sample after 4 weeks of culture inside the induction medium. GAG deposition with toluidine blue staining also showed higher amounts in microcapsules cultured under hypoxic conditions. Same trend was observed for collagen type II staining as higher deposition was observed around the cell aggregates. Finally, comparison of the porosity of the two type of microcapsules showed highest porosity in microcapsules made with collagen. This might be another reason for higher GAG deposition inside microcapsules as less GAG will be able to diffuse out compared to the higher porosity in microcapsules lacking collagen.
Theses microcapsules have the potential to generate large 3D cell aggregates which is one of the main objectives of the tissue engineering. To the best of our knowledge, this study has been the first to analyze the co-effect of 3 parameters including ECM, perfusion, and hypoxic conditions on chondrogenic differentiation of MSCs. We believe these microcapsules can be used as the modular components for developing an articular cartilage *in-vitro* which can replace the native damaged articular cartilage.
CHAPTER 6
Encapsulation and In-vitro Culture of Primary Rat Hepatocytes

6.1. Introduction

Hepatocytes are cells with high metabolic activity and high regeneration capacity *in-vivo*. Beside their *in-vivo* regeneration capacities, hepatocytes have shown low proliferation rate *in-vitro* which leads to their poor functionality and eventually loss of cell viability in these systems. Hence, any liver culture system should be able to retain the hepatocyte functionality in long-term. Co-culture of hepatocytes with non-parenchymal cells such as MSCs and VECs (vascular endothelial cells) is one way to preserve the metabolic activity of hepatocytes. Another key parameter which has shown to preserve the metabolic activity of the hepatocyte in long-term is the hepatocytes culture under perfusion conditions. Since perfusion facilitates the diffusion of nutrient and oxygen into the cells, these systems have great advantage over the static cultures. In this chapter the effect of these two parameters on the metabolic activity of hepatocytes was investigated both with quantitative and qualitative assays.

6.2. Aim and Rationale

The main specific aim of this chapter is to investigate the effect of co-encapsulation of hepatocytes with MSCs and VECs on metabolic activity of hepatocytes. The results will be compared with both collagen sandwich cultures which are well established hepatocyte culture systems and with microcapsules cultured under static conditions. This aim is significant because for any liver *in vitro* model to be used for DILI (drug induced liver injuries) applications, the hepatocytes should be able to preserve their metabolic activity over longer periods as many drugs show their side effect over longer periods on liver. By optimizing the hepatocyte culture conditions, we will also be able to expand the application of the culture system to other areas such as using microcapsules to build an engineered liver.

6.3. Experimental Approach

**Study 1:** Effect of co-encapsulation of hepatocytes with MSCs on metabolic activity of hepatocytes
Isolated rat primary hepatocytes were encapsulated in the optimized GAG formula and were cultured for four weeks under perfusion conditions to analyze the effect of both co-encapsulation and perfusion. Albumin, urea and CYP1A1 activity were measured as markers of the hepatocyte metabolic activity.

**Study 2:** Effect of seeding VECs inside of the microcapsules on hepatocytes metabolic activity

VECs were seeded inside of the microcapsules containing Hep + MSCs and the microcapsules were cultured on an orbital shaker for four weeks to investigate the presence of VECs on hepatocyte metabolic activity. In this study orbital shaker was used to mimic the perfusion as it is more convenient and practical compared to complex perfusion models. Same as the previous study, albumin, urea and CYP1A1 activity were measured as the hepatocyte markers. Vascularization inside the microcapsule was also analyzed using endothelial markers such as PECAM (platelet endothelial cell adhesion molecule). VECs adhesion and proliferation was also analyzed over time using phase contrast microscopy and H&E staining.

**6.4. Materials and methods**

The overall experimental procedure for this chapter is illustrated in fig. 6.1. All materials used were purchase from Sigma Aldrich or otherwise mentioned.

![Diagram](image)

**Fig. 6.1.** Illustration of the design of the experiments. Three types of cells were isolated from rat and were cultured, trypsinized, and encapsulated using the ES method. These three types of capsules were then cultured under perfusion culture for 28 days and the metabolic activity of hepatocytes under in each culture was assessed at certain time intervals.
6.4.1. Fabrication of Perfusion Bioreactor

The bioreactor for the culture of microcapsules was fabricated as explained previously in chapter 3 using the 3D printing method. The working volume of the bioreactor is 150mm³ and dimensions of the working section are 40mm×5mm×2mm (L×W×H).

6.4.2. Isolation and Culture of Rat Primary Hepatocytes on Collagen Sandwich Culture

Rat primary hepatocytes were isolated from female SD rats weighting 200-250g by two step collagenase perfusion method described by Seglen and modified by Dunn et al 140. Prior to isolation of primary hepatocytes, 60mm culture dishes were coated with 2ml of collagen solution containing 9 parts collagen (1mg/ml) solution and 1part 10X DMEM. The coated plates were transferred to incubator for 1hr to allow the collagen gelation. Isolated hepatocytes were then cultured at concentration of 2×10⁶ cells/2ml culture medium/dish. The hepatocytes complete culture medium composed of HDME supplemented with 10%FBS, 0.5U insulin/ml, 7ng/ml glucagon, 20ng/ml epidermal growth factor (Peprothech, NJ), 7.5µg/ml hydrocortisone, 50µg/ml gentamicin and 0.25µg/ml Amphotericin-B was then supplied to the plates. After 24hr of incubation, the medium on top of collagen layer was removed and second layer of collagen was added on top of the hepatocytes followed by 1hr incubation to complete the gelation. The medium was then added on top of the second collagen gel (2ml) with a daily medium change for four weeks.

6.4.3. Isolation and Culture of VECs

VECS were isolated and cultured from sheep aorta according to the established protocol and were cultured in VECs medium composed of M199 supplemented with 20% FBS141. VECs at passage four were used in all experiments.

6.4.4. Cell Labelling Using CellTracker™

CellTracker™ green was used for staining of the hepatocytes prior to encapsulation and CellTracker™ red was used for staining the MSCs prior to encapsulation according to manufacturer’s recommendation. In brief, a working solution of 25µM concentration for each of the dyes in serum free
medium was prepared and added to the hepatocyte and MSCs cell suspension. The dye solution containing the cell suspension was then incubated at 4°C for 30min followed by washing with PBS and centrifugation to remove the dye from the cell suspension. The labeled cells were then used for encapsulation and following analysis.

6.4.5. Co-Encapsulation of Hepatocyte and MSCs

Freshly isolated hepatocytes and MSCs at passage four were encapsulated using ES method as described in chapter 4. The optimized GAG formulation containing 1%HA,4%CSA and 1mg/ml collagen type I was used for encapsulation in all experiments. Hepatocytes to MSCs at a ratio of 7:3 was used for cell encapsulation based on previous studies\textsuperscript{142}. 7×10^6 hepatocytes/ml GAG was mixed gently with 3×10^6 MSCs/ml GAG and the solution was added to the syringe and transferred to syringe pump for encapsulation using ES method. All reagents and cells during the encapsulation process were kept at 4°C to minimize cell activity. Microcapsules without MSCs with a same concentration of hepatocytes were used as control to analyze the effect of MSCs on hepatocytes metabolic activity. In each set of experiments, 1 ml of GAG was used for making the microcapsules and with assumption of 10×10^6 cells used for encapsulation in the coculture system and average capsule diameter of 500µm, each microcapsule contains approximately 1000 cell initially which contains approximately 300 MSCs and 700 hepatocytes. This number will decrease to 700 hepatocytes in each capsule for monoculture system. Approximately 200 of mono microcapsules containing hepatocytes were transferred to one perfusion chip and the same number of co-encapsulated hepatocytes-MSCs microcapsules were also transferred to the second perfusion chip for culture over 28 days. Flowrate inside the perfusion chips was always kept at constant flow of 1ml/min using a peristaltic pump. Medium was collected at equal time intervals and the metabolic activity of the hepatocytes inside each perfusion chip was then compared with each other.
6.4.6. Seeding VECs Inside Microcapsules

For seeding the VECs inside the microcapsules, VECs were added to the hepatocytes + MSCs microcapsules at a ratio of 7:3:1 of hepatocytes, MSCs, VECs, respectively. These two types of microcapsules, one seeded with VECs and one without were transferred to an orbital shaker (70rpm constant rotation speed) as a convenient and reliable culture system to facilitate the diffusion of oxygen and nutrients into the microcapsules. Medium was collected at equal time intervals from the two system and the results were compared with each other.

6.4.7. Liver-Specific Function Assays

Culture medium from each study was collected daily for albumin, urea and CYP1A1 enzyme activity measurements. Albumin concentration was measured using ELISA with rat albumin (0-200μg/ml) as standard based on standard protocols \(^{140}\). Urea was measured using the chemical assay which uses diacetyl monoxime-glucuronolactone as color developing reagent based on established protocols \(^{143,144}\). Urea (0-20μg/ml) was used as standard. CYP1A1 activity was measured by induction with β-naphthofluorane (BNF) at a concentration of 10mM by dissolving the compound in DMSO. The enzymatic activity was then measured using the rate of conversion of ethoxy resorufin (ER) to resorufin (R) which was measured using spectrophotometer at Ex/Em of 530/585 nm based on established protocols \(^{145,146}\). Standard curve for R was done by dissolving in culture medium at concentration of 0 to 1000nm and measurement using spectrophotometer. Non-induced hepatocytes culture was used as control system.

6.4.8. Characterization of Seeded VECs Inside the Microcapsules

Immunohistochemistry using PECAM staining as marker of endothelial cells was also done to assess localization of VECs inside the microcapsules. To do the PECAM staining, paraffin embedded microcapsules were sectioned using microtome followed by dewaxing and dehydration in ethanol series, followed PBS-tween wash. Sections were then blocked with 1% bovine serum albumin for 30min and were then washed again three times with PBS and stained with anti PECAM-1 IgG1 antibody produced in mouse.
(EDM Millipore, Germany) diluted 1:50 in 1% BSA overnight at 4°C. After washing three times with PBS, sections were stained with anti-mouse IgG-FITC secondary antibody diluted 1:50 in 1% BSA for 2hrs. Color was developed using the DAB substrate kit (Abcam). Hematoxylin was used as the counter stain. Sections were then imaged under microscope to observe the transmembrane glycoprotein on the VECs using PECAM-1 and to observe the cell nuclei using hematoxylin.

