Chapter 1
INTRODUCTION

1 Introduction
Liver is a soft, pinkish-brown, triangular organ and is one of the largest internal organs in the human body with four lobes weighing about 1.4–1.6 kg. It is the key organ for regulating and maintaining the entire metabolism of the body and also carries out major biochemical functions [1]. The metabolic functions of the liver include detoxification, carbohydrate and lipid metabolisms, protein synthesis, erythropoiesis and production of biochemicals to aid digestion [2, 3]. Liver is made up of stellar cells, ito cells, Kupffer cells, sinusoidal endothelial cells of which hepatocytes occupy the major portion of about 75% [4]. As liver mainly constitutes hepatocytes, which are unipotent and possess greater replicative property, they hold immense promise for in vitro studies as they are expected to possess extremely short doubling times [5]. The secretory, metabolic and detoxifying mechanisms are all primarily carried out by the hepatocytes. Hepatocytes, the functional unit of liver have excellent replicative capacity and are capable of repopulating the liver incase of any injury [6].

Figure 1.1. Cellular architecture of the liver. (A) The schematic shows an adult liver (red), with the gall bladder and extra hepatic ducts (green), in relation to the stomach and intestine (yellow). The extra hepatic duct
system consists of the hepatic ducts (hd), which drain bile from the liver into the common hepatic duct (chd) to the gall bladder via the cystic duct (cd) and into the duodenum through the common bile duct (cbd).

(B) A schematic of the cellular architecture of the liver showing the hepatocytes (pink) arranged in hepatic plates separated by sinusoid spaces radiating around a central vein. Bile canaliculi on the surface of adjoining hepatocytes drain bile into the bile ducts (green), which run parallel to portal veins (blue) and hepatic arteries (red) to form the “portal triad”.

Any dysfunction in the liver leads to its failure, which in turn damages the cells involved in the metabolic activities [7]. Presently, liver transplantation is the leading clinical modality for treating patients suffering from critical liver complications. Transplantation is usually carried to replace the diseased liver with a cadaveric liver or a living donor graft and the survival rate is only 61% [8]. Moreover the shortage in the availability of donor liver and a long waiting list for the transplantation leads to the patient's death even before transplantation [9]. In split liver transplantation, the currently adopted procedure is that a part of the donor tissue is transplanted. However, this strategy is also have limited clinical implication because of the shortage of donor tissue and the highly expensive immunosuppressive treatment, making it unavailable to the patient majority [10]. On the other hand, cell transplantation, co-culture techniques and use of tissue engineered culture matrices promise an effective alternative to overcome the organ crisis but none of these are clinically proven as an enduring treatment for terminal stage liver failure [11]. A unique feature of liver is that it can perform its routine activities and can regenerate itself even after 70% damage arising due to numerous reasons [12]. Regeneration of liver occurs by DNA replication and mitosis through the action of different stimulators such as hepatocyte growth factor (HGF), epidermal growth factor (EGF), transforming growth factor-beta (TGF-β) and other potent stimulators of angiogenesis [13]. In the expansion and the subsequent proliferation phase, the hepatocyte population expands mainly due to HGF and Transforming Growth Factor-alpha (TGF-α). In vitro, the HGF secreted by the hepatocytes and supplementary HGF added in the media are both activated by proteolytic cleavage of urokinase-type plasminogen
activator (uPA), which is secreted in low concentrations by hepatocytes. STAT3 helps in further proliferation of the cells along with the activated HGF [14]. The cell proliferation is terminated in vitro as the concentration of HGF depletes in the media. Under in vivo conditions, the proliferation is terminated where TGF-β and activins seem to have a crucial role [15]. Research and development of extracorporeal bioartificial liver devices was thus emerged that provide necessary clinical support for the patients who are in advanced stage of liver diseases until an appropriate organ donor is available [16]. Approaches like hemodialysis, hemoperfusion, and plasma exchange using bioartificial liver devices are employed to improve patient survival time [17]. For development of bioartificial liver devices, ideal scaffold analogs have to be chosen as the cell loaded matrix. Scaffolds are temporary framework that mimics the extracellular matrix (ECM) to provide structural integrity of tissue and allow the cells to adhere, proliferate and migrate [18]. In addition, a three-dimensional matrix offers an environment that mimics the native tissue architecture and establishes the functional hepatocyte integrity [19]. Therefore, it is desirable to develop a scaffold that possesses similar structure and properties to match the ECM of the native tissues chosen to be cultured. Fibrin, a biopolymer naturally synthesized during the coagulation cascade is a less exploited natural material and is the body’s choice for the absorption and delivery of several factors. The use of fibrin as a biomimetic matrix protein is exploited in this research work, considering it as an ideal substrate for cell attachment, proliferation, extracellular matrix formation, eventual tissue regeneration, and can easily be degraded from the biological system. There are many techniques involved in the fabrication of 3D matrices with desirable properties for liver regeneration including solvent casting, phase separation, particulate leaching, freeze–thaw and electrospinning [20- 22] Degradable hydrogels have thus been used as vehicles for delivery of growth factors and for promoting regeneration of a damaged tissue [23]. In order to enhance activity of liver tissue cells (hepatocytes) seeded on a scaffold, one can activate them by interaction with the galactose-carrying synthetic or natural polymers [24]. Effect due to these interactions depended strongly on the geometry as well as on the density and orientation of the attached hepatocytes to PLGA, or to poly (acrylic acid), or to
alginate galactose are widely studied. Approaches involving the use of hepatocyte suspensions, liver microsomes or precision cut liver slices do not exhibit long-term stability since they do not mimic the \textit{in vivo} environment \cite{25}. Scaffolds such as nanofibers, films, and hydrogels that attempt to mimic the extracellular matrix topography have been studied extensively to maintain liver specific metabolic functions that can be employed for successful liver tissue regeneration \cite{26}.

\subsection*{1.1 Thesis Scope}
For developing an ideal bioengineered matrix for regeneration of complex tissues like liver, apart of having good biocompatibility and biodegradability, the matrices should also possess a 3D spatial architecture that promote cell growth and gene expressions related to liver-specific functions. The selection of polymer, fabrication technique, adhesion motifs and growth factors are essential for the cell attachment, proliferation, migration and differentiation of hepatocytes. For liver regeneration, the desirable topographical cues and porosity for nutrient transfer of the scaffold alone may not improve hepatocyte function. Similarly naturally derived polymers promote better hepatocyte adhesion than synthetic polymers, but fail to retain the liver-specific functions. Better adhesion of the cells to the scaffold is mediated by the addition of certain cell binding motifs like carbohydrate moiety and RGD. Carbohydrate modified scaffold minimizes the integrin signalling mechanism thereby controlling the de-differentiation. Cell proliferation after cell adhesion is mainly governed by the sustained delivery of growth factors. Hence, for the cells to regenerate, combinatorial factors like porosity, scaffold architecture, biomaterials, biocompatibility, biodegradability with non-toxic degradative products and controlled release profile provide necessary signalling cues to retain liver-specific functions.

Thus, 3D scaffolds embedded with protein nanoconstructs have drawn increasing interest in the biomedical arena owing to their additional ability to deliver growth factors at optimal doses, often resulting in increased efficacy of the released growth factors. Biodegradability, bioactivity, growth factor loading capacity as well as the controlled release kinetics of such chemokines from
protein nanoconstructs are of prime concern in processing them as novel nanocarriers in regenerative matrices for hepatic proliferation. Fibrin is one such natural bio-polymer, synthesized during the coagulation cascade and is also a less exploited research material particularly in its nano size scale as body’s choice of biomimetic agent for absorption and delivery of growth factors. Agarose is a natural biopolymer derived from red algae, which consists of a galactose-based backbone with alternating β-D-galactose and 3,6-anhydro-α-L-galactose units. Initial part of this research work explains the combinatorial strategy adopted for the synthesis of Agarose based cryogel matrix bed by incorporating growth factor loaded nano fibrin cell recognition moieties, its characterization and studies for proving the potential of using that cryogel matrix for inducing in vitro hepatic regeneration. Use of hepatocyte growth factor (HGF) loaded nano Fibrin (nF) moieties was thus experimented within such matrices for controlled proliferation of hepatocytes for prolonged durations. Since a major obstacle in liver regeneration studies is the limited availability of human cells, this research work also exploits the prospect of in vitro differentiation of umbilical cord blood derived human mesenchymal stem cells (hMSCs) to hepatocyte like cells (HLCs) and thus contributes an executable strategy to overcome liver tissue shortage. The practical inability of AnF cryogel matrix bed in maintaining prolonged hepatic functionalities owing to its weak mechanical properties experimented using a perfusion bioreactor leads us to develop a live cell loaded 3D hydrogel matrix bed for conducting prolonged perfusion experiments. A Live Cell based Bioartificial Liver Support System (LC-BALSS) was thus designed and developed in the final part of this research work by embedding metabolically active human hepatocytes inside alginate micro beads and positioned within a 3D hydrogel matrix bed framed of Agarose-nano Fibrin milieu. In order to study the prolonged response of embedded hepatocytes to the in situ liver fluid dynamics, the liver assisted system thus designed was positioned in a perfusion bioreactor system by giving a controlled fluid flow of cell culture media and body fluids (blood plasma and serum). Hepatocytes proliferated in this biological liver support system spontaneously formed functional cellular spheroids within the hydrogel matrices and retain their metabolic and detoxification activities for prolonged durations.
1.2 Specific objectives of this research work