6.5. Results

6.5.1. Effect of Perfusion on Hepatocytes Function Inside Microcapsules

A rule of thumb in design of the perfusion culture systems is to achieve a balance between the shear stress exerted on the cells and nutrient transfer to the cells\textsuperscript{147}. Applying higher flowrates would results into higher transport of nutrients but at the same time higher shear stress will be enforced on the cells\textsuperscript{148}. Most of the current liver-on-chip systems operate at lower than 1ml/min flowrates due to this criterion\textsuperscript{63,149,150}. By using microcapsules instead of cell monolayers or organoids, the limitation of flowrate on the design criteria will be withdrawn as the capsule wall will protect cells from the shear stress. Hence, a medium flowrate of 1ml/min was used in all conditions to provide enough nutrient and O\textsubscript{2} to the cells inside the microcapsule. Perfusion resulted into larger cell aggregates formation compared to static culture due to multiple reasons (fig. 6.2). One is that the perfusion facilitates cell movement inside microcapsule. Higher cell movement results in higher cell-cell attachment inside the capsule which is vital for cell growth and function. The larger cell aggregates formation can be observed from both fluorescence images and histology staining (fig. 6.2), nonetheless, in static cultures, cells are mostly spread around the microcapsule, not interested to form larger aggregates. This early aggregate formation was also observed in the chondrogenic capsules discussed earlier in chapter 5. Accordingly, it was observed that metabolic activity of the hepatocytes cultured under perfusion conditions were also higher compared to static conditions (fig. 6.3 and 6.4). The albumin synthesis rate for capsules cultured under perfusion was 1.4-fold higher than the static cultures at day one. This rate decreases sharply after day 1 to day 4 with 22% and 70% decline for perfusion and static cultures, respectively. We believe this decline is primarily due the damage in
hepatocytes during the collagenase isolation as the enzyme will affect the gap junction proteins on hepatocytes surface which are crucial for a proper cell-cell function. This sudden decline can also be caused by the aggregate formation inside microcapsules as there would be less oxygen available for the cells in the core of the aggregate resulting in sudden decrease in albumin synthesis rate. This rate observes a jump in co-encapsulated hepatocytes with MSCs cultured under perfusion from day 12 to 16 followed by a 16 % decrease from day 16 to day 20. We believe this decline might be partially due to the shear stress exerted on the hepatocytes over time or the functional loss of hepatocytes in long period. It seems in static culture, after day 4 up to end of culture, the hepatocytes gradually lose their albumin expression capabilities due to accumulation of toxins such as urea and nitrogen build up around the hepatocytes in addition to lack of nutrient and oxygen transfer to the hepatocytes. On the other hand, these toxins are removed in the perfusion culture and sustaining the hepatocytes function accordingly. The difference in the albumin production rate in the two perfusion conditions in mainly due to the presence of MSCs which will be explained in the next section. The effect of perfusion on urea synthesis rate can be seen in fig.6.4. As can be seen, both perfusion conditions have higher urea synthesis rate compared to static culture. This rate for the static culture drops to 75% of the base value and stays around the same value until the end of the study with a shallow decrease of around 33% from day 24 to day 28. On the other hand, in the perfusion cultures, regardless of 65% decrease of urea rate from day 1 to day 4, the urea synthesis rate bounces back to the original value of around 16µg/10⁶cells/day on day 24 for the co-culture system and to 7 µg/10⁶cells/day in monoculture system at day 20 which clearly shows the advantage of perfusion culture on urea synthesis rate. In conclusion, it was observed that perfusion is in complete favor of the metabolic activity of hepatocytes regardless of the presence of the MSCs or not.
Fig. 6.2. (A) Cell viability inside microcapsules cultured under different conditions over 28 days of the study. Cells were stained with Calcein-Am. As can be seen the largest cell aggregates and more interconnected cell-cell structure are observed in microcapsules containing both MSC and hepatocytes and cultured under perfusion conditions. (B) H&E staining of the microcapsule cultured under different conditions at day 7 and 28 of the study. Scale bar everywhere is 200µm. (C) How hepatocytes and MSCs co-encapsulated in microcapsules interact with each other over 10 days of culture; 1 day post encapsulation, hepatocytes and MSCs are spread around the microcapsule; After 3 days they start attaching to each other and emit yellow color appearance formed by merge of the red and green colors; They completely merge after 7 days and by day 10 the size of the aggregated also increases. It should be noted cell aggregates mostly attach to microcapsule membrane due to presence of GAG receptors on cells. Left column is the bright field images of the corresponding images on the right column (S=static, P=perfusion).

Fig. 6.3. Albumin synthesis rate in different culture systems; (A) Microcapsule containing hepatocytes alone and hepatocytes + MSCs cultured under perfusion or static conditions. (B) Collagen sandwich culture containing hepatocytes and hepatocytes + MSCs. (⁺P<0.05, ⁺⁺P<0.005, ⁺+++P<0.0005), ANOVA test.
6.5.2. Effect of MSCs Presence in Microcapsules on Metabolic Activity of Hepatocytes

Multiple researchers have found that soluble factors secreted by MSCs affects maintaining hepatocytes metabolic activity\textsuperscript{14,15}. Additionally, MSCs secrete multiple ECM proteins which help hepatocytes repair due to damage during the isolation process. In brief, MSCs assist hepatocytes during their recovery process by three means: 1) Secretion of cytokines such as TGF-\(\beta\) and TNF-\(\alpha\) which mediate hepatocytes growth and differentiation. 2) Secretion of ECM proteins such as collagen, fibronectin, vitronectin and laminin. 3) Expressing gap-junction proteins such as connexin-32 and LRP which facilitates cell-cell communication\textsuperscript{152}. Damage caused by collagenase perfusion during hepatocyte isolation leads to cell-cell and cell-ECM destruction which are vital for hepatocytes growth. It has been seen that MSCs can repair these connections by secreting growth factors and ECM proteins\textsuperscript{2}. As can be seen from fig. 6.3, the albumin production for both coulture and monoculture drops from day 1 to day 4 which is mainly due to the damage occurred during the isolation of hepatocytes. The drop in monoculture and static condition continues up to day 28 until there is 76% decrease in metabolic activity of hepatocytes, compared to 46% decrease in the co-culture system. The most promising results were observed in co-culture system in perfusion conditions which the cells regain their initial metabolic activity at day 16 with 94% retention of their initial metabolic activity. Notably, from day 16 to 20 there is a decrease in albumin production for both of the perfusion cultures but in the co-culture system at day 24, albumin production reaches its previous peak which is believed to be due to the restoring effect of MSCs on hepatocytes. At day 28 the albumin synthesis rate in the co-culture system increases further by 2.6% representing the complementary effect of MSCs on hepatocytes growth while at the same time in all other conditions synthesis rate decreased from day 24 to 28. The effect of co-culture on urea synthesis rate can be seen in fig. 6.4. As can be seen, the co-culture system has higher initial urea synthesis rate at day 1 in both static and perfusion conditions which represents the beneficial effect of MSCs. This value for all conditions has a negative slope till day 28 except for the co-culture system under perfusion. One might guess this would be because of perfusion but by comparing with the same monoculture under perfusion MSCs contribute to the bouncing back the urea rate.
to 16µg/10⁶ cells/day. Same results were observed in the sandwich culture system in which the rate of urea synthesis rate was higher compared to monoculture. These results show that co-culture of hepatocytes and MSCs will lead to repairing of the damaged hepatocytes and consequently regaining the original metabolic activity of hepatocyte.

Fig. 6.4. Urea synthesis rate in different culture systems; (A) Microcapsule containing Hepatocytes alone or hepatocytes + MSCs cultured under perfusion or static conditions. (B) Collagen sandwich culture containing hepatocytes or hepatocytes + MSCs. (*P<0.05, **P<0.005, ***P<0.0005), ANOVA test.

6.5.3. Effect of Internally Seeded VECs on Metabolic Activity of Hepatocytes

Forming a vascular network inside 3D tissue samples to facilitate oxygen and nutrient diffusion to the cells is of crucial importance. Without an adequate vascular structure, cells are prone to oxygen and nutrient deficiency as the oxygen diffusion is limited to 200µm from the closest blood vessel. This factor is of more importance inside the liver since the hepatocytes are cells of high metabolic activity degree, demanding high oxygen and nutrient \textit{in-vivo}. By adding sheep vascular endothelial cells into the microcapsule, larger cell aggregates were formed as can be seen from fig. 6.5. Larger cell aggregates imply higher cell growth and attachment which are of great interest in tissue regeneration. Analysis of H&E staining and Calcein-AM staining after 28 days of culture of the microcapsules with and without VECs illustrates larger, more compact cell aggregates with a formation of a vascular network inside the
microcapsules (Fig 6.5). Cell viability in both type of microcapsules was found to be above 95% using the CalceinAM-Ethd1 staining method.

**Fig. 6.5.** Analysis of cell growth and viability inside the two type of microcapsules. (A, B) H&E staining of microcapsules formed without VECs. (C) Viability of cells formed without VECs. (D, E) H&E staining of microcapsules formed with VECs. (F) Viability of cells formed with VECs.

PECAM staining (IHC) of the encapsulated VECs was also done using the established protocols. By analyzing the IHC images, it was observed that VECs form a vascular network inside the microcapsules (fig. 6.6A) in addition to surrounding the formed cell aggregates (fig. 6.6E). Moreover, higher magnification of the IHC images, revealed binding of the VECs to the interior membrane of the microcapsules (fig. 6.6D). Endothelial cells were also observed forming a vascular network inside the cell aggregates and as can be seen from fig.6.6E, F. These results put together, demonstrates the successful seeding and application of VECs in the interior of microcapsules.
Fig. 6.6. IHC staining of the seeded VECs using PECAM. (A, B) Formation of a vascular network inside the microcapsule. (C, D) VECs attachment to the capsule membrane. (E, F) VEC distribution around and inside the cell aggregates.