1. Synthesis of biocompatible fibrin nanoconstructs through surfactant free technique and its methodical physicochemical characterization.
2. Evaluation of \textit{in vitro} & \textit{in vivo} efficacy of drug / growth factor loaded FNCs as controlled delivery agents.
3. Development and characterization of Agarose nano fibrin (AnF) cryogel matrix bed and evaluating its potential for slow and sustained release of growth factors from nano Fibrin (nF) moieties.
4. Design and development of a live cell based bioartificial liver support system for prolonged hepatic functionality maintenance.
5. Standardization of \textit{in situ} hepatic simulation conditions in a perfusion bioreactor followed by the extensive metabolic and detoxification assays to prove hepatocyte functionalities.

1.3 Thesis outline

- In \textbf{Chapter 1}, the initial part is introduction of the thesis followed by a comprehensive review of literatures relevant for this research work.
- In \textbf{Chapter 2}, the materials and experimental methods adopted for this research work is explained under three major sections listed below with specific introductions and research question/s for each section.
  - Synthesis and characterisation of fibrin nanoconstructs loaded with candidate chemokines (drugs/growth factors) for their use as controlled delivery agents.
  - Development of 3D Agarose nano fibrin (AnF) cryogel matrix bed for differentiation and maintenance of human hepatocytes through sustained release of hepatocyte growth factor.
  - Design and development of a Live Cell based Bioartificial Liver Support System (LC-BALSS) for prolonged hepatic functionality maintenance.
- \textbf{Chapter 3} describes the results and discussion as three separate sections of this research work with relevant literature support.
- In \textbf{Chapter 4}, the research findings of this thesis are summarized and the future perspectives are discussed.
Chapter - I

Introduction

1.4. Review of literature

1.4.1 Regenerative Medicine and organ specific bio-engineering

The aim of regenerative medicine is to regenerate the soft and hard tissues, partial organoids, or even biologically active organ support systems that can be readily available for the patients in need with critical disabilities. Tissue engineering has been used to develop scaffolds to regenerate various tissues such as skin, nerve, bone, blood vessels, etc. [27-29]. Though the concept of regenerative medicine initially emerged as an extension of research progress on tissue engineering, it requires integration of emerging knowledge in the physical and life sciences with bioengineering and clinical medicine so as to understand how to trigger the failed human tissues and organs [30]. In the last two decades, regenerative medicine has shown the potential for "bench-to-bedside" translational research in specific clinical settings. The success of engineered matrices for tissue regeneration and organ development mainly depends upon the development of degradable extracellular matrix analogs that provide a temporary support for cell adhesion, proliferation, maturation and differentiation. In the case of liver tissue engineering, the scaffold needs to be degradable, possess unique properties like growth permissive environment for better cell adhesion, three-dimensionality for cell interactions and desirable porosity to facilitate the diffusion of nutrients and gaseous exchange [31-33]. In addition, the scaffolds should be able to induce vascularization, exhibit sufficient mechanical strength for transplantation and should be biocompatible to eliminate inflammatory response in addition to possessing appropriate cues to maintain hepatocyte characteristics without the loss of its phenotype and functions [34]. Progress made in cell and stem cell biology, material sciences and tissue engineering enabled researchers to develop cutting-edge technology which has lead to the creation of non-modular tissue constructs such as skin, bladders, vessels and upper airways. In all cases, autologous cells were seeded on either artificial or natural supporting scaffolds. However, such constructs were implanted without the reconstruction of the vascular supply, and the nutrients and oxygen were supplied by diffusion from adjacent tissues [35]. Engineering of modular organs (organs organized as functioning units referred to as modules and requiring the reconstruction of the vascular supply) is more
complex and challenging. Models of functioning hearts and livers have been engineered using ‘‘natural tissue’’ scaffolds and efforts are underway to produce kidneys, pancreata and small intestine. Creation of custom-made bioengineered organs, where the cellular component is exquisitely autologous and have an internal vascular network will theoretically overcome the two major hurdles in transplantation, namely the shortage of organs and the toxicity deriving from lifelong immunosuppression.

1.4.2 Regenerative medicine approaches for treating liver diseases

In humans, liver regeneration occurs most frequently after liver damage by ischaemia or hepatitis - an inflammation of the liver that is caused by insults such as toxins, viral infection or immune-mediated injury. Therefore, understanding liver regeneration in humans will help explain how the liver responds to toxic damage by alcohol and drug overdose, or infections like viral hepatitis. Humans with certain hepatic conditions, including cirrhosis (fibrosis of the liver), steatosis (fatty liver), and even those conditions that are due to old age, also have impaired liver regeneration that results in increased morbidity and mortality in response to liver transplantation or toxic chemicals [36]. At present, there is little insight into how the molecular pathways that are necessary for regeneration are altered in these disease states and pathophysiological conditions. Presently, liver transplantation is the leading option for the patients suffering from liver complications. Transplantation is usually carried out to replace the diseased liver with a cadaveric liver or a living donor graft and the survival rate is only 61% [37]. Hepatocyte transplantation is also considered as a valuable alternative to whole liver transplantation. Since its first attempt into a patient with familial hypercholesterolemia [38], several other cases have been performed to cure different livers diseases with non convincing results [39-46]. These failures may be attributed to the relatively small number of hepatocytes that engraft in the recipient because of the quality and quantity of transplanted hepatocytes, as well as immunosuppression- related toxicity [47, 48]. Nonetheless, transplantation of a number of hepatocytes corresponding to 1–5% of the total liver mass has been able to show a positive impact in transplanted patients [49].
Moreover the shortage in the availability of donor liver and a long waiting list for the transplantation leads to the patient's death even before transplantation [50]. In split liver transplantation, the currently adopted procedure, a part of the donor tissue is transplanted. However, this strategy is limited by the shortage of donor tissue and the highly expensive treatment, thus making it unavailable to all patients [51]. Other approaches like hemodialysis, hemoperfusion, and plasma exchange have failed to improve patient survival time [52]. On the other hand, cell transplantation, co-culture techniques and use of culture matrices promise an effective alternative to overcome the crisis but none of these are clinically proven as an enduring treatment for terminal stage of liver failure [53]. Extracorporeal bioartificial liver devices provide the necessary support to patients who are in an advanced stage of liver disease until a donor is identified [54].

1.4.3 Bio-engineered matrices for Liver tissue regeneration

Bioactivity of an engineered matrix for liver tissue regeneration is defined as the capability of the scaffold material to elicit a specific biological response at the interface of the material, which results in a formation of a bond between the tissue and that material. Recently bioactive scaffolds and various methods of incorporating bioactivity have gained considerable interest in the field of liver tissue regeneration. Many growth factors and cytokines have been implicated in regulating liver regeneration. The growth factors include hepatocyte growth factor (HGF), epidermal growth factor (EGF), transforming growth factors (TGFs), insulin and glucagons. And the cytokines include tumour necrosis factor (TNF)α and interleukin (IL)-6. There are several individual transcription factors or proteins including CREM (cAMP-response-element modulator), the Foxo proteins that are required for normal liver regeneration [55]. Therefore, it is desirable to develop a scaffold that possesses similar structure and properties to match the ECM of the native tissues chosen to be cultured. Scaffolds are temporary framework that mimics the extracellular matrix (ECM) to provide structural integrity of tissue and allow the cells to adhere, proliferate and migrate [54]. In addition, a three-dimensional matrix offers an environment that mimics the native tissue architecture and establishes the functional hepatocyte
integrity [56]. Approaches involving the use of hepatocyte suspensions, liver microsomes or precision cut liver slices do not exhibit long-term stability since they do not mimic the in vivo environment [57]. Scaffolds such as nanofibers, films, and hydrogels that attempt to mimic the extracellular matrix topography have been studied extensively to maintain liver specific metabolic functions that can be employed for successful liver tissue regeneration [58]. There are many techniques involved in the fabrication of scaffold with desirable properties for liver regeneration including solvent casting, phase separation, particulate leaching, freeze–thaw and electrospinning [59-62].