To further analyze the effect of VECs on hepatocyte metabolic activity, synthesis of albumin and urea in the two type of microcapsules was assessed over 28 days of culture. Approximately 200 microcapsules from each condition were transferred to a 10cm culture plate which were then placed on an orbital shaker inside incubator at a constant rotation speed of 70rpm. Microcapsules were cultured for 28 days with a medium change of every 2 days. Albumin and urea were measured every 4 days according to established protocols.
By analyzing the albumin synthesis rate under the two conditions (fig. 6.7A), it was observed that the initial albumin synthesis rate was 1.5-fold higher in microcapsules containing VECs which is mainly due to the positive effect of VECs on hepatocytes inside the culture as mentioned by others too$^{137,157}$. While the rate fluctuates over the 20 days of culture, the albumin rate in microcapsules containing the VECs reaches the highest rate at the end of culture while this rate is 2-fold lower in microcapsules without VECs. These results illustrate the benefit of co-culturing hepatocytes with VECs. We believe that VECs increase the hepatocyte metabolic activity by formation of vascular network around and inside the cell aggregates, facilitating the nutrient and oxygen transfer into the hepatocytes. Analysis of urea synthesis rate resulted in a different trend as the initial synthesis rate for microcapsules with VECs was lower compared to those without VECs (fig. 6.7B). Although the urea synthesis rate for capsules with VECs surpasses the ones without VECs after day 4, the rate still stays very close to each other in both conditions. This implies that urea synthesis rate is independent of the presence of the VECs which requires further analysis for providing a logic behind it.

6.5.4. Analysis of CYPIA1 Activity

To compare the enzymatic activity of microcapsules containing VECs with those without VECS, both type of microcapsules was induced for 2 days with BNF followed by CYPIA1 measurement. This procedure was done over 6 days to analyze the CYP1A1 at different time points. Standard controls were also carried out with non-induced microcapsules and induced microcapsules without cells. Comparison of the CYP1A1 activity from fig. 6.8B, it was observed that the highest induction was on day 2, with a decreasing trend over the 6 days of culture. This can be mainly due to hepatocytes death over the culture period either because of continuous BNF delivery to the hepatocytes or the decline in their overall activity. Long term exposure of BNF causes down regulation of CX32 gene which in turn results in liver injury,
inflammation and oxidative stress to hepatocytes as mentioned by others.\textsuperscript{158,159} Furthermore, presence of VECs inside the microcapsule didn’t affect the CYP1A1 activity to a noticeable degree which means that the hepatocyte enzymatic activity might be independent of the presence of other cell type present in the co-culture systems despite the beneficial effect of liver co-culture systems in preserving hepatocyte metabolic activity.\textsuperscript{81} Control test were also performed which demonstrated low or almost zero CYP1A1 activity under different conditions (fig. 6.8A).

![Graph](http://example.com/graph.png)

**Fig. 6.8.** (A) analysis of CYP1A1 activity in the presence and absence of VECs inside microcapsules over 6 days of culture. Standard controls containing the non-induced samples were also performed in at same time points. Data are after 2 days of induction with BNF (B) CYP1A1 activity inside the induced culture over 6 days. Samples were measured every two days. (*P<0.05, **P<0.005, ***P<0.0005), ANOVA test, (in=induced, non=non-induced)

6.5.5. **SEM Images of Microcapsule Interior**

Collagen remodeling inside the capsule was observed using SEM images. As can be seen from fig.6.9A, C, addition of collagen into the GAG formulation results in a formation of a fibrillar network which entraps and connect the cells inside the microcapsule. This fibrillar structure was also observed in other research which is believed to affect cell growth, migration and cell-cell interaction in a more *in-vivo* like assembly.\textsuperscript{160} Cell-cell connection and aggregation inside the microcapsules can also be observed in fig. 6.9B, D. Similarly, the effect of collagen in connecting cells and generation of a fibrillar network can
also be observed in these two images. In conclusion, SEM images further demonstrate the beneficial effect of collagen on cell remodeling, growth, and migration inside microcapsules.

Fig. 6.9. SEM images of the microcapsules containing hep+MSC+VEC. (A) Microcapsule interior. (B) Cell aggregate inside microcapsule. (C) Higher magnification of the fibrillar structure. (D) Higher magnification of the cell aggregate.

6.6. Summary and Discussion

Shear stress has been seen to associate with movement and morphological changes of hepatocytes in cell culture systems\(^\text{161}\). Multiple research have been conducted on the benefits of perfusion on preserving hepatocyte metabolic activity in long-term cultures. Nonetheless, most of these researches have cited the downside effect of perfusion which is inputting high shear stress on the cells\(^\text{86,150,162}\). In this chapter, the effect of perfusion and co-encapsulation of hepatocytes with MSCs, on hepatocyte metabolic activity in long-term cultures was investigated. It was observed that both perfusion and co-culture were in favor of preserving the hepatocytes metabolic function. Capsules cultured under perfusion conditions, either with or without MSCs, revealed higher albumin synthesis rate after 28 days of culture compared to static
conditions. It is believed that perfusion cultures results in higher oxygen and nutrient transfer, especially growth factors such as HGF which are vital to the hepatocytes viability\textsuperscript{137}. In most of the perfusion cultures studied before, cells are prone to high shear stress which damages hepatocytes membrane\textsuperscript{150,163,164}. In our study, capsule membrane protects the cells from the shear stress of the perfusion culture.

Moreover, it was observed that co-culture of MSCs and hepatocytes, preserves the metabolic activity of hepatocytes over the 28 days of the study. It is believed that MSCs preserve hepatocyte activity by secreting ECM proteins such as collagen type I, repairing the gap junctions between the hepatocytes such as connexin-32 and finally by secretion of cytokines such as TGF-\textbeta1 which supports hepatocytes proliferation and function\textsuperscript{142}. We observed that the albumin and urea synthesis rate at day 28 of the culture were 1.8 and 2.5 times higher than hepatocyte monoculture, respectively. Same trend was also observed in the collagen sandwich cultures as the co-culture system showed 4.6 times and 2 times higher albumin and urea synthesis rate compared to the monoculture, respectively. Apart from the advantages of the MSC regarding preserving the hepatocytes metabolic activity, there remains one big challenge which is the fast growth of MSCs inside microcapsules. This fast pace causes the domination of MSCs to hepatocytes over nutrient and oxygen consumption which is believed to be the reason behind the sudden decline of the albumin and urea synthesis rate at day 20 of the study. Although the ratio of the Hep:MSC is \textasciidetilde 2:1 inside the microcapsules, soon after 3 days of culture, the number of MSCs inside the microcapsules dominate the hepatocyte number. This domination leads to lack of nutrient and oxygen available for the hepatocytes. Even though the fast proliferation of MSCs has drawback on hepatocytes, it is at the same time advantageous as it will secrete more ECM proteins and cytokines which is believed to be the reason behind the rebounding of the albumin synthesis rate at day 24. Furthermore, MSCs does not have any fenestrae on their membrane which limits the diffusion and oxygen and nutrients into the cell membrane\textsuperscript{151}. This might be another reason for sudden decline of albumin and urea at certain time points. One way to overcome the pitfalls of MSCs is to use a more specialized organ cell type such as liver sinusoid endothelial cells (LSECs). It has been seen that LSECs are the highest fenestrated endothelial cells inside the body which facilitate
nutrient access to hepatocytes inside the liver sinusoid. Moreover, LSECs have been shown to react to shear stress in a productive way by expressing Kruppel-like factor 2 (KLF2) which mediates the effect of shear stress on hepatocytes. At the same time liver is also a highly vascularized organ with a special sinusoidal cell called liver sinusoidal endothelial cells (LSECs). In the next section of this chapter, in investigate the feasibility of formation a vascular network inside the microcapsules, sheep VECs were seeded alongside hepatocytes and MSCs inside the microcapsule at the specific ratio. Same set of metabolic activity tests were accomplished on these microcapsules explore the effect of VECs on hepatocytes. IHC staining of VECs using PECAM revealed the localization of VECs inside the microcapsules. It was observed that VECs were mostly arrange inside the capsule membrane in addition to surroundings and inside the cell aggregates. This phenomenon was of great importance as a proof of concept in formation of avascular network inside the microcapsule, facilitating the nutrient and oxygen transport into the hepatocytes. Accordingly, it was observed that the albumin and urea synthesis rate in microcapsules containing VECs was slightly higher compared to those lacking VECs which is a sign of higher oxygen and nutrient transfer. Furthermore, CYP1A1 enzyme activity as a major drug metabolizing enzyme was assessed in the microcapsules containing VECs and those without VECs and it was observed that the presence of VECs does not have a significant effect on the enzyme activity. As cited by other researchers, expression of CYP1A1 in VECs is greatly affected by the amount of shear stress imposed on these cells. Since in this study, microcapsules membrane protects the cells from high shear stress exerted on cells due to perfusion, the CYP1A1 synthesized by VECs is negligible and is insignificant compared to the CYP1A1 synthesized by hepatocytes.

Overall, we showed that both perfusion and MSCs will assist hepatocytes to regain their metabolic functionality during the 28 days of the study by easing the nutrient diffusion into the cells, production of ECM proteins and repairing the cell-cell and cell-ECM interactions. Additionally, adding VECs to the microcapsules further improved hepatocytes metabolic activity in addition to formation of tiny vascular structures inside microcapsules.
CHAPTER 7

FABRICATION OF AN IN-VITRO BILE DUCT

7.1. Introduction

Bile duct is an integral part of liver which is responsible for bile collection, modification and transfer\textsuperscript{166}. Biliary disorders such as biliary atresia and ischemic cholangiopathy are among the lethal disease which require complex treatments\textsuperscript{59}. Despite all the advances in the field of regenerative medicine, there is still a high demand for a tissue model which can mimic the bile duct physiology and structure in detail. Tissue engineered bile ducts can be used for multiple applications including the replacement of the extrahepatic bile duct as a treatment for biliary atresia and to study bile duct disease. In this chapter, a novel method for fabrication of tissue engineered bile duct (hollow fibers) has been explained in detail and the effect of multiples biomaterials including chitosan, collagen, heparin and chondroitin sulfate B (CSB) on structure and functionality of the hollow fibers was investigated. Cholangiocytes which are the epithelial cells of the bile duct were also isolated from rat and were seeded inside the hollow fibers to achieve a cell laden tissue engineered bile duct. Cell function inside the hollow fibers was analyzed and their transport properties were studied in detail. By finding the optimized hollow fibers material and maximizing the cell growth, we will be one step closer to the fabrication of the liver-on-chip system.

7.2. Aim and Rationale

The primary specific aim of this chapter is to fabricate hollow fibers internally seeded with cholangiocytes to mimic the liver bile duct. A unique fabrication method will be used to generate hollow fibers with uniform shape and structure. The properties of the hollow fibers including shape and mechanical properties will be analyzed in detail based on different biomaterials used for fabrication of the hollow fibers. This specific aim is of great importance as the tissue engineered bile ducts should be easy to fabricate, handle and have sufficient mechanical strength to be used for regenerative medicine applications. The second aim of this chapter is to successfully isolate cholangiocytes from rat liver and seed them into the lumen of the hollow fibers fabricated previously. Isolation of the primary cholangiocytes requires a specific
protocol and complex culture conditions as these cells only make up 2-3% of the total liver cells\textsuperscript{167}. Cell attachment and cell tight-junction formation inside the hollow fibers is another factor which should be considered in designing a tissue engineered bile duct. Therefore, the diffusion of FITC-dextran out of the seeded hollow fibers was analyzed to see if the fabricated bile duct recapitulates the barrier function of the native bile duct. Furthermore, the modifying properties of the tissue engineered bile duct was analyzed based on the absorption of the two primary components of the bile duct namely, taurocholic acid (TCA) and glycochenodeoxycholic acid (GCDCA). This specific aim is also of great importance as it will presents the activity of the cholangiocytes seeded on the hollow fibers.

7.3. Experimental Approach

**Study 1:** Fabrication and characterization of hollow fibers.

A novel dip coating method was used to generate hollow fibers with a uniform structure. Hollow fibers were made with different biomaterials including chitosan, collagen, chondroitin sulfate B (CSB) and heparin. CSB and heparin were crosslinked onto the chitosan surface and the crosslinking efficiency was evaluated. Mechanical properties of these fibers were also investigated in detail.

**Study 2:** Isolation, culture and seeding of the cholangiocytes into the lumen of the hollow fibers.

Cholnagiocytes were isolated from rat liver, cultured inside a collagen gel and after reaching confluency they were removed and seeded into the lumen of the hollow fibers fabricated previously. Isolated cells were characterize using multiple assays to assure the presence of the cholangiocytes. After seeding cells inside the lumen of the hollow fibers, their attachment and growth on the four types of hollow fibers was investigated and the most efficient hollow fiber with this regard was chosen for building the liver-on-chip system.
7.4. Materials and Methods

7.4.1. Isolation and Culture of Primary Intrahepatic Cholangiocytes

Isolation was done using the established protocols. Briefly, 6month old SD rats weighing 200-250g were anesthetized using isoflurane (2%). Abdomen was then incised, and the portal vein was exposed. Liver was then perfused through portal vein with 250ml of HBSS solution containing 0.5wt% BSA and 0.1g Na\textsubscript{2} EDTA followed by prefusion with 250ml of HBSS containing 0.05wt% collagenase type IV and 0.01wt% CaCl\textsubscript{2} at constant flow rate of 25ml/min. Liver was then shaken gently inside 37°C enzymatic solution to remove all the remaining hepatocytes. After assuring the removal of all hepatocytes from the liver, microscopic sized pieces of the intrahepatic bile duct were cut using scalpel and washed 5 times using HBSS solution at 4°C to remove all the remaining enzymatic solution inside the tissue. The bile ducts were cultured inside a thick collagen layer (2mg/ml) which was prepared as discussed elsewhere. Complete cholangiocytes medium containing HDMEM/F12(1:1), 5%FBS, 1% MEM non-essential AA, 1% ITS, 1% Na-pyruvate, 1% Chemically defined lipids, 1% Pen-strep, 0.04% gentamicin, 1% MEM vitamin solution, 1% soybean trypsin inhibitor, 1% L-glutamine, 0.1% dexamethasone, 0.1% 3,3',5-triiodo-L-thyronine, 10ng/ml EGF, 10ng/ml HGF was then added to the top of the gelled collagen. After 2 weeks of culture, hollow 3D cysts were observed forming inside the collagen gel with cholangiocytes branching from the side of the bile ducts. Subsequently, cells were subcultured after four weeks by first digesting the collagen with complete medium containing 0.05wt% collagenase IV for 30min. Biliary tree fragments alongside the cysts were then transferred to a 50ml conical tube and cysts were digested using 0.25% trypsin solution for 20min at 37°C. Trypsin neutralizing solution was then added and the solution was passed through a 70µm cell strainer to remove all the remaining bile ducts. In addition to the cholangiocyte culture inside the collagen, isolated cholangiocytes were cultured inside the Matrigel\textsuperscript{©} according to the manufacturer’s using a 48 well culture plate. Cultured cells were then isolated using Corning\textsuperscript{©} Dispase solution according to established protocols. Cells grown inside collagen and Matrigel\textsuperscript{©}
were then compared based on their growth and proliferation in each condition. Cells grown under most optimized condition were then seeded into the lumen of the hollow fibers previously made.

**7.4.2. Culture of Isolated Bile Ducts Inside Matrigel**

Matrigel is a well-known basement membrane extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma which is widely used in 3D cell culture systems\(^{171}\). To culture cells inside Matrigel, Matrigel was taken out of -20°C freezer and transferred to 4°C fridge the night before start of the culture. Culture plates and pipettes were all kept at 4°C before using to prevent gelation of Matrigel. 200µl of Matrigel was added per square centimeter of culture palate under cold (4°C) conditions and the microscopic sections of bile duct was then added to the Matrigel and culture plates were then transferred to incubator for 30min. Cholangiocytes culture medium was then added to the top of the Matrigel with a daily change of medium. Cells were grown for 2 weeks inside Matrigel\(^{®}\) with a medium change of every 3 days. Matrigel\(^{®}\) was then digested using the Corning\(^{®}\) dispase solution according to the manufacturer’s instructions. Isolated cells from Matrigel\(^{®}\) were then seeded inside the HFs.

**7.4.3. Characterization of Isolated Cholangiocytes**

Native cholangiocytes possess both secretory and adsorption properties. Therefore, one way to verify the presence of cholangiocytes is to analyze the secretion and adsorption potency of the isolated cells. To assess the secretion and absorption properties of cultured cholangiocytes, assay for transport of Rhodamine 123 was conducted. Rhodamine 123 is a fluorescent dye which can be transported into the cysts by multi drug resistant proteins (MDR1B). This transport can be obstructed by the verapamil which is the MDR1B inhibitor. Briefly, plates containing the cysts grown after 28 days were cultured in phenol red free/serum free medium the night before staining. The medium was then removed, and cysts were incubated inside phenol red free/serum free medium containing 100µM of rhodamine 123 for 5min followed by washing with phenol red free/serum free medium for three times. The cysts were imaged using and fluorescence microscope every 2min for total of 30min and change in the fluorescence intensity inside and
outside of the cysts were measured. The fluorescence inside the cyst was then normalized to the background fluorescence. Each experiment was repeated 4 times. Additionally, expression of major epithelial markers including cytokeratin 7 (CK7) and cytokeratin 19 (CK19) was also analyzed inside the isolated cysts using antibody staining.

### 7.4.4. Fabrication of Hollow Fibers Using Different Biomaterials

A complete schematic of the hollow fiber fabrication process is illustrated in fig. 7.1. Fibers were all made using 3wt% chitosan solution as the base material. In brief, 500µm in thickness and 7cm in length filaments were made using a 3D pen. PLA was used as the printing material in all experiments. The filament was then immersed inside the chitosan solution for 5sec followed by dipping into 28% ammonium hydroxide solution and vertical drying for 3min and 30min, respectively. This process was repeated three more times to increase the wall thickness and achieve sturdier hollow fibers structure. After the final dip into the ammonium hydroxide solution, hollow fibers were dried overnight followed by a dipping into ammonium hydroxide solution for another 2hr to complete neutralization. To remove the PLA filament, the hollow fibers were immersed in acidic PBS (pH=6.5) for 20min. By removing the PLA filament, a uniform hollow fiber will be left. To generate chitosan-collagen hollow fiber, a mixture of 1:1 of chitosan and collagen was prepared by adding equal volumes of 2mg/ml collagen type I solution to a 6wt% chitosan solution. This solution will have a final concentration of 3wt% chitosan and 1mg/ml collagen which will be used for generating hollow fibers as explained earlier. To generate chitosan-GAG hollow fibers, 1wt% heparin and 1wt% CSB were first prepared by dissolving in PBS. EDC was used as a crosslinker between chitosan and GAGs as is explained in fig. 7.2. EDC crosslinker was prepared at a concentration in which the molar ratio of EDC to GAG would be 10:1 (10 time extra)\(^\text{172}\). Each type of GAG and EDC were mixed in equal volumes for 15min on an orbital shaker. This activated GAG solution was then perfused into the lumen of the hollow fibers previously made using the 3wt% chitosan solution for 24 hr using a peristaltic pump at room temperature (fig. 7.3). Crosslinked HFs were then washed three times with PBS, sterilized for 12hrs in 70% ethanol and were stored in sterile PBS for further use.
**Fig. 7.1.** Schematic of the hollow fiber (HF) fabrication using the dip coating method. (A): making the PLA filaments using a 3D pen. (B): dipping the PLA filaments into 3% chitosan and 3% chitosan+Collagen solutions separately. (C): chitosan gelation by dipping into the ammonia solution. (D): removal of the PLA filament by immersing into acidic PBS solution. (E): storage of HF in PBS for further analysis. (F), (G): easy handling and flexibility of the HFs make them convenient tool in TE applications.

**Fig. 7.2.** Schematic of carbodiimide crosslinking reaction. Reaction between carboxylic acid groups of Heparin or CSB with EDC and formation of active ester intermediate which is displaced by primary amine groups. The amine groups of chitosan bonds to the original carboxylic group and urea is produced as the by-product.
Fig. 7.3. Illustration of GAG immobilization and cell seeding onto chitosan HF. (a) heparin and CSB were immobilized onto chitosan fibers using a perfusion system running for 24hrs. (b) fabricated HFs were seeded internally with cultured cholangiocytes. (c) a schematic of the seeded cholangiocytes into the lumen of the HF and GAG immobilized onto the chitosan surface.

7.4.5. Analysis of Hollow Fibers Structure

To verify the immobilization of GAG onto the chitosan surface, two methods including dimethyl methylene blue (DMMB) assay and GAG staining were used. For histology staining of immobilized heparin and CSB on hollow fibers, safranin O and toluidine blue staining were used, respectively. In brief, immobilized HFs were fixed in 10% neutral buffered formalin overnight followed by dehydration in ethanol series, clearing in xylene and finally embedding in paraffin. Embedded hollow fibers in paraffin were then sectioned to 7µm thickness using microtome and were mounted on charged slides. 0.1wt% safranin O and 0.04wt% toluidine blue solutions were used for staining the immobilized heparin and CSB, respectively. To quantify the amount of GAG immobilized onto chitosan HFs, GAG content inside the perfusion medium before and after perfusion was measured and the difference was reported as the immobilization percentage.
In brief, 1ml of the crosslinking solution was collected before and after perfusion and was mixed with 1ml of papain solution (300µg/ml). The resulting solution was incubated at 60°C overnight to digest the interfering proteins. Afterward, 100µL of the digested sample was mixed with 200µL of the DMMB reagent followed by reading the absorbance at 525nm using SpectraMax250 instrument. Chondroitin sulfate A(CSA) was used to generate the standard curve representing the amount of GAG in the sample.