Topography of the substrate, both morphology as well as dimension, play a major role in hepatocyte adhesion, migration, and proliferation [63]. Silicon wafers processed by micromachining technology promoted three-dimensional growth of the tissue through vascularization and formed capillary networks thereby leading to formation of hepatic lobules [64]. A multi-array of channels cultured with primary rat hepatocytes were found to possess spheroid-forming capability and were viable for two weeks [65]. Human liver and porcine hepatocytes cultured on polyurethane foam substrates developed spherical multi-cellular aggregates with 80% recovery rate in rats with liver failure due to proteoglycan accumulation and pores present on the scaffold [66]. The pore size and porosity of scaffolds play an important role in the diffusion of growth factors and induces vascularization thereby aiding maintenance of liver specific functions. Pore size is a crucial factor, which controls the mass transport of oxygen and nutrients into the interior of the scaffold, thereby supporting cellular growth in the region [67]. Cells do not migrate when the pore size is greater than 500 μm as they do not recognize the surface and hence porous scaffolds having pore size ranging from 50 to 150 μm, with high inter-pore connectivity are desirable for the culture of hepatocytes [68]. The ideal characterestics needed for a bioengineered matrix for liver tissue regeneration can be summarized as represented in Figure 1.2
Figure 1.2: Ideal characteristics needed for a bioengineered matrix for liver regeneration. More emphasis was given to explain cell-biomaterial interactions

Porous scaffolds provide high surface-to-volume ratio for the attachment of hepatocytes, which are essential for sufficient cell seeding and proliferation. Further, it improves the nutrient transfer, oxygen and other metabolic exchanges since hepatocytes consume 5 to 10 folds more oxygen compared to other cells [69]. Hepatocytes when cultured in 2-D monolayer conditions tend to dedifferentiate rapidly and lose their liver-specific functions such as gluconeogenesis during the culture process [70]. Therefore, three-dimensional culturing of cells with high cell density are required for tissue reconstruction as it would enhance the interaction and communication between hepatocytes for their differentiated functions when compared to the two dimensional system.

Hepatocytes grow as multi-cellular aggregates with spheroidal morphology [71]. The presence of ECM proteins like collagen, laminin, fibronectin has been reported to regulate hepatocyte behavior and gene function [72]. Hepatocytes cultured on decellularized porcine liver sheets have been shown to promote cell
attachment; viability and the liver specific functions were maintained for 21 days [73]. Matrigel® is a commercially marketed scaffold widely used for culturing hepatocytes since it contains many basement membrane proteins [74]. The expression of liver specific functions in the hepatocytes cultured on Matrigel® were maintained for longer time than those on stromal ECMs such as type I collagen and fibronectin, suggesting that the basement membrane proteins are effective in preserving liver-specific function of the cells for long term [75]. The general classifications of biomaterials and cells used for liver tissue regeneration was elaborated in a detailed review [75] is tabulated below as Table 1.1

Table 1.1: Classification of bioengineered matrices for liver tissue regeneration focusing on cell-material interactions. The highlighted references are the research reports stating the use of alginate based matrices and peptide based hydrogels for in vitro hepatocyte proliferation.

The use of synthetic polymers for liver tissue regeneration applications are limited as they lack cell recognition motifs that aid in hepatocyte attachment, as cell adhesion is a key function of scaffolds [76]. Thus, naturally derived polymers such as collagen, chitosan fibrin and alginate have been used for their cell adhesive property due to their close resemblance to the native ECM environment [77]. Since collagen is the most abundant ECM protein in liver cells, it has been used most extensively for liver cell adhesion [78]. Both
monolayer and sandwich forms of collagen gel have been found to maintain hepatocyte function for ten days. Hepatocytes when cultured on sandwich gel maintained the morphology and viability [79]. The signals from the spatial attachment and the tension caused by cell-ECM or cell–cell adhesions promoted the maturation of small hepatocytes (SHs) and altered the cell shape from small and flat to large and cuboidal structure leading to the formation of organoids, which resembles the hepatic lobules that are formed by the cell aggregation [80]. Angiogenic factors such as vascular endothelial growth factor, acidic or basic fibroblast growth factor (FGF) and TNF-α was been administered in combination with polymeric carriers to improve the organoid formation [80].

Chitosan, a naturally derived polymer from the partial N-deacetylation of chitin, is biodegradable, biocompatible and shows structural similarity to glycosaminoglycans, a native liver ECM component [81]. Though naturally derived polymers show better adhesion, lack of mechanical integrity and source variation limit their application in liver regeneration [82]. Cells on TiO2/chitosan composite scaffolds formed spheroids with prolonged viability and improved liver-specific functions in culture, mediated by cell-to-cell interaction [81]. The survival and morphological integrity of hepatocytes were maintained up to 14 days on hyaluronic acid based non-woven scaffold HYAFF-11 due to secretion of extracellular matrix by the hepatocytes [83].