To investigate the mechanical properties of the HFs, they were first rehydrated in PBS for 6hrs followed by testing the fibers under strain using MTS Bionix 100(MN, USA) testing device with a constant gauge length of 15mm at constant strain rate of 2mm/min. Modulus of elasticity, ultimate tensile strength (UTS) and maximum strain for each type of HFs made previously were determined using the stress vs strain graph. Data represents mean ±SD from seven separate samples (n=7).

7.4.6. Seeding Cholangiocytes Inside Hollow Fibers

Prior to seeding cholangiocytes, sterile fibers with a 2cm length were washed three times with PBS and then soaked in complete medium for 60min. One end of the hollow fiber was blocked by adding a droplet of collagen solution and letting it gel at 37°C for 30min. Cholangiocytes in complete medium at concentration of 0.4 million cells/ml medium were then infused into each fiber followed by blocking the other end with the collagen. The blocking is critical as it prevents the cell movement out of fiber prior to complete cell attachment. The fibers were transferred to separate culture plates and were rotated to cover the whole 360-degree angle, every 30min for total of 4hrs to ensure uniform cell distribution inside the lumen. Hollow fibers containing cells were then cultured for 24hr followed by removal of collagen from the ends using a 22G needle. Fibers were again transferred to incubator and were cultured inside the cholangiocytes medium for 2 weeks with a medium change of every 3 days to obtain a uniform cell monolayer inside the HFs.
7.4.7. Characterization of Seeded Cholangiocytes

Barrier function of cholangiocytes was analyzed using FITC-dextran (MW=40kDa) at a concentration of 20µg/ml in PBS. Briefly the fluorescence solution was perfused into the seeded and unseeded hollow fibers at a constant flow rate of 1ml/min for 14min in the perfusion chip illustrated in fig. 7.4. Fluorescence intensity of the medium collected inside the dish was measured every 2min at Ex/Em =490/520nm. Since FITC-dextran cannot be transported by epithelial cells but at the same time can diffuse out of the hollow fibers, the absence of FITC-dextran in the collection medium after 14min represents the successful barrier function of seeded cholangiocytes inside the hollow fibers. Another key property of cholangiocytes is their transport function. In this study, the transport of taurocholic acid (TCA) in presence and in absence of glycochenodeoxyxholic acid (GCDA) was investigated (GCDA is known as TCA blocker). Briefly, TCA at a final concentration of 7µM was added to the HBSS solution which was then perfused into the lumen of the HFs for 2hr at constant flowrate of 1ml/min. The perfusate at the exit of the hollow fibers was collected every 10min and the TCA concentration was measured using the total bile acid kit (Cell Biolabs, MET5005, San Diego, CA) according to the manufacturer protocols. Concentration of TCA at the same time points in the inlet solution was also measured. To investigate the effect of GCDA on inhibition of TCA, GCDA at final concentration of 7µM was added to HBSS solution containing 4µM of TCA which was then perfused into the lumen of the hollow fibers at constant flowrate of 1ml/min. Identical procedure was done for measurement of concentrations as mentioned before. It should be noted that in all above tests, the perfusate does not circulate inside the HFs and it is a one-way direction flow.
Fig. 7.4. Perfusing the lumen of the HFs in a 35mm culture dish. The advantage of this perfusion system is the ease of fabrication, simple handling, cost effective and ability to image under microscope. The HF is attached to the end of two needles and based on different experiment it can be perused with a variety of reagents for different assays.

7.5. Results

7.5.1. Fabricated Hollow Fibers Represent Uniformity, Integrity and High Mechanical Strength

The dip coating method used in this study represented high potency in rapid and uniform fabrication of HFs which are essential for regenerative medicine applications. As can be seen from the SEM images (fig. 7.5), hollow fibers represent smooth surface both on outside and inside. These smooth surfaces can later be modified with different techniques to generate grooved surfaces for better cell attachment and growth depending on the application\textsuperscript{74}. Another advantage of the dip coating method is the ease in adjustment of hollow fibers dimensions including length and inner diameter by adjusting the length and diameter of the PLA template. Moreover, the wall thickness of the hollow fibers can also be adjusted by number of coatings on the PLA template. Fig. 7.5E represents the wall thickness of the hollow fibers used in this study. As can be seen, crosslinked HFs have thicker walls which is due to the formation of a GAG layer on the surface of the chitosan. Chitosan-collagen fibers on the other hand represented thinner walls which is due to the shrinkage of chitosan after binding to the collagen\textsuperscript{175}. We also observed a successful
immobilization of GAG on the chitosan surface as can be seen from the safranin O staining of the heparin layer and toluidine blue staining of the CSB layer in figure 7.6. To verify the histology results, quantitative analysis of immobilized GAG was done using the DMMB assay which showed 83.4% and 79.8% GAG immobilization for heparin and CSB, respectively (fig. 7.6G).

**Fig. 7.5.** SEM images of the fibers without cells showing uniform structure formed using the dip-coating method. (A): the whole structure of the HF. (B): chitosan membrane. (C): chitosan + collagen membrane. (D): chitosan + heparin membrane showing thicker and denser membrane. (E): chitosan + CSB membrane. Scale bar in magnified images is 10µm. (F) wall thickness comparison of the HFs fabricated in the study. Data are ±SD of 7 different measurements (n=7). (Chi=chitosan, Col=collagen, Hep=heparin, CSB=chondroitin sulfate B). (*P<0.05, **P<0.005, ***P<0.0005), ANOVA test.
Fig. 7.6. Histology of the hollow fibers fabricated using the dip-coating method. (A): staining of the chitosan fiber with Eosin-Y stain. (B): GAG staining of the chitosan+heparin hollow fiber using the safranin-O stain. Arrows show the thin GAG layer immobilized on chitosan surface with the magnified rectangle area depicted in (C). (D): chitosan+collagen hollow fiber stained with Eosin-Y dye. (E): chitosan+CSB hollow fiber stained with toluidine blue-fast green stain reveal the GAG deposition on chitosan surface with magnified section shown in (F). (G): quantification of GAG immobilized on each surface using the DMMB assay showing high percentage of GAG initially used was immobilized onto chitosan surface.

In general, fibers made of chitosan are well studied in multiple literature regarding their mechanical properties[39,176,177]. In this research, for the first time, the effect of adding collagen and immobilization of two different GAGs on the surface of chitosan HFs were investigate. HFs made with 3% chitosan solution revealed high UTS of 6 MPa with a Young Modulus of 43 MPa and strain of 0.14 (Fig. 7.7). In all samples it was observed that stress would increase linearly with increasing the strain. By addition of 50% collagen
into the chitosan solution, the UTS and the Young modulus increased by 17% and 13% meaning that the chitosan-collagen HFs are both stronger and stiffer. It is believed that the mechanical properties of HFs are greatly affected by how packed and tight the membrane structure is. By addition of collagen into the chitosan at 50% final concentration, a complex will be formed between amine groups of chitosan and carboxyl groups of collagen with a high binding degree\textsuperscript{178,179}. Additionally it has been reported that a hydrogen bond is formed between the chitosan and collagen molecules which explains the increase in the mechanical properties of these type of HFs\textsuperscript{175}. Furthermore, it has been seen that different ratios of chitosan to collagen will also affect their mechanical properties\textsuperscript{180}. Crosslinking of the chitosan HFs with heparin and CSB decreased their mechanical strength (fig. 7.7C) by 30% and 13% respectively compared to chitosan HFs. This decrease in UTS was accompanied by 70% and 30% increase in stiffness of the heparin and CSB crosslinked HFs, respectively, compared to chitosan HFs. One reason affecting the decrease in the strength of the crosslinked HFs is due to increase in wall thickness. Besides, the increase in stiffness of the crosslinked HFs is chiefly due to the formation of strong amide bond between the GAGs and chitosan after the crosslinking.
**Fig. 7.7.** Mechanical properties of HFs made using the dip coating method. (A): stress-strain curve for the four type of HFs studied. (B) Comparison of modulus of elasticity. (C) comparison of the ultimate tensile strength. (D) Comparison of the maximum strain for the four type of the HFs used in the study. Data are ±SD of 7 separate measurement (n=7). Legend colors same everywhere. (*P<0.05, **P<0.005, ***P<0.0005), ANOVA test.

7.5.2. Epithelial Morphology, Cyst Formation and Transport Function of Cholangiocytes

Since cholangiocytes form only 2-3% of liver cells, it is of crucial importance to characterize these cells prior to their application in HFs. Cholangiocytes form a continuous cell monolayer when cultured in 2D cultures with a cobble stone structure. Their proliferation in 3D culture also results in the formation of hollow cysts which is their primary signature. In this study, we observed the formation of cobble stone cell monolayer on the collagen surface after two weeks of culture (fig. 7.8). The size of these monolayer increased over the 28 days of culture.

**Fig. 7.8.** Cobblestone, monolayer structure formation of cholangiocytes after 14 days of culture inside the collagen coated plates. Note the size of the cobblestone-like structure increases from day 14 to 21 (A &B) and reached the maximum after 4 weeks of culture (C). Scale bar everywhere is 50µm.

Cysts are the primary morphogenesis of bile ducts which represents the same polarity and transport function as native cholangiocytes in bile duct. In this study, cyst formation inside 3D collagen culture was observed after two weeks (fig. 7.9). It was also observed that the size of the cysts would increase by 50% from day 14 to day 28 which represents the viability and proliferation of cells forming the cysts (fig. 7.9D).
Fig. 7.9. Cyst growth from the side of the isolated microscopic bile ducts. (A) after 7 days of culture cysts have thin membrane which is not completely formed. (B) after 14 days the size of the cyst formed increases and the membrane become thicker which is a sign of cell growth. (C) cyst acquires maximum size in the last day of the culture in addition to the formation of a thicker membrane. (D, E, F) cyst formation and growth after 7 days of culture inside Matrigel® represents the earlier cyst formation compared to cells grown inside collagen gel. (G, H) quantitative measurements of cyst diameter at different time intervals for the cells grown in collagen and Matrigel® (n=4).