Hepatocytes when cultured on an injectable scaffold made of fibrin matrix prepared with fibrinogen and thrombin showed a decrease in cell number from day 3 till day 7 due to sub-optimal nutrition and lack of oxygen. The fibrin matrix degraded as the cells proliferated. The degradation of the fibrin matrix did not impair hepatocyte survival and differentiation because differentiated hepatocytes were detectable in the explanted livers completely during the observation period [84]. Eventhough, different types of fibrin based biomaterials are used for bioengineering applications as listed in Table 1.2, the use of fibrin based hydrogel matrices for liver regeneration was the only reported attempt of using this much promising biomimetic protein [85].
Table 1.2: Reports of the use of fibrin based matrices for various bioengineering applications. The highlighted references are the only research reports to the date that exploited the usage of fibrin based hydrogel matrices for liver tissue regeneration. [85]

Another strategy to improve functional regeneration of liver attempted to micro-encapsulate liver cells. In this process, hepatocytes were micro-encapsulated with commercial bovine dermal collagen matrix into sodium alginate copolymer membrane. *In vivo* studies reported the effective transplantation of hepatocytes into the matrix resulting in reduction of hyperbilirubinemia for a period of 4–6 weeks [86]. A non-woven polyurethane fiber matrix coated with collagen by fiber extrusion having pore diameter of 200 μm supported the morphology and functionality of hepatocytes through the formation of small aggregates on and between the microfibers [87]. The extensive intercellular contact established through the extracellular matrix proteins maintained liver specific activity [88]. Primary rat hepatocytes formed spherical multi-cellular aggregates (spheroids) on the pores of a polyurethane foam (PUF) and expressed high cell activity for a period of six weeks [89]. Proteoglycan (ECM component) accumulation on the internal surface of the PUF was observed and the affinity of PUF and proteoglycan promoted the spheroid formation.
Hepatocytes were cultured on RGD modified poly(N-isopropylacrylamide-coacrylic acid) hydrogel poly(NiPAAm-co-AAc) and the modified gel maintained higher viability [90]. Further, the cells produced albumin and urea at constant rates for a period of 28 days when compared to unmodified gel [91]. Thus, the RGD motifs for hepatocyte culture constitute a potentially useful three-dimensional cell system for application in bio-artificial liver devices and modifying the scaffold with cell recognition motif improves cell adhesion on the polymeric scaffolds. Figure 1.3 summarizes the applications of bioengineered liver matrices reported to the date that are used for translational research.

![Figure 1.3: Applications of bioengineered liver matrices for translational research. The usage of engineered liver tissue for cell based therapies and in vitro screening applications.](image)

1.4.4 Bioactive factors and biomaterials for Liver tissue regeneration

Regeneration of liver occurs by DNA replication and mitosis through the action of different bioactive growth factors. In the liver, production and release of growth factors are part of a complex interplay between mature and immature hepatocytes, non-parenchymal cells and recruited inflammatory cells.
Hepatocyte growth factor (HGF), a mesenchyme-derived protein, has been found to play a central role in liver development and regeneration after injury. HGF is a pleiotropic morphogen that has been shown to have mitogenic, motogenic, and antiapoptotic effects [92, 93]. In addition, HGF is being explored as an anti-fibrotic agent and may have applications for treatment of liver fibrosis [93]. The other potent stimulators of liver tissue regeneration include epidermal growth factor (EGF) and transforming growth factor-beta (TGF-β) that contributes mainly for inducing angiogenesis [94]. As liver mainly constitutes hepatocytes, which are unipotent and possess greater replicative property, they hold immense promise for in vitro studies as they are expected to possess extremely short doubling times [94]. In the expansion and the subsequent proliferation phase, the hepatocyte population expands mainly due to HGF and Transforming Growth Factor-alpha (TGF-α). In vitro, the HGF secreted by the hepatocytes and supplementary HGF added in the media are both activated by proteolytic cleavage of urokinase-type plasminogen activator (uPA), which is secreted in low concentrations by hepatocytes. STAT3 helps in further proliferation of the cells along with the activated HGF [94, 95]. The cell proliferation is terminated in vitro as the concentration of HGF depletes in the media. Under in vivo conditions, the proliferation is terminated where TGF-β and activins seem to have a crucial role [96].