Transport function of cysts was analyzed using rhodamine 123 fluorescent dye which can be transported by MDR1B protein. Cholangiocytes which are in the membrane of the cysts, uptake rhodamine 123 and will pump it into the cysts. It was observed that cysts were able to uptake rhodamine 123 and transport into the cyst as can be seen from fig. 7.10A. Presence of verapamil in the medium, which is an inhibitor of the MDR1B protein, resulted in the absence of fluorescence dye inside the cysts (fig. 7.10B).
To quantify these results, fluorescent intensity inside the cysts was measured and was observed that in the presence of verapamil the intensity decreases from 98% to 18% (fig. 7.10C). These results together, confirm the transport function of the formed cysts in 3D culture. To further verify the morphology of the cysts, IF staining was done using two primary epithelial cell markers including CK7 and CK19. As can be seen from fig. 7.11, cysts were able the express these two epithelial markers inside the 3D culture. Together, these results verify the morphology, polarity, and transport properties of the isolated cells as the epithelial cells of the liver which will be used later in seeding the HFs.

**Fig. 7.10.** Functional characterization of cultured cholangiocytes. (A) Rhodamine 123 (Rho) which is a substrate for MDR1 is secreted into the lumen of the cysts. (B) This secretion is inhibited by the presence of verapamil (Ver) which is an inhibitor of MDR1 protein. (C) Quantification of the absorption of the Rho in (A) & (B) normalized against the background. Data are ±SD of three independent experiments.
**Fig. 7.11.** IF staining of the cysts formed inside the thick collagen layer. (A), (B): Bright field and fluorescence images of the cysts formed after 28 days of culture using the CK7 antibody. (C), (D): Bright field and fluorescence staining of the cyst using the CK19 antibody.

### 7.5.3. Cholangiocytes Demonstrate Attachment to the Hollow Fibers

Improving cell attachment on tissue engineered structures is of great importance in design of the *in-vitro* systems. Cell attachment inside the lumen of the fabricated HFs was analyzed by qualitative and quantitative assays including fluorescence staining and Hoechst, respectively. As can be seen from fig. 7.12, the highest number of cells attached to the lumen of the HF was observed in Chitosan crosslinked with heparin HFs. Accordingly, CSB, collagen and chitosan HFs were in the next order regarding the number of cells attached. DNA quantification using Hoechst verified the fluorescence staining as the highest DNA content was recorded in heparin HFs. Heparin is found in large amount inside liver, additionally they can seize or start the secretion of growth factors which are crucial for cell binding through heparin binding sites\(^\text{182}\). We believe these factors contribute to the higher cell attachment in heparin HFs.
CSB, another GAG found in the liver also facilitates cell-cell interaction and sequester proteins release which are required for activation of cell binding sites\textsuperscript{30}. Collagen also presented higher cell attachment compared to chitosan which is mainly due to abundance of integrins present in the collagen\textsuperscript{183}.

\textbf{Fig. 7.12.} Cell attachment to the lumen of the four types of HFs fabricated in the study after 24hr. Cells stained with Calcein-Am (A) lowest cell attachment was observed in chitosan HF. (B) Collagen HFs (C) Heparin represented the highest number of cells attached. (D) CSB hollow fibers. Scale bar everywhere is 200µm. (E) Quantitative analysis of cell attachment using Hoechst 33258, as can be seen heparin HFs represented the highest number of cells attached. (\textdagger P<0.05, \textasteriskcentered P<0.005, \textasteriskcentered\textasteriskcentered P<0.0005), ANOVA test.
7.5.4. Seeded Cholangiocytes Barrier and Transport Function

Barrier function of seeded cholangiocytes inside the lumen of the HFs was analyzed using FITC-dextran (40kDa). FITC-dextran at concentration of 20µg/ml in PBS was perfused into the lumen of the HF for 14 min and the concentration of labeled dextran outside of the HF inside the dish was quantified using fluorometer. By analyzing the data (fig. 7.13), it was observed that in the HFs without cells, there is a sudden jump of FITC-dextran from 0 to 8 µg/ml in the first minute of perfusion. This concentration gradually increases till the 14 min until reaches the maximum value if approximately 10 µg/ml. On the contrary, HFs seeded with cholangiocytes, showed 2.5-fold less diffusion of FITC-dextran out of the HFs. Highest diffusion rate was also observed in the first minute of the perfusion which became plateau and constant around 3 µg/ml. These results demonstrated the successful barrier function of cholangiocytes inside the lumen of the HFs with lower diffusion of FITC-dextran out of the lumen of the HFs seeded with cholangiocytes.

![Graph](image)

**Fig. 7.13.** Analyzing the barrier function of seeded cholangiocytes into the lumen of the HFs. Seeded cholangiocytes restrict the diffusion of FITC-dextran out of the HFs which suggests the successful barrier function of cholangiocytes inside the lumen.
One main role of cholangiocytes *in-vivo* is the transport of bile components from apical to the basal surface and vice versa. To study this phenomena in the seeded hollow fibers with cholangiocytes, taurocholic acid (TCA) at concentration of 7µM in HBSS was perfused throughout the HFs at constant flow rate of 1ml/min and the concentration of TCA at the exit of the HF was measured every 30min for total of 2hrs. To assess the TCA transport more in detail, glycochenodeoxycholic acid (GCDA) which is a known to inhibit the TCA uptake inside the bile duct, was added to the HBSS solution containing TCA at a final concentration of 4µM and were perfused in throughout another HF seeded with same number of cells 173. Analyzing the obtained results (fig. 7.14), revealed a sudden increase in the TCA concentration in the HF perfusing without GCDA in the first 30min which then reduces over the 2hr of the study. Regardless of sudden increase in the first 30min, it can be observed that the TCA concentration in the output is 2-fold lower compared to the input TCA concentration. We also observed that GCDA successfully inhibits the uptake of TCA by cells as can be seen from fig. 7.14, TCA concentration was observed to be 2-fold higher compared to the group without GCDA which reveals the less uptake of TCA by cholangiocytes. At 90min, the TCA concentration in collector reaches the maximum of 5µM which is almost close to the input TCA concentration. This shows that the seeded cholangiocytes, successfully represented the transport function of native cholangiocyte which is of crucial importance in characterizing the bile duct.
Fig. 7.14. Analyzing the transport function of seeded cholangiocytes inside the lumen of the HF's. Two separate HF's seeded with same number of cholangiocytes were used for this experiment. One perfused with 40µM TCA for 2hrs and the other perfused with 40µM TCA+ 70µM TCA for same period. As can be seen, HF's successfully transported the TCA from the apical to the basal of the bile duct as the concentration of TCA decreases over time. GCDA also inhibited the TCA uptake as can be seen from middle graph. Top graph represents the TCA concentration inside the inlet which is constant during the test.

7.6. Summary and Discussion

In this chapter, we demonstrated a practical, rapid, and versatile method for fabrication of HF's which can mimic the liver bile duct structure. These HF's can later be used as part of the liver-on chip system. Fabricated HF's represented uniformity and high mechanical strength which are of crucial importance for tissue engineering applications. Heparin and CSB were successfully immobilized on the surface of the chitosan HF's with 80% efficiency as seen both by histology results and DMMB, GAG quantification assay. Chitosan + Collagen HF's showed the highest UTS and strength compared to the other three type of HF's due to strong bonding between chitosan and collagen. Cholangiocytes were also isolated from rat liver and prior to seeding on the lumen of the HF's, they were characterized on both 2D and 3D culture systems. Cobblestone monolayer of cholangiocytes was observed after 14 days of culture on collagen surface. The size of the monolayer increases over the 28 days of the study which illustrates the retention of cell viability and functionality over the culture period. Cyst formation in 3D cultures which is another characteristic of cholangiocytes was also observed in 3D collagen culture after 14 days. Furthermore, the size of the cysts increased over the culture period from 190µm to 280µm which shows higher growth in size compared to other studies. Formed cysts also presented the transport function as was seen through transport of rhodamine 123 into the cyst from the medium. This transport was blocked by the presence of verapamil which is an inhibitor for MDR1 protein on the apical surface of cholangiocytes. CK7 and CK19 which are the two primary epithelial cell markers presented positive expression in the formed cysts. These results verified the presence of cholangiocytes in the culture plates which were then used for seeding into the lumen of the HF's.
Isolated cholangiocytes were seeded into the lumen of the HFs using an optimized technique to assure complete attachment of the cells into the HFs. Cell attachment to the HFs was investigated using Calcein-Am staining and DNA quantification method. Heparin HFs illustrated highest number of cells attached which is mainly due to the highest capability in activation of cell binding sites and induction of cell attachment\textsuperscript{182}. CSB, collagen and chitosan were in next places regarding cell attachment efficiency. According to the results obtained in this chapter, it was concluded that the HFs fabricated have a great potential to be used in liver-on-chip system and the chitosan crosslinked with heparin was the most optimized one regarding cell attachment and growth. To further characterize the seeded cholangiocyte inside the fibers, the barrier and transport function of seeded cholangiocytes was investigated. It was observed that the cells successfully presented the barrier function feature as the HF without cells passed most of the dextran through the membrane. Analysis of the transport function also illustrated the transport of TCA from the apical to the basolateral membrane which agrees with the native cholangiocyte function.

By the studies done in this chapter, we showed that the seeded cholangiocytes can function as the native cells in the bile duct and can be used as an \textit{in-vitro} bile duct inside the liver-on-chip system.
CHAPTER 8
ASSEMBLY OF THE BILE DUCT-ON-CHIP

8.1. Introduction

Drug induced liver injuries (DILI) are among the main reasons of drug removal from both clinical trials and market. Liver in-vitro models are one of the key components in testing new drugs and assessing their toxicity level. Liver is the main organ of target in DILI with one third of total drug withdrawals due to liver failure. Therefore, testing newly developed drugs on high throughput in-vitro systems which mimic the liver physiology is of great importance. Also, these in-vitro models can lessen the number of animals used in stage II of drug development phase to a great extent. Studying liver disease and liver physiology is another application of these systems. Up to now, most of these systems rely on 2D and 3D culture systems incorporating multiple liver cells including hepatocytes and liver sinusoidal endothelial cells under static and flow conditions. Apart from all the progresses, the liver-on-chip models still do not mimic the liver bile duct structure in detail. These systems mostly lack hepatocytes which are the primary cells producing the bile. In this chapter, we propose an approach for assembly of liver bile duct and hepatocyte in a bioreactor, mimicking the physiology of the bile duct. To achieve this goal, the hollow fibers seeded with cholangiocytes and hepatocytes inside microcapsules will be used inside the bioreactor under perfusion culture. To the best of our knowledge, this is the first complete bile duct-on-chip system which employs both hepatocyte and cholangiocytes inside the system under perfusion conditions.