The influence of nanoscale features on cell behaviours has been reported by numerous cytokines and growth factors that are involved in the regulation of hepatocyte proliferation [97]. Primary hepatocytes were cultured in Williams Eagle media along with EGF on polyethylene terephthalate fabric coated with biodegradable PLGA and poly(N-p-vinylbenzyl-4-O-β-D-galactopyranosyl-D-gluconamide [98]. After 48 h of culture, significant aggregation was observed in the presence of EGF when compared to media without EGF [98]. However, due to the short half-life of growth factors in plasma circulation, growth factors have to be supplemented with the media frequently and hence become an expensive procedure [99]. Therefore, sustained release of growth factors from the scaffold has been attempted to overcome the problem of maintaining the cell functionality for long term [100]. Heparin based three-dimensional
hydrogels were fabricated to release HGF in a sustained manner resulting in the up-regulation of albumin and urea synthesis [101]. It was hypothesized that the heparin in the hydrogel protected HGF from proteolytic degradation and also retained the liver specific functions by the controlled and sustained release of HGF [101]. Hepatocytes cultured on HGF/ECM proteins formed three-dimensional (3-D) spheroids and were found to express significantly higher levels of albumin [102]. A number of recent studies suggest that hepatocytes cultured in 3-D configuration such as a spheroid become more functional as compared to standard monolayer cultures [102]. The organization of hepatocytes into 3-D spheroids prolongs liver specific functions in the presence of HGF [102]. Hepatocyte proliferation and albumin secretion increased twice in poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) microspheres when compared to other 2-D scaffolds [103]. Cell proliferation was improved further when the microspheres were covalently conjugated with type I collagen, laminin, and fibronectin [104]. The PLGA/PHBV composite micro-carriers incorporating HGF exhibited sustained delivery of HGF which maintained bioactivity and a moderate degradation rate for 40 days [105]. Localized direct delivery of HGF to the cells through the microspheres protected it from proteolytic cleavage thereby solving the problem of using growth factors into the media frequently and hence could be an effective component in the development of a scaffold for liver tissue [106]. Alginate scaffolds with high porosity and pore size of 100–150 μm have been prepared by freeze-drying to deliver vascular endothelial growth factor (VEGF) [107]. The sustained local delivery of VEGF from alginate scaffolds increased the microvascular density and showed a 35% increase in the formation of capillaries with the formation of larger and more mature blood vessels promoting the vascularization when implanted on the liver lobes [108]. PLGA was melt blended with PEG and encapsulated with growth factors HGF, EGF, Fibroblast Growth Factor 1 (FGF-1), and Fibroblast Growth Factor 2 (FGF-2) and liver derived extra cellular matrix (L-ECM). An enhanced tissue penetration into the pores of the scaffold was observed and hepatocyte and non-parenchymal cell proliferation in the scaffold was promoted [109]. The vasculature was established and the cells migrated into the pores and differentiated liver tissues appeared on the scaffold. Though the influence of the
individual responses of these growth factors was not studied, the effect of multiple growth factors helped in the proliferation of the liver cells in the scaffold [109]. Hepatocytes established an optimal interaction with the exogenously applied extracellular matrix, which is responsible for the restoration and the maintenance of liver-specific functions clearly indicating the need for growth factors for the long-term cell survival and stability [110].

1.4.5 Liver support systems.

Liver failure is the inability of the liver to perform its normal detoxification, biosynthesis, and/or biotransformation functions. The clinical presentation of liver failure includes a prolonged prothrombin time, encephalopathy, and jaundice. Regardless of the etiology, liver failure can be divided into two categories: acute (ALF) or acute-on-chronic (AoCLF) [111]. Both are accompanied by high mortality [112] Transplantation is still the only ultimate solution for end-stage liver failure, but its application is hampered by a worldwide scarcity of donor organs. In this context, extracorporeal liver support systems have been expected to provide a bridge to transplantation or to provide an opportunity for the native liver to regenerate [112, 113].

Cell sources that have been previously used in extracorporeal BAL treatment in patients and/or large animal models include primary pig hepatocytes, primary human hepatocytes, and human liver tumor-derived cell lines. Primary pig hepatocytes are the biological components of all of the BALs currently under clinical trials, except for ELAD. For nearly 30 years, extracorporeal BAL support systems have raised great expectations for the treatment of liver failure. However, so far, none of these systems is ready for routine clinical use. BAL systems experience bottlenecks in several areas, including cell sourcing, bioreactor design, convenience, and efficacy assessment.
The liver biomass is comprised of hepatocytes and a variety of non-parenchymal cells such as Kupffer cells, sinusoidal endothelial cells and stellate cells. These cells communicate with each other and maintain the complete physiological functions of the liver. Depending on whether they are loaded with metabolically active hepatocytes, the supporting non-parenchymal cells or not, the liver support systems developed to the date can be broadly classified into two types: artificial or bioartificial liver (BAL) systems. It is widely accepted that an artificial liver, which can only detoxify, is insufficient to support liver failure patients, while in theory an ideal hepatocyte-based BAL could provide most or even all normal liver functions [115]. However, it has to be recognized that the BAL is still far from being ready for routine clinical application. BAL systems currently under clinical trials include ELAD,[116] HepatAssist,[117] BLSS,[118] AMC-BAL,[119] MELS,[120] RFB,[121] and HBAL/TECA-HALSS.[122]. Among these, the HepatAssist system was the first and initially reported in the 1980s.[123] All of these systems were found to be safe in phase I clinical trials (Table 1.3). However, to date, only two randomized controlled clinical trials exploring the effectiveness of BALs have been reported,[124] and the results were not encouraging, suggesting that the development of an effective BAL system with widespread clinical acceptance must be quite difficult.
Table 1.3: Overview of Liver support systems currently under clinical trials.