8.2. Aim and Rationale

The primary specific aim of this chapter is to assemble microcapsules containing hepatocytes and hollow fibers seeded with cholangiocytes together inside a perfusion bioreactor to mimic the liver bile duct. Building a liver-on-chip is of great importance due to its multiple applications including drug toxicity tests and studying liver. The focus of this chapter will be primarily to build a bile duct-on-chip. Capability of the
system in production, collection, and modification of the bile inside the bioreactor will be investigated in detail.

8.3. Experimental Approach

**Study 1**: Feasibility of assembling microcapsules and hollow fibers in a perfusion bioreactor

Hepatocyte microcapsule and hollow fibers seeded with cholangiocytes will be transferred to a perfusion bioreactor and the stability of the system under the perfusion culture will be investigated. This study is of great importance as is the first step in building a bile duct-on-chip system.

**Study 2**: Capability of the hollow fiber to collect bile was analyzed

The primary goal of the bioreactor is to demonstrate the capability of the system to collect and modify bile. Hence, the bile concentration at the exit of the hollow fiber and exit of the bioreactor was measured and compared. Any changes in the concentration of bile components was considered as the successful function of epithelial cells inside the hollow fiber.

8.4. Materials and Methods

8.4.1. Liver-on-chip Bioreactor

The bioreactor used for assembly of the hollow fiber and microcapsule is illustrated in fig. 8.1. Same procedure as described earlier in chapter 4 with a slight modification was used to make the bioreactor. The volume of the working area was about 500mm$^3$ which could embed a 5cm in length hollow fiber alongside 500 microcapsules inside the bioreactor.
Fig. 8.1. Fabrication of the liver-on-chip bioreactor. (a) 3D printed template was made using 3D printer. (b) Template was attached to glass slide (25mm×75mm). (c) PDMS solution was added followed by degassing and curing inside oven (70°C for 1hr). (d) PDMS removed from oven and glass slides attached to the top and bottom alongside the needles and tube. (Working volume of the bioreactor is 500mm³)

8.4.2. Microcapsules and Hollow Fiber Assembly Inside Bioreactor

All steps were done under sterile conditions to minimize the chance of contamination. In brief, after fabrication of the hollow fiber seeded with cholangiocytes as explained before, a 4cm section was cut and was put inside the bioreactor chamber and a 22G needle was attached to the output of the hollow fiber for bile collection. The bile duct output was then attached to a collection reservoir for bile collection. After securing the hollow fiber inside the bioreactor, microcapsules previously made containing hepatocytes, were seeded around the hollow fiber in a manner to completely fill the remaining gaps inside the bioreactor. Following the seeding of the microcapsules inside the bioreactor, the two inlets and one outlet of the bioreactor were connected to the tubes and tubes were connected to the pump setup illustrated in fig. 8.2.
Fig. 8.2. (A) Illustration of the bile duct-on-chip; the hollow fiber seeded with cholangiocytes displayed in orange was first transferred to the bioreactor followed by adding the hepatocytes microcapsules shown in red. Bioreactor has two inlets and two outlets. (B) Bioreactor was then attached to the peristaltic pump and the medium was circulated throughout the system over 7 days of the study.

8.4.3. Bile Synthesis and Collection Analysis from the Bioreactor

Bile synthesis and collection was analyzed during the culture period. To do so, after assembling the microcapsules and the hollow fiber inside the bioreactor, the flow of the perfusate was adjusted to 1ml/min and the output of the system at the outlets (fig. 8.2) was collected daily for total bile acid analysis (Cell Biolabs, San Diego, CA). Bile duct synthesis rate at these two points were compared with each other in which higher concentrations of bile acids at the exit of the bile represents the success of the bioreactor setup in collection of the bile duct. Moreover, the synthesis of the bile by the encapsulated hepatocytes inside the perfusion chip was also considered as the proper function of the system.
8.5. Results

8.5.1. Bile synthesis and collection from the system

After securing a 4cm of the HF bile structure and approximately 500 microcapsules, the top section of the chip was attached and the system was perfused initially for 1hr to test the stability of the system regarding the microcapsules and hollow fiber dislocation inside the system. It was observed that the microcapsules stayed stationary and were not exiting from the system during the 1hr of the study. The HF structure also was completely sealed around the 22G needle without any leakage and represented a stable and uniform structure during the 1hr of the study. With this observation the first objective of this chapter was accomplished which was to assess the stability of the system regarding the stability of the perfusion chip.

Following the stable function of the system, we started collecting medium samples from the outlet of the perfusion and outlet of the bile duct every 2hr for total of 10hr. Afterward, the total bile concentration was measured as explained before using the Cell Biolabs total bile acid kit. First, the flowrates at the exit of the chip and bile duct was measured and recorded as 1ml/min and 0.083ml/min, respectively, which is approximately 12 times lower than the exit flowrate of the system. This implies that the system is functioning properly, and the bile duct installed in the chip is capable of bile transport to the reservoir. By comparing the total bile concentration inside the chip with the bile collected from hollow fiber (fig. 8.3), it was observed that the perfusion had 2-fold higher bile concentration after 2hrs (0.553µM). The bile concentration collected from the HF increased during the first 8hrs of the study which represent the successful function of seeded cholangiocytes inside the HF. This value surpasses the bile concentration collected from the perfusion after 6hrs which is due to the lag time required for the cholangiocytes to adopt to the new microenvironment and culture conditions. It was also noted that the bile concentration collected from the perfusion was staying around the constant value of 0.55µM during the 10hr of the study which implies that hepatocytes are producing bile at a constant and stable flowrate which is partially collected from the perfusion system.
Fig. 8.3. Total bile concentration measured from the medium out and the bile of the system over the 10hr of the study.

The decline in the concentration of total bile collected from the bile duct from 8hr to 10hr can be due to multiple reasons including reduction in hepatocytes metabolic activity or lack in the proper function of cholangiocytes which requires further investigation.

8.6. Summary and Discussion

In this chapter, we presented a rapid and convenient method to fabricate a perfusion culture bioreactor for culturing the encapsulated hepatocytes and HF seeded with cholangiocytes. The HF seeded with cholangiotes represents the in-vivo bile duct and the encapsulated hepatocytes mimic the in-vivo hepatocytes lining the bile duct. The flow direction inside the system is opposite each other inside the HF and the perfusion culture which exactly mimics the in-vivo physiology of the bile duct. In the first step of the experiments we observed that the microcapsules and the HF could be assembled inside the prefusion with high degree of stability. Furthermore, it was seen that the bile could be synthesized by the hepatocytes and was also measured from the system. This implies the high degree of viability and functionality of hepatocytes in the system. We also observed that the initial bile concentration inside the perfusion was higher compared to the bile duct but after 6hrs of the culture, the bile concentration collected from the HF surpasses the perfusion culture which represents the proper adoption of cholangiocytes to the new
microenvironment and the stable function of the cells in bile collection and transport. We also further observed that this concentration decreased from 8hr to 10hr of the culture which is mostly due to the decline in metabolic activity of hepatocytes or cholangiocytes inside the system.

In conclusion, we showed that the bile duct structure can be mimicked using microcapsules containing hepatocytes and HF seeded with cholangiocytes inside the perfusion culture. Bile could be synthesized by the encapsulated hepatocytes and was measured from both perfusion culture and HF inside the system which implies the successful function of cholangiocytes and hepatocytes inside the system. These results prove the successful function of this perfusion model with an application in multiple liver systems including drug toxicity testing models and studying liver diseases outside of the body.
CHAPTER 9
CONCLUSION

Cell microencapsulation is a promising tool in tissue engineering to fabricate dense, multicellular, and functional tissue structures *in-vitro*. Meanwhile, the method used for cell encapsulation is also of crucial importance as it will affect cell viability and function during the process. In this study, a novel electrospaying (ES) method was proposed for cell encapsulation. MSCs encapsulated in GAG-chitosan microcapsules demonstrated above 90% viability 28 days post encapsulation. The ES method could generate microcapsules in the range of (200-500µm) diameter with high uniformity in short period of time. Since many parameters affect the microcapsule size and cell viability in this method, the ES technique was optimized in detail based on the three affecting parameters of voltage input, needle size and GAG composition. It was observed that microcapsules made using 12kV power input, 22G needle size and GAG composition of 1%HA+4%CSA, resulted in the most uniform microcapsules with highest degree of cell viability compared to all other conditions. Therefore, for the rest of the project these conditions were used to generate the microcapsules. To evaluate the effect of ES on MSCs multilineage capacity, encapsulated MSCs were cultured under adipogenic, osteogenic and chondrogenic culture to assess the differentiation to these three lineages. It was observed that MSCs could differentiate to adipocytes by staining with BODIY stain and differentiation to osteocytes was also confirmed by the mineralized microcapsules. Expression of adipogenic and osteogenic markers including FABP4, PPARG, OC and ALP further verified our qualitative data. Apart from these two lineages, differentiation of MSCs into chondrocytes has attracted a great attention due to the great potency of MSCs in treating articular cartilage damages including osteoarthritis. Hence, we did a comprehensive study on methods to accelerate and facilitate the chondrogenic differentiation of MSCs inside microcapsules. Since collagen is one of the main components of the cartilage, the effect of collagen presence inside microcapsules on MSCs differentiation to chondrocytes was investigated. It was observed that collagen increases cell growth, GAG deposition and collagen type II synthesis inside microcapsules compared to microcapsules lacking collagen. Nutrient and growth factors
transport (TGF-β1) to MSCs for their proper function is of crucial importance. In the next step, we analyzed the effect of culturing MSCs under perfusion conditions to see how it affects their differentiation efficiency. We observed that perfusion was in favor of chondrogenic differentiation as we noticed from both qualitative and quantitative data. There has been evidence by other researches that hypoxic condition accelerate the differentiation of MSCs into chondrocytes by upregulation of SOX9 gene. Hence, in the next phase we transferred the perfusion chip containing the encapsulated MSCs into a hypoxic incubator (5% O₂) and we cultured them in the chondrogenic culture for 28 days. It was observed that combining the perfusion and hypoxic conditions results in the highest amount of GAG and collagen type II deposition inside the microcapsules compared to all other conditions. These results were further verified by observing the higher expression of chondrogenic markers including SOX9, COL2A1 and Aggrecan inside these microcapsules. These results together, illustrated the great strength of microcapsules in providing a growth microenvironment for the encapsulated cells. By employing these microcapsules in different culture conditions one can generate 3D tissue structures in-vitro which can later be implanted in-vivo.