Even though a high degree of metabolic similarity was observed between human and pig hepatocytes, the pig hepatocytes have not the ability to synthesize coagulation factors that function in the human body. [125, 126] Primary human hepatocytes have only been used in three clinical trials, based on ELAD. There are two single-case studies and one phase I clinical trial that used human hepatoblastoma cell lines during the developmental phase of ELAD and it was discussed in those literatures that such applications of using primary human hepatocytes are very difficult because both yield and quality are poor [127-130]. Another serious concern is that, the availability of healthy donor livers are scarce so that only organs or tissues discarded at transplantation (i.e. with fibrosis and steatosis) are available for BALs. Reports are there saying that primary human hepatocytes do not proliferate efficiently in vitro and demonstrate a serious loss of viability after the freeze-thaw process [131]. Among non-primary cell sources, only the C3A cell line and HepG2 hepatoma subclone was used for development and clinical trials of the ELAD system.[129] and unfortunately no improvement in either survival or biochemical parameters was demonstrated in a pilot clinical trials.[132] Several other human liver tumor-derived cell lines, such as GS-HepG2 (glutamine synthetase, GS), HepG2-GS-3A4, and FLC-4, were used in BAL support in large animal models [133]. Prolongations of survival without statistical significance were achieved in these studies. However, none have been so far applied in clinical trials. Poor differentiation and the potential risk of metastatic tumor formation might be the
main hurdles [134]. In addition, a hepatocyte line with high glutamine synthetase expression, for ammonia removal, would potentially increase the production of glutamine. This may further disturb brain function in patients with liver failure [135]. Cell sources that have not yet been tested in extracorporeal BAL systems include immortalized fetal human hepatocytes, immortalized adult human hepatocytes, and human stem cell-derived hepatocytes. More than a decade ago, some researchers claimed that their immortalized fetal or adult hepatocyte lines were promising cell sources for BALs.[135] However, the follow-up research and applications are still absent, which may suggest that they encountered insurmountable difficulties. Recently, a new immortalized human fetal hepatocyte line, cBAL111, was established by overexpression of the reverse transcriptase of telomerase (hTERT)[134]. However, this cell line fell under scrutiny because it was found to have considerable variations at the genetic level, compared with primary hepatocytes in BALs in vitro [135]. The method of reversible immortalization was once encouraging. In this process, the immortalizing genes, i.e., simian virus 40 large T antigen (SV40LT) or hTERT, can be excised using a Cre/LoxP site-specific recombination. Then, an increase of liver-specific functionality can be shown later [136]. Although stem cells from different tissues have the potential to differentiate into hepatocyte-like cells, some issues, such as insufficient quantity, incomplete functionality, ethical controversy, and safety still challenge the clinical availability of these cells.[137] From all of this information, a relevant conclusion can be drawn. As the biological component of extracorporeal BAL support systems, an appropriate cell source should combine the following characteristics: (i) nearly full functionality of mature human hepatocytes, (ii) unlimited life-span and proliferative capacity in vitro, and (iii) no potential risk of metastatic tumor formation, zoonotic transmission, or immunogenicity. Unfortunately, no such cell source has yet been found. Some thought that a highly differentiated human hepatocyte line was most likely to be competent in BALs. Others, however, argued that it was difficult to replace liver functions with a single cell line. Coculture techniques, therefore, are considered promising for obtaining cell sources for BALs in vitro. [138] While it is possible to obtain an ideal cell source ultimately, the existing bioreactor design remains flawed. An ideal BAL
bioreactor should also provide an in vivo-like environment, where the viability and functionality of a large number of hepatocytes can be optimally maintained in vitro. Hence, in this research work, we aimed to address most or all of the above-discussed critical issues restricting the development of high density cell laden bio artificial liver support systems.

References


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