Using the ES technique to encapsule hepatocytes was also investigated in the next phase of the study. Since the liver is a highly complex, vascularized organ with multiple cell types present at the same time, any in-vitro model which is used to mimic liver should also address these characteristics. Here, to preserve and maintain hepatocyte metabolic activity and to form a vascular structure inside the microcapsule, we encapsulated hepatocytes alongside with MSCs and VECs. This illustrated another key characteristic of the microcapsules in making a multicellular structure. Hepatocyte presented higher metabolic activity expressing higher amounts of albumin and urea when co-encapsulated with MSCs and VECs inside the microcapsules. VECs were able to form a vascular network around and inside the cell aggregate inside the microcapsules as was observed through the PECAM staining. On the other hand, presence of VECs inside the microcapsule did not affect the CYP1A1 enzyme activity compared to the capsules without VECs. These results demonstrated the successful encapsulation three cell type at the same
time inside microcapsules forming a dense liver tissue structure in-vitro which can be used inside liver-on-chip models for drug toxicity tests or studying liver diseases.

Hollow fibers, as the next component of our modular construct were fabricated using the dip coating method. Chitosan was used as the base material of the hollow fiber and two different types of GAG including heparin and chondroitin sulfate B were immobilized on the chitosan surface to analyze their effect on cell attachment and growth. These fibers demonstrated high mechanical properties and at the same time high elasticity which are crucial for their in-vivo implantation. Isolated cholangiocytes from rat liver were then seeded into the lumen of these hollow fibers and was observed that the hollow fibers coated with heparin showed higher cell attachment and growth compared to all other conditions. This is mainly due to the activation of GAG receptors on the surface of cholangiocytes by heparin resulting in stiffer bindings. It was also observed that these hollow fibers are capable of bile modification and possess the barrier function which are two main characteristics of the native cholangiocytes inside the liver. At last, the hepatocytes microcapsules and the seeded hollow fiber with cholangiocytes were assembled inside a liver-on-chip model and the potential of the system to collect bile under perfusion conditions was analyzed.

We believe the methods, techniques and designs proposed in this research are novel, efficient and can address multiple challenges currently present in the field of tissue engineering including formation of dense, multicellular, and functional tissue structures. The modular nature of our work permits the easy scale up of the system for fabrication of organs or tissues using 3D bioprinting methods or other assembly techniques. Although the 3D bioprinting field has a long road ahead in fabrication of a fully functional organ, we believe microcapsules are a promising starting point to build more complex tissue structures. Apart from the in-vivo application of the microcapsules for implantation, they are a great component to be used inside the in-vitro culture systems to study organs and different tissues as they can be assembled with hollow fiber structure to mimic more complex tissue structures.
CHAPTER 10

FUTURE WORK

This work demonstrated a detailed application of microcapsules in multiple regenerative applications including cartilage tissue engineering and liver tissue engineering. In the first part of the project we applied microfluidic techniques to generate GAG-chitosan microcapsules which was not completely successful as a high-throughput assay for generation of uniform and small (<200µm) microcapsules. Droplet microfluidic techniques have shown great potential in formation of w/o or o/w droplets in a short period of time. We believe, to be able to fabricate GAG-chitosan microcapsules using the microfluidic technique, more optimization for the fluid flow rates and the GAG and chitosan concentrations should be done to obtain uniform and small microcapsules. Utilizing a high throughput technique such as microfluidics will pave the road for the commercial application of microcapsules to be used at the point of care. The ES method introduced in this research for generation of GAG-chitosan microcapsules, demonstrated high yield and performance compared to the microfluidic method. The smallest microcapsule diameter we reached in this project was approximately 200µm. Since smaller diameter microcapsules are more efficient regarding oxygen and nutrient diffusion, one can utilize more optimizations in the ES method to reach these range of diameters. These methods include the application of ring electrodes and using other GAG concentrations. Following the successful encapsulation of MSCs using the ES technique, differentiation of MSCs to chondrocytes was analyzed in detail. One area that can be studied more in this section would be to analyze co-culture systems of MSCs and other progenitor cells such as SMCs capability inside the microcapsules to differentiate into chondrocytes. Effect of other growth factors such as TGF-β3 and other biomaterials such as gelatin are another parameters which can be analyzed more in depth. One other key parameter which should be investigated in detail is the assembly of the chondrogenic microcapsules together and formation of a vascular network between them. In-vivo test of structure and examination is another crucial section which is a comprehensive project for a group of graduate students.
Aggrecan is one of the key chondrogenic markers in which in this research was not studied in detail as one can study the expression and production of this protein in our study more in detail.

Regarding the application of microcapsules in encapsulation of hepatocytes, co-culture of hepatocytes with MSCs and VECs resulted in higher metabolic activity and cell growth and aggregation inside the microcapsules as we observed both from qualitative and quantitative results. Use of organ specific VECs is of great importance in the growth of tissue and cell function. In this research VECs from sheep aorta were used for the co-culture system as a proof of concept to assess the effect of VECs on hepatocytes. LSECs have shown a great potential to be used as the endothelial cell source in the hepatocyte cultures which improve cell function and preserves hepatocyte viability in long-term cultures\textsuperscript{190}. These cells can therefore be used in the future works instead of the sheep aorta endothelial cells. More work can also be done with the use of the encapsulated hepatocytes inside the liver on-chip device proposed here to analyze nonalcoholic fatty liver disease and drug induced liver injuries (DILI)\textsuperscript{191}. With this regard, the activity of more enzymes such as CYP3A4, CYP3A5 and, CYP2C9 as the major drug metabolism enzymes can also be assessed in detail \textsuperscript{192}. In addition to the application of the chip for drug toxicity applications, one can also assemble the microcapsules in a 3D form structure to be implanted and applied to the damaged liver, replacing the diseases and dead hepatocytes. Hence, the use of the microcapsules as a 3D bioprinting ink is another area of research which one can do requiring lot of optimization and analysis. Another section which we believe requires more in detail research and study in this project is a establishing an efficient method for isolation and culture of intrahepatic cholangiocytes. One of the major challenges in this project has been the low growth rate and isolation efficiency of the isolated intrahepatic cholangiocytes either in Matrigel or collagen. Use of optimized commercially available culture mediums and optimizing the culture conditions of the intrahepatic cholangiocytes may address these aforementioned issues\textsuperscript{167}. Uniform cell seeding inside the hollow fibers is another section which requires more work to be done to obtain a uniform cell distribution. This parameter is also of great importance as the hollow fibers should be able to replicate the bile duct structure accurately and precisely.
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ABSTRACT

DESIGN AND ASSEMBLY OF MICROCAPSULES AND HOLLOW FIBERS FOR IN-VITRO CULTURE SYSTEMS AND ORGAN-ON-CHIP SYSTEMS

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Every year thousands of people in U.S die due to lack of organ available for transplantation. The goal of tissue engineering is to fabricate and generate functional, multicellular tissue structures in-vitro, capable of mimicking the organ or tissue specific function. These tissue structures can later be transplanted in-vivo to replace the damaged organ and tissue or can be used inside the in-vitro culture and organ-on-chip systems to study the specific organ and organ related disease. The traditional approach in tissue engineering or the top down approach possess multiple drawbacks including lack of vascularization and inability to form large 3D tissue structures. On the other hand, modular tissue engineering approach can address these limitations by generating smaller modular components such as microcapsules and hollow fibers to be assembled to form a larger 3D tissue structure.

In this study, we investigate the design and fabrication of two type of tissue structures namely microcapsules and hollow fibers using efficient, high throughput techniques which make them great options to be used in the point of care. Electrospraying method was studies as a rapid and convenient technique to encapsulate mesenchymal stem cells (MSCs) and their differentiation into cartilage tissue was studied in detail. Microcapsule created a genuine microenvironment for MSCs growth and differentiation by providing the required ECM proteins such as collagen and proteoglycans for cells. We further demonstrated
that MSCs differentiation into chondrocytes could be accelerated by using a perfusion culture system and providing a low oxygen concentration for MSCs inside the microcapsules. This platform has the potential to be used as a cartilage tissue regeneration using MSCs as a cell source for treatment of osteoarthritis.

We also demonstrated the application of microcapsules in liver tissue engineering by creating a multicellular structure inside the microcapsules. In this study, hepatocytes alongside MSCs and vascular endothelial cells (VECs) were co-encapsulated inside the microcapsules to create a vascular and dense tissue mimicking the liver structure. Hepatocytes showed to preserve their metabolic activity up to 28 days of culture in the perfusion culture which can be used for drug toxicity tests and liver disease studies. VECs also formed a vascular network inside the microcapsules facilitating the transport of oxygen and nutrient into the hepatocytes. Aside from the application of hepatocytes microcapsule in building liver-on-chip models for drug toxicity tests, they also have the potential to replace the damaged part of the liver. Hollow fiber is another component of our modular structure which can mimic multiple tissues inside our body including vein, arteries, and bile ducts. In this study, hollow fibers were fabricated using a novel dip coating method and were seeded internally with liver epithelial cells to mimic the liver bile duct. These hollow fibers represented the bile modification characteristic same as the native bile ducts inside the liver which verified their successful function and activity in our in-vitro culture system. The artificial bile ducts fabricated in this study are the first step in building a fully functional bile duct to replace the damaged bile duct in patients suffering from biliary atresia and ischemic cholangiopathy. We further assembled our hepatocyte microcapsules with the artificial bile duct fabricated inside a liver-on-chip model to mimic the physiology and function of the native bile duct. To the best of our knowledge this is the first study done on mimicking the physiology of the bile duct using both the parenchymal and non-parenchymal liver cells. This research represents the promising potential of microcapsules generated using the electrospaying method and hollow fibers fabricated using the dip-coating method to create dense, multicellular 3D tissue structures to be used inside the in-vitro culture systems for organ related studies in addition to their application in generating dense 3D tissue to replace the damaged organ or tissue.
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